This Volume of Advances in Chitin Science

is devoted to the memory of

Professor Henryk Struszczyk

who passed away on April 15th, 2005

Edited by

Henryk Struszczyk
Alain Domard
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ADVANCES IN CHITIN SCIENCE

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PREFACE

The 6th International Conference of the European Chitin Society, EUCHIS '04 was arranged in Poznań, Poland, in 2004, two years after our Conference EUCHIS '02 held in Trondheim, Norway. This period resulted in many important innovations in chitin fundamental science as well as its application.

The unique properties on chitin and chitosan, particularly their bioactivity, biodegrability and biocompatibility, were the base for development of the original research and applications, mainly in medicine, biotechnology, agriculture and food preservation. Therefore, almost 130 participants, scientists and specialists representing Academia and Industry, interested in chitin science from around the world presented 47 oral contributions, and 85 posters were exhibited during this Conference. Most of these presentations are included in this Monograph.

Our most important task was to publish this volume of Advances in Chitin Science as soon as possible in the satisfactory quality.

We are most grateful to Dr. Wojciech Folkman for scientific and linguistic verification of the submitted manuscripts and his valuable assistance in preparing the issue of this book.

Henryk Struszczyk

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CHAPTER I

INVITED AND KEYNOTE ARTICLES

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A GENERAL LAW OF BEHAVIOR FOR THE PHYSICOCHEMICAL PROPERTIES OF CHITOSAN

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Key words: chitosan, laws of behavior, physicochemical properties

INTRODUCTION

The literature on physicochemical properties of chitosan has been made essentially on chitosans deacetylated in heterogeneous conditions and then, in almost all the cases, on polymers of both different molecular weights and molecular size distributions, and different distributions of acetyl groups.

The studies on the variation of the physicochemical properties of chitosan necessarily need to work on homogeneous series of polymers by the distribution of their molecular dimensions and the repartition of the two kinds of residues. Up to date, this can only be achieved if we start with a highly deacetylated chitosan of relatively low polydispersity, then, re-acetylated in sufficiently soft conditions to allow the production of homologous series of copolymers having the same size distribution and only differing by their DA, or their average molecular weight.

The two essential parameters when we operate this kind of studies are the DA and the molecular weight distributions. Then, their accurate determination is very important. For the first case, NMR is now the most simple and precise method. In the second case, an absolute method such as light scattering is certainly the most appropriate. For that, the refractive index increment, a very useful parameter must also be determined with a very great accuracy and precision.

If this is done, it becomes possible to study the variation of a property with DA for a constant chain length distribution and, for different average molecular weights, with a same polydispersity index and a same DA. In the first case, whatever the context, we observe the same law of behavior, even when the molecular weight is not involved. In the second, particularly interesting results can also be evidenced. This law of behavior allows us to define 3 ranges corresponding to DA<25%, DA>50% and within 25 and 50% in relation with the polyelectrolyte range, the hydrophobic domain, with the formation of aggregates and a transition range, respectively.

ELECTROSTATIC AND HYDROPHOBIC PARAMETERS

Chitosan as almost all natural polymers is a non thermo-plastic structure and then, the processing of materials such as gels or solid forms needs the use of solutions. These solutions only exist in water or mixtures of water with some other polar solvents. As a consequence, the properties of aqueous solutions of chitosan must be particularly well understood.

Chitosan is a term used for the series of the linear copolymers of D-glucosamine and *N*-acetyl-D-glucosamine, which can be soluble in water. This property depends on whether the polymer is constituted of a block or a random distribution of the two kinds of residues.

The solution properties of this series are strongly related to the cationic polyelectrolyte behavior illustrated by the Katchalsky's equation [1]

$$pK_a = pH + \log(1-\alpha)/\alpha = pK_0 - \varepsilon\Delta\psi \cdot (a)/KT_{pK}$$

This equation illustrates that the ionization state of chitosan represented by pK_a depends on various parameters: the intrinsic pK (pK_0), the dielectric constant of the medium (ε), the temperature, the apparent charge density and the chain length. Some of these parameters are themselves depending on others such as pH, ionic strength and the chemical structure. As a consequence, the electrostatic properties should be strongly sensitive to both the chemical structure and environmental parameters.

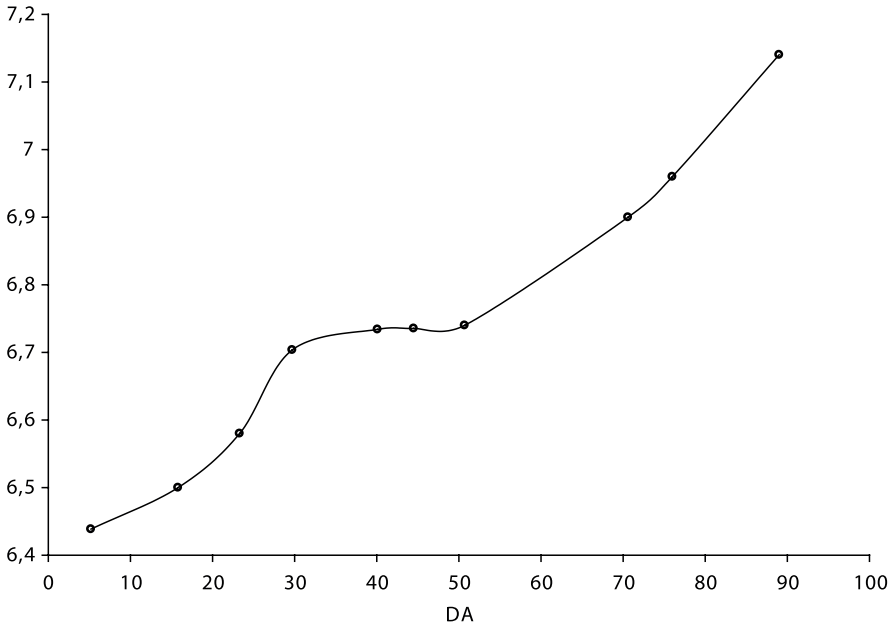

Figure 1. Variation of pK_0 as a function of DA

pK_0, is a parameter sensitive to the structural environment of the ionic sites of the chain and the ionic strength. Thus, for a homogeneous series of chitosans of

same molecular distribution, only differing by their DA, the study of the variation of pK_0 with DA reflects a typical behavior with 3 ranges defined by: DA ≤ 25%, 25% ≤ DA ≤ 50% and DA ≥ 50% [2].

The first range is attributed to the behavior of a polyelectrolyte which apparent charge density decreases on increasing DA. This is in favor of the formation of hydrogen bonding partly responsible for the pK_0 increase. It is noteworthy that the upper limit of 25% of this polyelectrolyte range also corresponds to the limit of the Manning condensation [3], where the Manning parameter ξ becomes below 1. Over 50%, the charges are statistically comparable to isolate charges on polymer chains becoming more and more hydrophobic as DA increases. In between these two ranges, we notice a transition. If DA is a very important parameter, for a given DA, as shown on figure 2, other parameters must be considered for example μ, the ionic strength [2]. But, there also, we demonstrate that DA plays an important part since the role of μ on the value of pK_0 is still influenced by DA. Thus, as observed on figure 2, for DAs in the first range, pK_0 varies significantly with μ although it remains almost constant in the transition range and varies again in the 3rd one. In the first range, we observe the classical role of the charge screening operated by μ and in the 3rd, the role of μ on hydrophobic interactions. We also observe that the nature of the counter-ion has also to be considered.

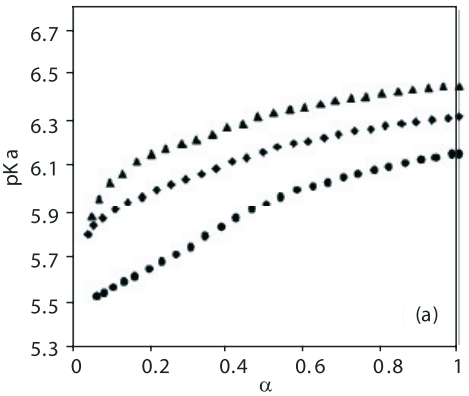

Figure 2. Variation of pK_a for a chitosan of DA5.2%, at 3 different ionic strengths: ▲ 0.1M, ◆ 0.05M and, ● 0,01M

As a conclusion, we may consider that all the properties of chitosan in solution should depend on all the parameters influencing either electrostatic properties or hydrophobic interactions. Therefore there is no property we may consider as independent of these two behaviors.

SOLUTION STABILITY

It is known that chitosan solutions give rise to a kinetic process of precipitation over a given pH close to that of the half neutralization [4, 5]. This critical phenomenon is strictly related to the equilibrium between hydrophilic and hydrophobic interactions depending on various parameters, especially, those already mentioned, with a particular role for DA. If we represent the variations of the critical pH of precipitation or, as in figure 3, the corresponding neutralization degree, $\alpha_{crit.}$, we find again a typical law of behavior with the two extreme ranges of DA and a transition in between. The higher values for the first range illustrate a higher hydrophilicity in relation with the polyelectrolyte character and, for DA over 50%, the low solubility in relation with the hydrophobic character of *N*-acetyl groups, although an increase of pK_0 with DA occurs (see figure 1).

SOLUTION PROPERTIES

Electric polarisability of chitosan chains

The properties of chitosan in solution should also reflect the behaviors discussed above. They necessarily have a consequence on the conformations and the formation of self-association of polymer chains. Light scattering is a particularly interesting technique for the study of this kind of properties. It depends on both the molecular dimensions and the electric polarisability of the solution and then on that of the solvent and the polymer chains, in relation with their respective refractive indexes or dielectric constants. This is illustrated by the well known equation [6]:

$$\alpha_p = \left(\frac{n_0 V}{2\pi N}\right)\left(\frac{dn}{dC}\right) = \frac{\varepsilon - \varepsilon_0}{4\pi(N/V)}$$

with α_p, the electric polarisability of the polymer, n_0, the refractive index of the pure solvent, N, the Avogadro's number, dn/dC, the refractive index of the polymer in the considered solvent, and $\varepsilon - \varepsilon_0$, the difference of dielectric constant between the solution and the pure solvent, representing the dielectric constant of the polymer. Therefore, dn/dC is characteristic of a given polymer in a given solvent and is in direct relation with the electric polarisability of the considered polymer chains. It is necessarily sensitive to the dielectric environment of the polymer chains and thus, to the apparent charge density and the proportion of *N*-acetyl glucosamine residues. As a consequence, it should be particularly dependent on DA. The results reported in figure 4 confirm quite well this assumption. We notice a continuous decrease of dn/dC with the apparent charge density λ_a. The variation of λ_a can be obtained by various ways corresponding to work with a constant DA and change the neutralization degree α or the ionic strength, or, to work at a given α and ionic strength and change DA. In the two first cases, we notice a monotonous decrease of dn/dC [7, 8]. On the contrary, in the second case, the variation of dn/dC illustrates again the law of behavior in three ranges. The role of electrostatic parameters is particularly important in the first range and disappears at a DA close to 25%. Nevertheless, the role of DA remains important all along the scale of DAs. It reflects both the electrostatic behavior in the first range and the hydrophobic role of *N*-acetyl residues in the 3rd one. It is noteworthy that at the limit of the water solubility of chitosan, close to DA=75%, dn/dC becomes near that of a neutral polysaccharide such as dextran and then close to 0.145cm^3/g, independently of the ionization state and ionic strength. The typical law of behavior of dn/dC as a function of DA is cru-

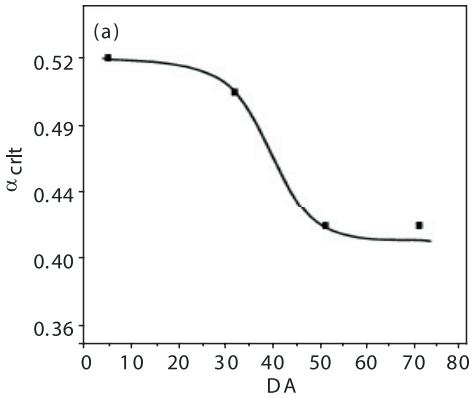

Figure 3. Variation of α_{crit}, the critical pH where precipitation arises, as a function of DA

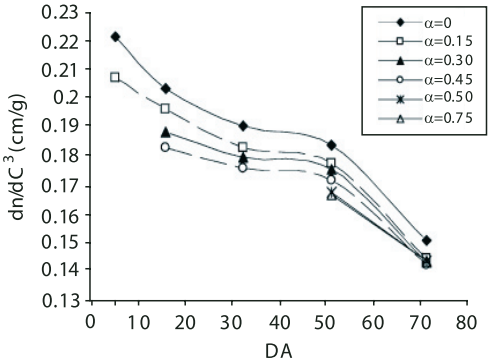

Figure 4. Variations of the dn/dC of chitosan solutions in $KclO_4$ 0.1 M, at different α and DA values

cial for the determination of molecular characteristics determined by light scattering. The authenticity of this law has been indirectly checked in different salts [7, 8] and ionic strengths and different series of reacetylated chitosans. Indeed, both the number- and weight-average degrees of polymerization were the same in all cases in a large range of DAs between 2.5 and 71%.

Solubility and conformational parameters deduced from viscometric measurements

In order to study the role of both the molecular weight and DA on the viscometric power law $[\eta] = KM^{\alpha}$, we prepared 4 homogeneous series of chitosans. Inside a given series, the weight-average degree of polymerization (DP_w) and the polydispersity index were almost constant and only DA was varying from near 1 to 70%. Then four series with DPs ranging from 650 to 2600 were prepared. The polydispersity index was necessarily low and generally below 1.4 to limit its influence on the parameters of the studied power laws [9].

To minimize the electrostatic properties of the polymer chains and their influence on the studied properties, we used an additional ionic strength of 0.15M brought about by an acetic acid/ammonium acetate salt at pH near 4.3. This salt also had the advantage to limit the chain self-associations by hydrogen bonding and hydrophobic interactions [10].

To limit the influence of aggregates essentially present in solutions of chitosan with both a high DA and DP_w, an additional filtration on a 0.22μm pore-size membrane could be performed allowing to considerably decrease the distortion of the zimm plots [9].

The studies on the variation of the reduce viscosity as a function of the concentration always allowed the drawing of straight lines and then to deduce the intrinsic viscosities in good conditions. It was then possible to consider the variation of the intrinsic viscosity with DA and thus, to deduce the law of behavior for the parameters K and α of the viscosity law. First of all, the representation of the intrinsic viscosity as a function of DA, for each series of chitosan, reproduced the typical general law of behavior independently of DP_w. The three domains corresponding to DA below 25, over 50% and in between these values were still observed. It was also interesting to notice that the role of DA was less important on increasing DP_w. Nevertheless, it was always significant and had to be considered especially when C*, the critical concentration of chain entanglement had to be evaluated. Moreover (not shown), this parameter also followed the general law of behavior as a function of DA.

On figure 5 are represented the variations of K and α.

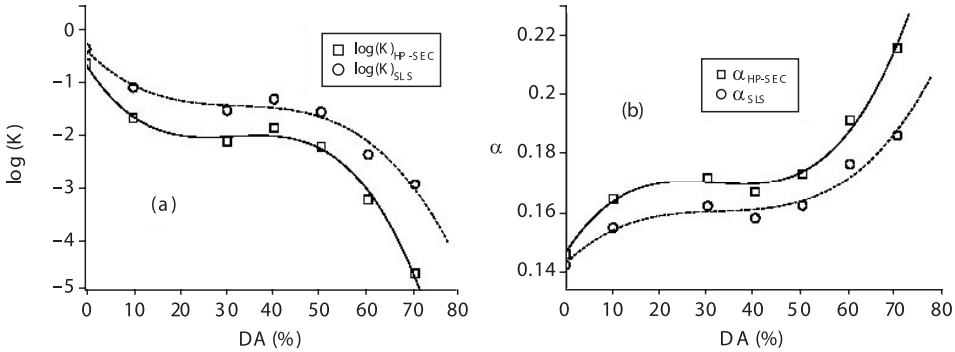

Figure 5. Variations of log K(a) and a(b) as a function of DA from values of DPw deduced from steric exclusion chromatography on line with a light scattering detector(SEC) and from multi-angle laser-light scattering experiments in batch (SLS)

Whatever the parameter or the method used to evaluate DP_w, we still observed the general law of behavior with the same ranges of DA values. The shift of K and α to higher values when comparing the evaluation made from SLS in batch and SEC respectively must be related to an overestimation of DP_w by SEC, in relation with a loss of chromatographic separation for the highest molecular-weights. We first notice a continuous decrease of K on increasing DA, except in the transition range. As we shall see, this behavior illustrates the variation of the solubility, which also decreases in a similar manner. On increasing DA, the α parameter of the MHKS equation increases continuously with for a DA close to zero, a value of near 0.65 in agreement with a conformation of random coils in a good solvent, to a value close to 1.05 corresponding to wormlike chains in perturbed conditions.

If the ionic strength was sufficiently high to screen the electrostatic repulsions and then to allow the chain depletion up to a conformation of random coils (for low DA's), the "α" parameter remained still far from 0.5 and as often claimed for polyelectrolytes the true unperturbated conditions could only be achieved at infinite ionic strength [11].

Our solvent, strongly inhibiting the formation of hydrogen bonding and hydrophobic interactions, could be considered as responsible for our typical law of behavior but, as already seen above for the variation of the refractive index increment, measured in $KClO_4$ and for other properties studied in quite different conditions, this argument cannot be considered. What is also particularly interesting is that this law of behavior is observed in a very large range of DP_w values.

Conformational parameters deduced from light scattering measurements

Static light scattering measurements made in batch were particularly interesting to study some conformational parameters, especially the z-average value of the giration radius ($R_{g,z}$) and the persistence length ($L_{p,t}$). In the case of the highest molecular weights, and high DA's it was necessary to add a filtration over 0.22µm. membranes to limit the role of aggreagates.

The results reported on figure 6 show the important role played by both DA and DP_w. In the first case, the law of behavior with the 3 domains still exists and in the second case, we observe a critical value of DP_w near 1600 over which, there is a strong change in behavior concerning the role of DP for a given DA. In the range of the lower values, as DA increases, we notice a continuous fall of $R_{g,z}$ al-

though the chain stiffening with DA often claimed in the literature should induce an increase of this parameter. The presence of a critical value of DP_w ($DP_{w,c}$) suggested that in addition to the electrostatic contribution (β_{el}) to the total excluded volume (β_{tot}) a steric contribution had to be considered (β_{st}). The latter term represents the involvement of short- and long-term interactions on the excluded volume and are depending on both DA and DP_w. Then, the total contribution can be evaluated thanks to the following equation

$$\beta_{tot} = \beta_{el} + \beta_{st}$$

The steric contribution is a particularly important term since when we study the modeling of the variation of the conformation of chitosan chains in solution with DP_w, we notice that the electrostatic term (β_{el}) can be reasonably neglected, especially for DAs over 25%.

The persistence length could be calculated for each chitosan assuming the Benoit-Doty equation:

$$R^2_{G,z} = \frac{LL_{p,tot}}{3} - L^2_{p,tot} + \frac{2L^3_{p,tot}}{L} - \frac{2L^4_{p,tot}}{L^2}(1 - e^{-L/L_{p,tot}})$$

where L corresponds to the contour length of the polymer chain, considering the monomer size as equal to 0.49 nm. The determination of L_p by SLS in batch (figure 7) led to more reliable results than from HP-SEC/MALLS. Figure 7 still reproduces the classical law of behavior as a function of DA. In addition, it allows us to show for the first time a continuous raise of L_p along with DA. It is important to underline the very important role played by the polydispersity index in the evaluation of the total persistence length and then the necessity to work with chitosans of relatively sharp size distributions. This is particularly the case of the sample of DP_w 650 which polydispersity index decreased on increasing DA. The role of the polydispersity index on the flattening of the law of behavior is much more evidenced on the results obtained with the samples of Schatz et al. (figure 7a). For DP_w over 1600, the variations of both $R_{g,z}$ and L_p as a function of DA were in good agreement and could be described by the theory of the excluded volume, in relation with long distance interactions becoming preponderant to the detriment of the others. Thus, on increasing DA, β_{st}(DA,DP) could be compared to β_{el} and considered as becoming successively negligible for DA < 25%, then, similar for DAs between 25 and 50% and higher for DAs over >50%.

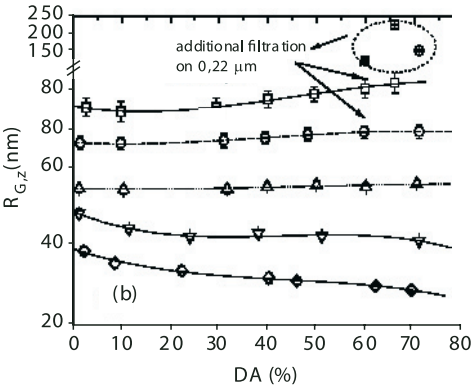

Figure 6. variation of $R_{g,z}$ for the four series of chitosan as a function of DA: DP_w = 650 (◇), 1600 (△), 2200 (○), and 2600 (□); results of C. Schatz et al. [8] for a DP= 1000 (▽)

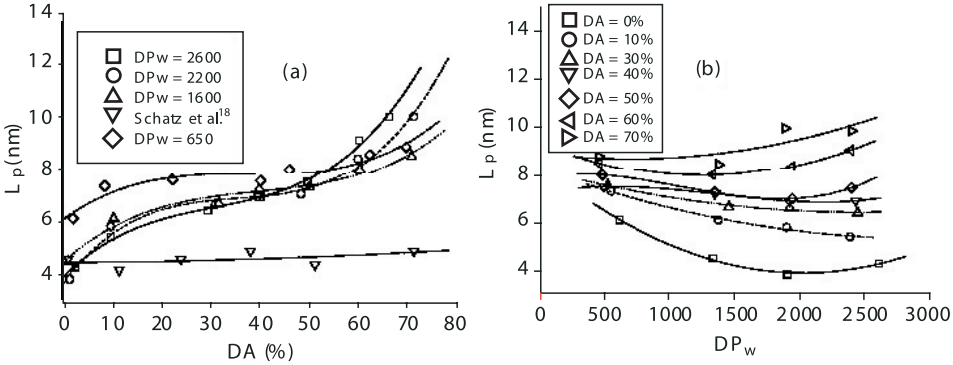

Figure 7. Variation of L_p as a function of DA(a) and DP_w (b)

This simple consideration can be used to explain the conformation changes of chitosan chains as a function of DA.

Figure 7b gives some general information. Thus, whatever the DA, up to a critical value of DP_w, we term $DP_{w,c}$, the increase of the chain size is responsible for a decrease of the chain flexibility. This result agrees with the fact that below the critical DP_w, the chain stiffness is essentially due to short-distance interactions. Therefore, in this range, we can consider that both β_{st} and β_{el} remain almost constant whatever DA. On the contrary, for DP_w over $DP_{w,c}$, due to the majoritary role played by long-distance interactions, depending on DA, two kinds of behaviors could be observed. For DA below 50%, L_p was almost constant in relation with the fact that the two contributions to the excluded volume remained unchanged. This has also to be related to the influence of the electrostatic interactions. On the contrary, on increasing DA from 50 to 70%, due to the increase of the number of *N*-acetylated residues, the electrostatic contribution disappeared and the steric contribution to the excluded volume became majoritary. The consequence was an increase of the chain stiffness as DA increases. We also notice a slight shift of $DP_{w,c}$ from 1800 to 1300 when DA increases from 0 to 70%. It is finally important to mention that for the highest DAs, the self-association of chitosan chains should artificially emphasize the apparent stiffness of chitosan chains and then influence the variations of L_p as a function of DA or DP, in this domain.

OTHER EXAMPLES OF APPLICATION OF THE LAW OF BEHAVIOR

We recently showed the possibility to form true physical hydrogels of chitosan without any chemical cross-liking agent. In a first case, the gels were formed during the evaporation of a hydro-alcoholic solution of an acetic acid chitosan salt initially over C*. The general law of behavior was observed in various circumstances such as: the variation of the time necessary to reach the gel point as a function of DA, the role of DA on the apparent neutralization of chitosan on the gel point, the proportion of water in the mixture on the gel point, the role of DA on the storage modulus at the gel point [12, 13].

We also studied the direct gelation of a pure aqueous solution of chitosan thanks to the use of gaseous ammonia. The same laws of behavior were still observed when we studied the role of DA on the time to reach the gel point or on

the variation of the storage modulus on the gel point. But, as above in the case of the role of DP_w, we noticed the important role played by the initial concentration on the molecular organization in solution, responsible for the formation of precursors of gelation [14].

CONCLUSIONS

In this paper we showed that thanks to homogeneous series of chitosans with a random distribution of *N*-acetylglucosamine residues, it was possible to evidence a general law of behavior, when a given property was studied as a function of DA. This law illustrates the presence of three domains we may now consider as being located below 25%, between 25 and 50% and over. This law is observed in a very large range of DP_w. We also demonstrated the important role of the polydispersity that must be as low as possible to correctly study and interprete some properties. Merover, whatever the DA, we evidenced a critical DP_w below which only short-distance interactions were counterbalancing electrostatic interactions, although, over this value, long-distance interactions became preponderant thus influencing largely the stiffness of the polymer chains. It was also highlighted that over a DA of 50% polymerchain self-association could also artificially influence this parameter. Then, the three domains of the general law of behavior could be successively attributed to the polyelectrolyte domain, the transition from electrostatic to long-range interactions and the range of molecular associations.

When C was over C* the law of behavior was observed in various circumstances and the role of the initial concentration of polymer chains was also shown as playing an important role on the molecular organization responsible for the formation of precursors of gelation.

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PREPARATION AND SOME PROPERTIES OF HYDROGELS OF CHITIN AND CHITOSAN

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Key words: Chitin, Chitosan, Hydrogel

INTRODUCTION

Although regenerated chitin and chitosan have been expected to apply for biomedical materials owing to low toxicity and biodegradability in animal body, drastic conditions are requested to regenerate either on the dissolution or on the coagulation [1, 2]. Much milder conditions to regenerate both polysaccharides would participate to keep environment clean. A calcium ion was suggested to interact with chitin because Crab or Shrimp shells, main resources for chitin, were covered by calcium carbonate originally. Since formic acid [3], one of solvent for Nylon family, was reported as a solvent for chitin, calcium chloride dihydrate saturated methanol, also one of good solvent for Nylon 6 or 6, 6 [4], was suggested to be a solvent for chitin among various metal ions-organic solvent systems. α-Chitin was recovered as a hydrrated chitin gel from the solution of calcium chloride dihydrate-saturated methanol by precipitation in large excess of water or methanol. The chitin hydrogel contains about 95% of water. Since the suspension of α-chitin in water looks like pulp in water, applying a paper manufacturing would result in chitin nonwoven fabrics. This is a nonwoven fabrics made of hundred percent of α-chitin, suggesting a perfect biomedical device.

However, β-chitin from squid pen was found to conduct the lower solubility and the higher viscosity than that by α-chitin from Crab or Shrimp shells. Several differences were observed between α- and β-chitins on the adsorption of metal ions, ionic dyes and ionic polymers even after the regeneration into fiber or nonwoven fabrics. There was one way to prepare nonwoven fabrics for α-chitin following to dissolution into calcium chloride solvent, but there were a couple of method for β-chitin following to similar way to α-chitin and to swelling with water by mechanical agitation. The methanol-acetone combination was proposed to apply for the regeneration of both α- and β-chitins due to such a high solubility of calcium chloride against methanol.

Chitosan hydrogel was also prepared from the organic acid solution. Neutralization of the solution gave the chitosan hydrogel which is stable in wet state for long period.

PREPARATION OF HYDROGEL

The calcium chloride saturated methanol (Ca-MeOH) has been reported to be a good solvent system to dissolve nylons [4]. The dissolution mechanism of nylon is considered to imply the destruction of hydrogen bonds by the chelation of calcium ion to amide bonds. Chitin has also an amido bond to stabilize chitin by hydrogen bonding network. Thus, we have anticipated that chitin would be also dissolve in the calcium chloride saturated methanol, an extremely mild solvent than ever, without depolymerization. Variously *N*-acetylated chitosans were applied to the solubility test together with different molecular weights. As seen in Figure 1, solubility of *N*-acetylated chitosan depended both on the degree of *N*-acetylation and also molecular weight. The limitation of solubility by the chitin of higher molecular weight seems to be such a high viscosity in spite of fast dissolution of *N*-acetylated chitosan. Viscosity measurement of chitin solution had proved high stability of the chitin solution of calcium chloride dihydrate saturated methanol compared to the conventional strong acid solution.

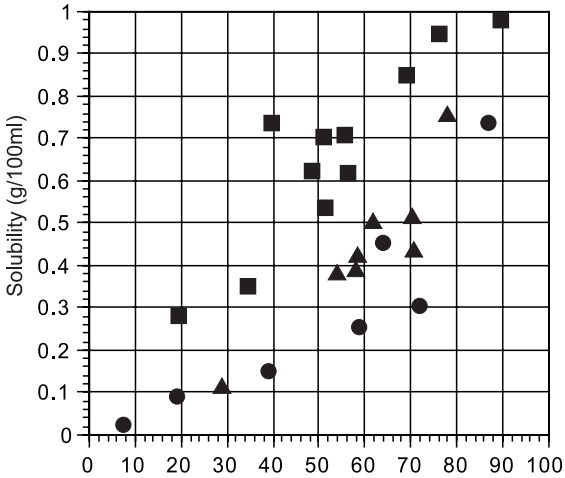

Figure 1. Dependence of degree of *N*-acetylation on the solubility of chitin; Molecular weight of chitin; ■: 1.2×10^4, ▲: 4.0×10^4, ●: 1.6×10^5.

A swollen fibrous chitin (chitin hydrogel) was successfully prepared following by addition of chitin solution into large excess of water at room temperature to remove calcium completely. The gel contains 95–97% of water. The process was monitored by the transmittance measurement. The result is shown in Figure 2. Transmittance of chitin solution decreased drastically by the addition of water. About 0.2 fold water against chitin solution is sufficient for the completion of the precipitation. The result suggests that dissolution of chitin in calcium chloride dihydrate saturated methanol is persisted in the precise balance of the component.

β-chitin can be highly swelled in water by the vigorously stirring by blender, probably due to the lose packing of polymer chain. *β*-Chitin exists in a crystalline hydrate which accounts for its lower stability since water can penetrate between the chains of lattice [5]. Thus, due to the lose packing of polymer chain, *β*-chitin can be highly swelled by the vigorously stirring by blender.

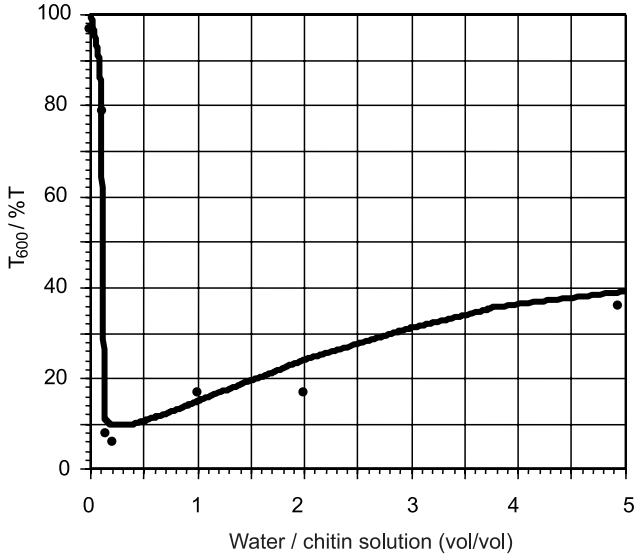

Figure 2. Transmittance measurement adding water into the chitin solution of calcium chloride dihydrate saturated methanol.

Superior character of chitin hydrogel was evaluated by the enzyme digesting activity. Figure 3 shows the chitinase digestion activity measured by the transmittance profile. Chitin hydrogel was very sensitive for chitinase even under the low concentration of enzyme condition. In contrast, chitin powder was not digested at all. The results suggests that chitin hydrogel is highly hydrated and swollen.

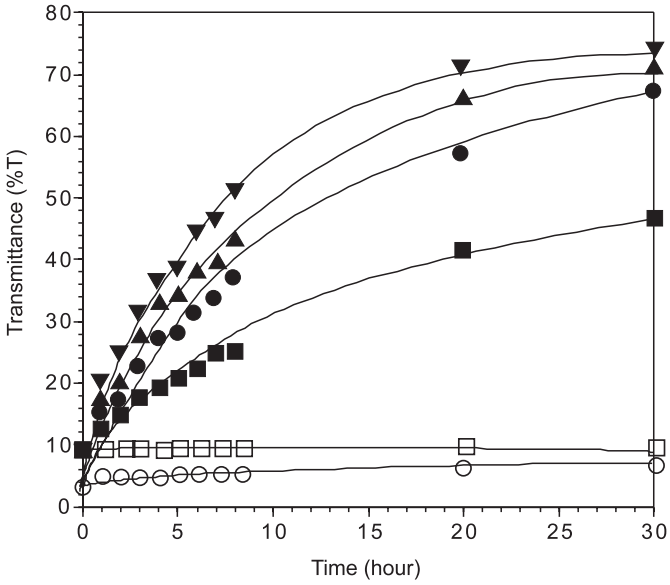

Figure 3. Chitinase digestion experiments using chitin samples as a substrate. □:chitin gel control, ○:chitin powder using 0.8 units of chitinase, Chitin gels using 0.2 (■), 0.4 (●), 0.6 (▲) and 0.8 (▼) units of chitinase.

PREPARATION OF SHEET

Since the suspension of chitin in water looks like pulp in water, applying a paper manufacturing resulted in chitin sheet. This is a sheet made of hundred percent of chitin, suggesting a perfect biomedical device. The thickness of the sheet can be easily controlled by the amount of chitin hydrogel. Both the α- and β-chitin hydrogel can be applied for sheet. In addition, β-chitin can be easily to be a hydrogel by mechanical agitation with water. This swollen hydrogel was also possible to prepare sheet in the same manner. The XRD experiments revealed interesting crystal structural results. The crystal structure of raw material was maintained in the sheet using precipitated α-chitin hydrogel and swollen β-chitin hydrogel, whereas that of sheet prepared from precipitated β-chitin hydrogel was α-chitin. Although mechanical agitation of β-chitin broke down hydrogen bonding network partially, dissolving β-chitin led to a completely destruction of hydrogen bonding network. Thus, α-chitin type crystal structure was regenerated by the hydrogen bonding network accompanying by removal water with filtration. Dye adsorption property of the sheet revealed that chitin sheet adsorbed anionic dye much stronger than cationic dye. The result suggests that chitin has cationic character. In addition, chitin sheet is useful for DDS because most of drugs have anionic functionality to maintain water solubility.

PREPARATION OF CHITIN-GELATIN COMPOSITE

Both α-chitin from Crab or Shrimp shells and β-chitin from squid pen were found to dissolve in calcium chloride dihydrate saturated methanol and regenerated to chitin hydrogel prepared by precipitation with addition of large excess of water to the chitin solution. In addition, β-chitin slurry was also prepared by repeated mechanical agitation of β-chitin powder in limited amount of water. The hydrogel is very useful for several areas, such as preparation of sheet, substrate for graft polymerization and so on. Gelatin, on the other hand, is obtained by a controlled hydrolysis from the fibrous insoluble protein collagen, which is widely found in nature being the major constituent of skin, bones and connective tissue of animal. Being a protein, gelatin is composed of a unique sequence of amino acids and is an excellent biodegradable and biocompatible material as well as the chitin. Since chitin sheet shows somewhat brittle character arising from strong hydrogen bonding, composite of gelatin with the chitin hydrogel will improve the property. The material is suitable for artificial soft tissue which require biodegradable, biocompatibility and appropriate strength. Pig skin gelatin was used because of prevention BBS problem. Three types of chitin hydrogels were mixed with gelatin and cast on a stainless plate. Concentration of gelatin was 20–50% and crosslinking was done by glutaraldehyde. Thickness of the composite sheet was 0.2–0.5 mm. Swelling ratio of the sheet was 4–7 and the values depended on the type of hydrogel used. Equilibrium swelling was completed after 24 hours immersion in the medium. Comparing the chitin alone sheet, tensile stress of composite sheet in dry state was remarkably improved using regenerated chitin hydrogel.

CHITOSAN NEUTRAL HYDROGEL

Chtitosan powder was firstly dissolved in aqueous acetic acid solution under mechanical agitation followed by filtration to remove insoluble material. Chitosan solution was brought to alkali condition by the addition of sodium hydroxide aqueous solution followed by dialysis against distilled water or extensive filtration to remove sodium acetate. This chitosan neutral hydrogel was stable in wet condition for long period even under high temperature. Thus, the hydrogel is useful for the preservation of chitosan.

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CHITIN DEACETYLASE, A TOOL FOR CHITIN AND CHITOSAN DEACETYLATION

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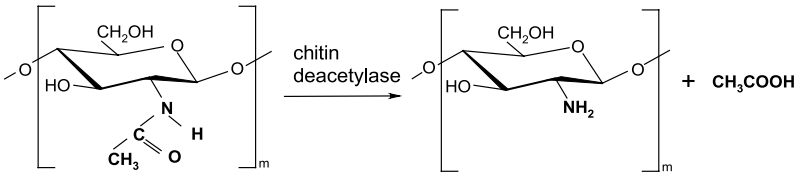
Chitosan is produced on an industrial scale by the chemical deacetylation of chitin. In a single process a polymer with high acetylation degree (40–45%) and middle molecular weight is produced. More rigorous conditions during the process or its repetition several times causes not only further deacetylation but also hydrolysis of polymer chains; chitosan with a lower acetylation degree but also with low molecular weight or even chitosan oligomeres are produced [1, 2].

The process of chemical deacetylation of chitin has several disadvantages, as it generates highly concentrated wastes and the resultant chitosan often has a fishy odor. In order to eliminate these problems several new methods of chitosan production are being developed, such as chitosan production from fungi [3–6] and enzymatic deacetylation of chitin [7, 8].

Enzymatic chitin deacetylation is carried out by enzyme chitin deacetylase, which may be also used for enzymatic deacetylation of chitosan [9–11] to reduce the degree of acetylation.

Chitin deacetylase hydrolyses the links between the acetyl group and the amine group in *N*-acetylglucosamine mers of chitin and chitosan:

As a result of this action, mers of GlcNAc are transformed into mers of GlcN (decrease the acetylation degree) and an increasing concentration of acetic acid can be observed.



N-acetylglucosamine glucosamine

Chitin deacetylase is an enzyme that can be separated either from fungal mycelium or from culture medium. Two types of enzymes are cited in the literature: intracellular (*Mucor rouxii,Absidia coerulea, Absidia orchidis, Absidia glauca, Rhizopus stolonifer, Saccharomyces serevisieae*) and extracellular (*Mucor rouxii, Mucor he-*

malis, Absidoa coerulea, Absidia glauca, Colletotrichum lindemuthianum, Gongronella butleri, Rhizopus oryzae, Aspergillus nidulans).

Intracellular chitin deacetylase is an enzyme connected with chitin and chitosan formation in the fungal cell walls. Chitosan is produced by a complex action of two enzymes:

– chitin synthase; responsible for building the chain of chitin and chitosan,
– chitin deacetylase; responsible for deacetylation of nascent chitin and its transfer into chitosan; responsible for the acetylation degree of the biopolymer.

In both cases, chitin and chitosan, uridino-di-phosfo-N-actyloglucosamine (UDP-GlcNAc) is a precursor for each of these biopolymers and is used by chitin synthase in the polymerisation process – a chitin chain is formed. Next, in the moment of formation, this chitin chain is deacetylated by chitin deacetylase and transformed into chitosan chain.

This mechanism of chitosan formation was described by Araki and Ito [12, 13] and explained in detail by Davis and Bartnicki-Garcia [14] and Bartnicki-Garcia [15] (1989) and presented for the fungus *Mucor rouxii*.

The role of extracellular chitin deacetylase was not satisfactory explained so far. It is connected with diminishing plant resistant against the pathogens elicited by the chitin oligomers for *Colletotrichum lindemutianum* [7, 16], or autolysis of the fungus *Aspergillus nidulans* [17], or growth of the Zygomycetes mycelium [8].

PROPERTIES OF THE CHITIN DEACETYLASE

Molecular weight

It can be clearly observed that extracellular chitin deacetylase has lower molecular weight (27–33 kDa) than intracellular enzyme (75–80 kDa). The molecular mass of intracellular enzyme was independent of the source. But in two cases (*Mucor rouxii* and *Rhizopus stolonifer* [18]) the molecular mass approx. 38–40 kDa was also observed. This value is one-half of the value reported for chitin deacetylase. Kafetzopoulos et al [19] observed the activity of this „smaller" enzyme in the range of 10–15% of the "larger" one and suggested that this lower value is connected with the enzyme that is a product of enzyme degradation. Looking at the data presented it can be thought that chitin deacetylase is not a monomer as the majority of authors suggest but is built with two subunits with molecular weight of 38–40 kDa each.

Optimal pH

The optimal pH of chitin deacetylase strongly depends on the origin of the enzyme. The extracellular chitin deacetylases have optimal pH in neutral (pH 7.0, *Aspergillus nidulans* CECT 2544) or alkaline range (pH 11–12, *Colletotrichum lindemutianum* ATCC 56676) while intracellular chitin deacetylases have optimal values in the acidic range (pH 4.0–5.8).

Optimal temperature

The optimal temperature is similar to all enzymes and has been found to be 50°C for nearly all enzymes, except the enzyme secreted by *Colletotrichum lindemuthianum* ATCC 56676, whose optimal temperature was slightly higher (60°C).

Substrate specificity

Chitin deacetylase hydrolyse the linkage between amine and acetyl groups in N-acetylglucosamine mers. Glycol-chitin was usually used as a standard for enzymatic assay, but chitin and chitosan were mainly used as a substrate for the enzymatic process of deacetylation. It was found that the soluble or colloidal forms of these polymers were faster deacetylated than the crystalline forms [9, 20, 21]. This is obvious when one take into account the compact crystalline form of chitin and chitosan where the GlcNAc mers are less accessible for the enzyme. Chitosan with a high acetylation degree (39–51%) was readily deacetylated than the polymer with low AD [20, 22]. This observation suggested that the enzyme starts its attack when there is proper sequence of GlcNAc mers. The investigations with chitooligomers suggested that chitin deacetylase needs no less than $(GlcNAc)_3$ – $(GlcNAc)_4$ consecutive mers [7, 9, 17, 20]. The activity of enzyme increased with an increase in successive N-acetylglucosamine mers and was the highest for chitohexose (the largest chitooligomer tested).

Requirement of cations

Requirement of cations as activators for chitin deacetylase depends on the origin of the enzyme.

It was shown that some cations at low concentrations can act as activators for chitin deacetylase: Zn^{2+} (1 mM), Ca^{2+} (1 mM), Co^{2+} (1 mM). It was also observed that Zn^{2+} can act as an activator for the intracellular enzyme from *Mucor rouxii* ATCC 24905, but also as an inhibitor for the extracellular enzyme [23]. Some cations can also act as inhibitor when their concentration increase: Co^{2+} and Zn^{2+} for *Colletotrichum lindemutianum* ATCC 56676 (increase of concentration from 1 mM to 10 mM [7])

Influence of organic acids

It was clearly showed that most organic acids acts as inhibitors for chitin deacetylases: acetic, formic and propionic acids in concentration of 250 mM decreased the activity to 10%, 50% and 15% respectively for the enzyme from *Mucor rouxii* ATCC 24905 [19]. But for the enzyme separated from *Aspergillus nidulans* CECT 2544 [17], the activation by acetic acid in the range of concentration of 0.4 to 4.0 mM was observed. The influence of acetic acid on chitin deacetylase from *Colletotrichum lindemutianum* ATCC 56676 [7] was less significant than for the enzyme separated from *Mucor rouxii* ATCC 24905 [19]. The enzyme lost only 4% of initial activity for the concentration of sodium acetate of 100 mM and only 30% for the concentration of 800 mM

The mechanism of inhibition was not determined. Our preliminary data suggest that it is non-competative or even mixed inhibition. In such case the effect of inhibition by acetic acidcan not be eliminated and can cause the deactivation of chitin deacetylase.

KINETICS OF CHITIN AND CHITOSAN DEACETYLATION

It must be clearly emphasized that there are only few data on kinetics of chitin, chitosan and chitooligosaccharides enzymatic deacetylation. Chitin deacet-

ylation was investigated by Stevens et al. [24], who stated that the process is biphasic for enzyme separated from *Absidia coerulea* IFO 5301: very fast during the first 30 min and much slower during the next 2–3 hours. These observations were confirmed by investigations of Win and Stevens [25] for the enzyme separated from *Absidia coerulea* and excreted by *Colletotrichum lindemuthianum* ATCC 56676. The preliminary (faster) phase of deacetylation last about half an hour while in the second phase of process a slow increase in the concentration of liberated acetic acid was observed for up to 10 hours of the process. Win et Stevens noticed that deacetylation levelled off when 5% of the acetyl groups were liberated. They explained this observation by deacetylatin of external acetyl groups accessible for the enzyme first and next a much slower process when the internal acetyl groups were deacetylated. The internal acetyl groups were much less accessible for the enzyme due to the very firm crystallographic form of chitin.

The authors did not suggest any mechanism for the process.

Contrary to enzymatic deacetylation of chitin, it was suggested that the deacetylation of chitosan follows the Michaelis-Menten mechanism [26]. They investigated the chitosans with a different acetylation degree in the range of 8% to 62%. They also found that the relative rate of reaction (relative to a rate for chitosan with acetylation degree 8%) is in linear relationship with acetylation degree.

The same mechanism was proposed for chitooligosaccharides, Table 1.

Table 1. The constants of Michaelis-Menten equation of enzymatic deacetylation by chitin deacetylase.

Origin	Substrate	K_M	V_{max}	Ref.
Colletotrichum lindemuthianum ATCC 56676*	$(GlcNAc)_2$	18.4 mM	16.2**	[7]
	$(GlcNAc)_3$	11 mM	11.3**	
	$(GlcNAc)_4$	0.6 mM	184**	
	$(GlcNAc)_5$	0.4 mM	158**	
Colletotrichum lindemuthianum ATCC 56676*	$(GlcNAc)_4$	81.6 μM	–	[27]
	$(GlcNAc)_5$	69.9 μM	–	
Mucor rouxii ATCC 24905	Chitosan AD 8%	2.1 mg/mL	–	[26]
	Chitosan AD 35%	1.7 mg/mL	–	
	Chitosan Ad 62%	2.1 mg/ mL	–	

* – extracellular chitin deacetylase,
** – the reaction rate express as [μM of GlcN/ (min*mg protein)]

Martinou, Kafetzopoulos and Bouriotis [9] and Tokuyasu et al. [27] evaluated the K_M constant on the basis of Lineweaver-Burk

Contrary to these observations, Dunkel and Knorr [28] and Jaworska and Konieczna [29] suggested some deviations from the Michaelis-Menten mechanism for chitosan deacetylation. Dunkel and Knorr observed for the enzyme from *Mucor rouxii* DSM 1191 a linear increase of concentration of acetic acid with time

of reaction during the first 60 min. (chitosan with acetylation degree 50%). The kinetics differed significantly from the Michaelis-Menten mechanism.

Investigations by Jaworska and Konieczna for the enzyme from *Absidia orchidis vel coerulea* NCAIM F0642 [29] suggested a more complex mechanism. Data presented in the form of Lineveawer-Burk plot did not give the linear relationship, but linearity was obtained for the plot of 1/(reaction rate) *vs* 1/(concentration)^2. This suggests that chitin deacetylase from *Absidia orchidis vel coerulea* NCAIM F 0642 can operate with two subunits and with positive cooperation between them. This mechanism is possible when you take into account the data for molecular weights of chitin deacetylases: they were reported to be about 75–80 kDa but also about 33–35 kDa, half of the higher value – probably a value of a single unit of chitin deacetylase.

The kinetics of chitin and chitosan deacetylation is not satisfactory explained so far and should be the subject of further investigation.

ENZYMATIC DEACETYLATION OF CHITIN AND CHITOSAN

The process of enzymatic deacetylation of chitin was worked out by Win and Stevens [22]. They checked several physical and chemical factors influencing the deacetylation process and finally found that chitin should be ground down to a particle size of around 75μm (before or after deacetylation). Next, the super fine (SF) chitin was obtained by dissolving in methanol saturated with calcium chloride dihydrate ($CaCl_2 \times 2H_2O$, 83% w/v) and after precipitation was next dissolved in formic acid (18%). Dissolved chitin was adjusted to the optimal pH for enzyme, and finally deacetylated by chitin deacetylase from *Absidia coerulea* at optimal conditions. As a result of enzymatic deacetylation, a decrease in the acetylation degree from 85% to 10–20% was observed. They also observed the reduction of viscosity of the solution to 5% of the initial value.

A method for enzymatic deacetylation of chitosan proposed by Marinou, Kafetzopoulos, Bouriotis [9] was tested only on a laboratory scale (1.0 mL of chitosan sample), but they reported that chitosan was deacetylated from 42% to 2% during 13 hours and from 28% to 3% during 6 hours. The process was carried out in a glutamate buffer (pH 4.5) at 50°C. They also suggested the use of dialysis membrane tubes (cut-off 10 kDa) for the deacetylation process to prevent the inhibition of the chitin deacetylase by the acetic acid, a product of the reaction [30].

Slightly poorer results were presented by Rzodkiewicz, Kolodziejska and Wojtasz-Pajak [10]. They reported deacetylation of chitosan from acetylation degree of 32% to 18% in glutamate-HCl buffer (pH 4.0) at 50°C or in Tris-HCl buffer (pH 5.8) at the same temperature during 24 hours of incubation. They also observed the decrease of viscosity of chitosan solution during continuation of the process.

A deacrease of the viscosity of the reaction mixture was reported, for chitin as well as for chitosan. None of the authors explained the nature of this process: decrease of acetylation degree, hydrolysis of chitosan chains by chitosanolytic enzymes or by the action of buffer, aging of chitosan solution, increase of solubility of the polymer

CONCLUSIONS

Chitin deacetylase is an enzyme presented mainly in the mycelium of fungi belonging to Zygomycetes and after separation can be used for enzymatic deacetylation of chitin or chitosan. A significant majority of the papers presented so far are focused on the separation methods and characterisation of chitin deacetylase from different origins. There are only few papers presenting the kinetics of chitin or chitosan deacetylation and only on a small laboratory scale. Among them the method of chitin deacetylation proposed by Win and Stevens [25] confirm that chitin deacetylase is a useful tool for laboratory scale and that the process can be scaled up to an industrial scale.

Enzymatic deacetylation of chitosan was investigated only on a small laboratory scale, although this process can play a more important role in the future. Loosening of chitin crystallographic structure due to chemical treatment in the process of chemical deacetylation makes the acetyl groups more accessible for the enzyme. The process of production of chitosan with very low acetylation degree (close to 0%) and medium or high molecular weight can be planned to follow two consecutive steps: chemical and enzymatic deacetylation.

However, more investigations should be done before the process will be ready for scaling-up to an industrial level.

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TRIMETHYLSILYLATION OF CHITIN AND CHITOSAN AND EVALUATION OF THE PRODUCTS AS PRECURSORS FOR MODIFICATION REACTIONS

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Key words: trimethylsilylation, desilylation, modification reaction, solubilization, molecular weight, acetylation

INTRODUCTION

Of many kinds of polysaccharides, chitin and chitosan are particularly interesting because of their unique physicochemical and biological properties [1–3]. The distinguishing characteristics are closely related to the amino groups of these amino polysaccharides that are structurally similar to a glucan counterpart, cellulose, having only hydroxy groups. Chitin and chitosan are therefore specialty biopolymers in view of their high potential in utilization in various fields. Furthermore, the presence of the amino groups at the C-2 position would be advantageous in structural modifications by chemical reactions that will make possible development of new types of materials with advanced functions [4]. Special emphasis has thus been put on the chemical modifications of these amino polysaccharides to explore their full potential.

The reactions of chitin and chitosan are, however, generally difficult due to the lack of solubility in ordinary solvents suitable for conducting modification reactions. Although a wide range of chemical modifications of chitin and chitosan have been attempted thus far, the reactions under heterogeneous conditions are usually accompanied by serious problems associated with the poor extent of reaction, difficulty in regioselective substitution, structural ununiformity of the products, and partial degradation under harsh reaction conditions.

In our preliminary study, chitin was found to be trimethylsilylated to give an organosoluble derivative, 3,6-*O*-bis (trimethylsilyl) chitin [5]. Chitosan also underwent trimethylsilylation resulting in the improved affinity for solvents [6, 7]. Trimethylsilylation of chitin and chitosan was therefore studied in detail to

establish efficient and reliable procedures and to elucidate the influences on the solubility, molecular weight, and reactivity of the resulting derivatives.

EXPERIMENTAL

Materials

α-Chitin and β-chitin were isolated from shrimp shells [8] and squid pens [9] by the reported methods as white powdery and fibrous materials. Chemicals were of reagent grade and used as received.

Trimethylsilylation of chitin

Hexamethyldisilazane and chlorotrimethylsilane (10 equivalents each to pyranose) were added to a suspension of chitin in pyridine, and the mixture was stirred at room temperature or at 70°C. After the reaction, the mixture was added to acetone. The acetone extract was concentrated and poured into water to give silylated chitin as a white fibrous material.

Modifications of silylated chitin

Silylated chitin was dissolved in pyridine, and triphenylmethyl (trityl) chloride (10 equivalents to pyranose) or acetic anhydride (100 equivalents to pyranose) was added. The reactions proceeded in homogeneous solution, and the tritylated or acetylated product was precipitated in water.

Trimethylsilylation of chitosan

Chitosan was dispersed in pyridine, quinoline, or sulfolane, and hexamethyldisilazane and chlorotrimethylsilane were added. The mixture was stirred in the presence or absence of 4-dimethylaminopyridine and poured into acetone to isolate silylated chitosan.

Acetylation of silylated chitosan

To a dispersion of silylated chitosan in pyridine was added acetic anhydride. After stirring at room temperature for a given period, the mixture was added dropwise to ice water to give a fibrous precipitate.

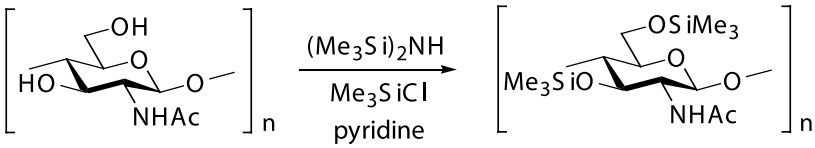
RESULTS AND DISCUSSION

Trimethylsilylation of chitin

Chitin was once reported to be trimethylsilylated with hexamethyldisilazane in formamide at 70°C, and partial substitution (ds 0.6) was attained under the conditions where cellulose was fully substituted [10], indicating the poor reactivity of chitin. In order to study trimethylsilylation behavior, both shrimp α-chitin and squid β-chitin were subjected to the reaction with a mixture of hexamethyldisilazane and chlorotrimethylsilane in pyridine (Scheme 1).

When the reaction was carried out at room temperature, silylation was slow, and no distinctive difference in the extent of substitution was observed between α- and β-chitins as summarized in Table 1. At 70°C, however, the reaction was quite facile. α-Chitin was silylated steadily, and the degree of substitution (ds)

was 0.99 in the reaction of 24 h. After 96 h, a fully silylated product was obtained. With β-chitin as a starting material, the substitution was much more easy as expected, and full silylation at OH could be accomplished in 16 h.



Scheme 1

Table 1. Trimethylsilylation of chitin[a]

chitin	temperature (°C)	time (h)	ds for TMS[b]
shrimp α-chitin	r. t.	24	0.16
shrimp α-chitin	r. t.	48	0.39
squid β-chitin	r. t.	24	0.16
squid β-chitin	r. t.	48	0.32
shrimp α-chitin	70	24	0.99
shrimp α-chitin	70	48	1.45
shrimp α-chitin	70	96	2.04
squid β-chitin	70	7	1.61
squid β-chitin	70	16	2.06

[a] With hexamethyldisilazane and chlorotrimethylsilane (10 equivalents each to pyranose) in pyridine
[b] Degree of substitution for the trimethylsilyl group per pyranose unit determined from the C/N value of elemental analysis

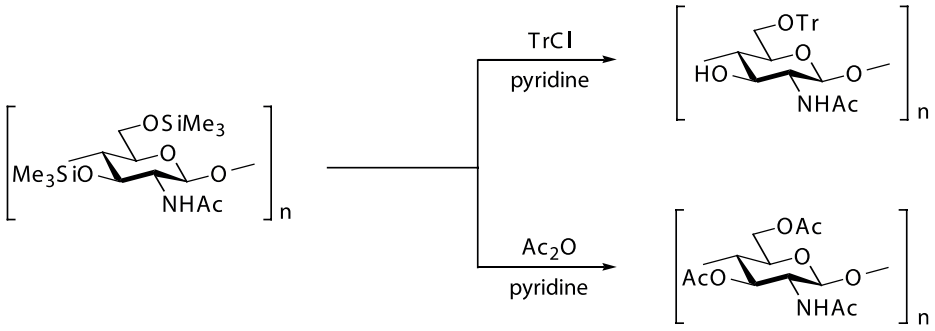
The resulting trimethylsilylated chitin showed a highly improved affinity for organic solvents and was soluble in acetone and pyridine when the ds was above 1.5. It was almost soluble in dimethyl sulfoxide and *N*,*N*-dimethylacetamide, leaving small amounts of highly swollen transparent gels. Although the silylated product was reasonably stable in neutral water and aqueous alkali, the *O*-trimethylsilyl group was rather labile under acidic conditions and removed readily in 10% aqueous acetic acid at room temperature to regenerate chitin.

Protection reactions often degrade the polymer main chain considerably, and thus the influence of silylation on the molecular weight was elucidated by comparing the molecular weight of the original chitin and that of the regenerated chitin prepared via silylation and desilylation. The viscosity data as well as gel permeation chromatography data supported that the chitin main chain was not hydrolyzed significantly during the silylation and desilylation process.

Tritylation and acetylation of silylated chitin

The silylated chitin was soluble in pyridine and thus expected to undergo various modification reactions efficiently in solution under mild conditions. Though chitin was rather resistive toward tritylation with trityl chloride even at elevated temperatures, silylated chitin gave rise to 6-*O*-tritylchitin with ds 1.0 at 90°C in 24 h (Scheme 2). The tritylated chitin was soluble in polar organic solvents such as pyridine, dimethyl sulfoxide, and *N*,*N*-dimethylacetamide.

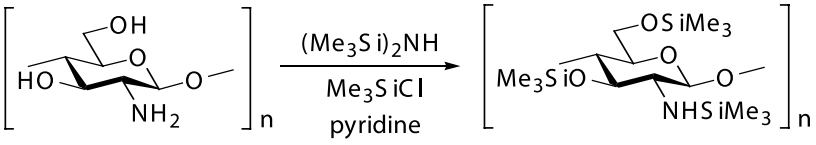
Acetylation with acetic anhydride also proceeded easily with silylated chitin in pyridine. In the 24 h reaction at room temperature, the degree of *O*-acetylation was 1.77, while the ds values were 0.22 and 0.85 with α- and β-chitins. The ds became 2.0 in 8 h with silylated chitin in the presence of 4-dimethylaminopyridine as the catalyst (Scheme 2). The acetylated product was soluble in pyridine and dimethyl sulfoxide, and highly swelled in *N*,*N*-dimethylacetamide.



Scheme 2

Trimethylsilylation of chitosan

Chitosan was similarly treated with a mixture of hexamethyldisilazane and chlorotrimethylsilane in pyridine. It was, however, somewhat less reactive than chitin and required more harsh reaction conditions. Compared to the chitosan prepared from α-chitin (chitosan (α)), that from β-chitin (chitosan (β)) exhibited a slightly enhanced reactivity at room temperature, but similar ds values could be achieved at elevated temperatures (Scheme 3). As the catalyst, 4-diemthylaminopyridine was effective, though only to a limited degree, and silylated derivatives with a ds up to 2.8–2.9 could be obtained under appropriate conditions as listed in Table 2. Quinoline and sulfolane were less suitable as a solvent than pyridine.



Scheme 3

The introduction of the trimethylsilyl group into chitosan enhanced affinity for organic solvents markedly, and the resulting silylated chitosan was almost soluble in pyridine. It swelled considerably in various solvents such as acetone, tetrahydrofuran, dimethyl sulfoxide, and *N*,*N*-dimethylacetamide. The trimethylsilyl group could be removed easily with aqueous acid as in the case of silylated chitin.

Table 2. Trimethylsilylation of chtiosan[a]

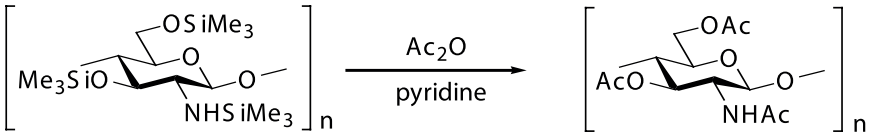
chitosan[b]	solvent	DMAP/ pyranose (mol/mol)[c]	temperature (°C)	time (h)	ds for TMS[d]
chitosan (α)	pyridine	–	rt	24	0.19
chitosan (α)	pyridine	–	100	24	2.50
chitosan (β)	pyridine	–	rt	24	0.49
chitosan (β)	pyridine	–	100	24	2.64
chitosan (β)	pyridine	–	100	48	2.69
chitosan (β)	pyridine	5	100	48	2.82
chitosan (β)	quinoline	5	120	72	2.46
chitosan (β)	sulfolane	5	100	72	0.08

[a] Silylating agents, 10 equivalents
[b] Chitosan (*α*), chitosan prepared from *α*-chitin; chitosan (*β*), chitosan prepared from *β*-chitin
[c] DMAP, 4-dimethylaminopyridine as the catalyst
[d] Degree of substitution for the trimethylsilyl group calculated from the C/N value of elemental analysis

Acetylation of silylated chitosan

The resulting silylated chitosan was then evaluated as a precursor for modification reactions. As a typical example, acetylation was examined in detail in pyridine. On treatment with acetic anhydride at room temperature, silylated chitosan gave highly acetylated products (Scheme 4), and the reaction proceeded facilely compared to that of the original chitosan. 4-Dimethylaminopyridine was effective as the catalyst as in the acetylation of silylated chitin.

In addition to *O*-acetylation, *N*,*N*-diacetylation appeared to take place with silylated chitosan to some extent even under such mild conditions as evidenced in Table 3. Silylated chitosan was thus confirmed to exhibit a considerable reactivity in pyridine and to be useful as a precursor for conducting the reactions in organic solvents.



Scheme 4

Table 3. Acetylation of trimethylsilylated chitosan[a]

chitosan[b]	temperature	time (h)	ds for Ac[c]
chitosan (α)	rt	48	1.53
chitosan (β)	rt	48	2.22
TMS-chitosan	rt	48	2.55
TMS-chitosan	rt	6	2.85
TMS-chitosan	rt	16	3.05
TMS-chitosan	rt	48	3.16

[a] Acetic anhydride, 100 equivalents to pyranose; catalyst, 4-dimethylaminopyridine
[b] Chitosan (*α*), chitosan prepared from *α*-chitin; chitosan (*β*), chitosan prepared from *β*-chitin; TMS-chitosan, trimethylsilylated chitosan
[c] Degree of substitution for the acetyl group calculated from the C/N value of elemental analysis

CONCLUSIONS

Trimethylsilylation of chitin and chitosan proceeded smoothly with hexamethyldisilazane and chlorotrimethylsilane in pyridine, and chitin was more readily silylated than chitosan. β-Chitin and the derived chitosan were more reactive than ordinary α-chitin and the derived chitosan. The resulting silylated derivatives exhibited high affinity for organic solvents, especially in pyridine that is suitable as a solvent for modification reactions. They were subjected to some reactions including tritylation and acetylation and have proved to have high potential as precursors for modification reactions of chitin and chitosan under mild conditions.

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A NOVEL SOLVENT SYSTEM FOR CHITOSAN

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Abstract: Chitosan solvents are conventionally dilute aqueous solutions of either organic or inorganic acids such as acetic, lactic, hydrochloric or perchloric acid. The use of such solvents has a number of drawbacks including gradual degradation of the polymer chain through acid hydrolysis and, since the chitosan exists in solution in these solvents as a cationic polyelectrolyte, incompatibility with anionic polyelectrolytes, a polymer class that includes many biologically important polysaccharides. The current paper reports on a new solvent system in which the chitosan is solubilised through formation of an anionic condensation derivative. Although this derivative is stable enough to be isolated and characterised, the formation step is reversible so that the chitosan may be regenerated if required. As the solvent system has a neutral pH the chitosan does not undergo hydrolysis on standing, and since the dissolved chitosan carries a negative charge it is fully compatible with anionic polyelectrolytes such as sodium alginate and carragheenan.

Key words: Chitosan, solvent, sodium formaldehyde bisulphite, anionic derivative, polyelectrolyte blends

INTRODUCTION

The most frequently used solvent systems for chitosan are dilute aqueous acid solutions, acetic acid being the most common [1]. In such systems the chitosan interacts with the solvent, forming cationic groups along the polymer chain through protonation of the amine groups. Although this cationic character is important in a number of proposed applications for chitosan, in many cases the uncharged chitosan is required and this can be readily regenerated by treatment of the solid chitosan acid salt with alkali. In addition chitosan has been reported as being soluble in DMF-N_2O_4 [2] in which medium it is presumably present as a non-ionic polymer.

Recently a new aqueous solvent system for chitosan has been reported [3] in which the chitosan chains are solubilised through formation of temporary anionic groups along the chain. Thus the chitosan is present in solution as an anionic polyelectrolyte, rather than as a cationic polyelectrolyte as is the case in aqueous acid systems. This new solvent system offers a number of benefits and opportunities in comparison with the traditional acid-based solvent systems.

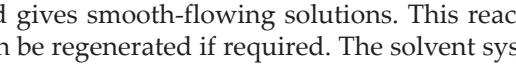
RESULTS AND DISCUSSION

Preparation of chitosan solutions

The solvent system is aqueous sodium formaldehyde bisulphite ($HOCH_2SO_3Na$) formed by the reversible addition reaction between formaldehyde and sodium metabisulphite:

$$2CH_2O + Na_2S_2O_5 + H_2O \leftrightarrow 2\ HOCH_2SO_3Na$$

The amine groups on the chitosan chains react with the sodium formaldehyde bisulphite, forming the condensation product sodium *N*-(sulphonomethyl)chito san:



$$\text{Chit-}NH_2 + HOCH_2SO_3Na \leftrightarrow \text{Chit-}NHCH_2SO_3Na + H_2O$$

which is water-soluble and gives smooth-flowing solutions. This reaction is reversible and the chitosan can be regenerated if required. The solvent system is non-degradative, unlike aqueous acid solvent systems, and solutions of chitosan prepared using sodium formaldehyde bisulphite are stable and do not undergo chain hydrolysis even on standing for prolonged periods.

There are several routes for the preparation of solutions of sodium *N*-(sulph onomethyl)chitosan.

a) Stirring chitosan flakes/particles in water containing sodium formaldehyde bisulphite; typically 1 g of chitosan in 100 ml water containing 2 g $HOCH_2SO_3Na$ will give a solution within 6–8 hours, although low molecular weight chitosan will dissolve up more rapidly.
b) The sodium *N*-(sulphonomethyl)chitosan may be isolated as a stable solid by precipitation on addition of a solution, prepared as described above in (a), to acetone or other water-miscible non-solvent. The precipitate, after washing with aqueous acetone, may be dried and stored until required, when it may by dissolved by addition to water at neutral pH.
c) Solid sodium *N*-(sulphonomethyl)chitosan may be prepared directly by reacting chitosan with sodium formaldehyde bisulphite under heterogeneous conditions, typically by stirring chitosan particles in 60% (v/v) aqueous methanol containing sodium formaldehyde bisulphite, followed by rinsing with aqueous methanol to remove excess sodium formaldehyde bisulphite, and drying.

The solid sodium *N*-(sulphonomethyl)chitosan prepared as described in b) or c) above has a shelf life in excess of 3 years when stored at room temperature.

Solubility *versus* F_A relationships

The range of F_A values over which solubility could be achieved was determined for two sets of chitosan samples using the standard conditions of 1 g chitosan/2 g sodium formaldehyde bisulphite/100 ml distilled water. The mixture was stirred overnight at room temperature and the solution quality was assessed visually using crossed polarising sheets. The first set of chitosan samples was prepared by heterogeneous deacetylation of crab chitin and covered the range $\sim 0.0 < F_A < 0.51$ as determined by dye adsorption [4]. The second set, which cov-

ered the range ~0.09 < F_A < 0.80, was prepared by homogeneous *N*-acetylation in 0.1 M acetic acid/methanol of a chitosan[0.09] sample previously prepared by heterogeneous deacetylation of shrimp chitin. This technique of re-*N*-acetylation has been shown to affect only the F_A value and to have no effect on the chain length of the chitosan molecules [5].

The results show (Table 1) that for heterogeneously deacetylated chitosan samples the maximum F_A value allowable is 0.40–0.45 if good solutions are to be obtained in the solvent system:

Table 1. Solubility *versus* F_A for heterogeneously prepared samples.

F_A	Solubility
~0.00	Soluble – clear solution
0.15	Soluble – clear solution
0.26	Soluble – clear solution
0.32	Soluble – clear solution
0.40	Soluble – clear solution
0.45	Soluble – some swollen gel particles
0.51	Insoluble – particles only partially swollen

whereas for homogeneously prepared *N*-acetylchitosans (Table 2) the upper limit of F_A values giving good solutions is 0.70–0.75.

Table 2. Solubility *versus* F_A for homogeneously prepared *N*-acetylchitosans.

F_A	Solubility
0.09	Soluble – clear solution
0.18	Soluble – clear solution
0.33	Soluble – clear solution
0.48	Soluble – clear solution
0.58	Soluble – clear solution
0.70	Soluble – clear solution
0.73	Soluble – some swollen gel particles
0.80	Insoluble – particles only partially swollen

Similar results for solubility as a function of F_A were found for both the heterogeneously and the homogeneously prepared samples using 0.1 M acetic acid as solvent.

The greater solubility range for the *N*-acetylchitosan samples is to be expected, due to their greater uniformity of structure. However it is likely that the greatest F_A value at which solubility is obtained will depend to some extent on the initial F_A value of the chitosan that is homogeneously *N*-acetylated. Such a dependence was found [6] for the extent of swelling of chitosan[0.5] samples in water at neutral pH, and a similar trend would be expected for solubility in either aqueous sodium formaldehyde bisulphite or aqueous acetic acid. Furthermore the maxi-

mum F_A values permitting solubility for both types of chitosan materials may increase with decrease in the molecular weight of the sample, in accordance with the general principle that the solubility of a polymer increases with decrease in molecular weight.

Structure of the water-soluble anionic complex

Since the sodium *N*-(sulphonomethyl) group is very hydrophilic it was initially thought likely that aqueous solubility would be attained at a relatively low DS value – where DS is defined as the mol fraction of amine groups that have reacted with sodium formaldehyde bisulphite. Two methods were used to determine DS values for several samples. The first was a gravimetric method [7] based on the weight loss of a sample on hydrolysis in 2 N H_2SO_4, and the second was a modified version [7] of the standard protocol for analysis of chitin and chitosan by dye adsorption [4]. Both methods of analysis were in agreement and indicated that a DS of ~ 1.0, that is reaction of almost 100% of the available primary amine groups, is necessary for aqueous solubility. Although disubstitution of the amine groups is theoretically possible;

$$\text{Chit-NH-CH}_2\text{SO}_3\text{Na} + \text{HOCH}_2\text{SO}_3\text{Na} \leftrightarrow \text{Chit-N(CH}_2\text{SO}_3\text{Na)}_2 + \text{H}_2\text{O}$$

no evidence of this was found with any of the samples analysed.

This requirement of a very high DS value for water-solubility appears strange at first when compared with the DS values of ~0.5 required for water-solubility in *N*-carboxymethylchitosan [8], *N*-carboxyethylchitosan [9] or carboxymethylcellulose [10]. However the first two are prepared under homogeneous conditions which allow random distribution of the solubilising groups along the chain, whilst carboxymethylcellulose is prepared heterogeneously but under strongly alkaline conditions that induce extensive swelling of the cellulose, thereby disrupting the crystalline regions and enabling more uniform substitution. In comparison the reaction between chitosan and sodium formaldehyde bisulphite occurs heterogeneously and under conditions that do not disrupt the residual crystalline regions thus a more highly substituted derivative is required for solubility.

Solution properties

Much of the behaviour of solutions of chitosan in aqueous sodium formaldehyde bisulphite is analogous to that of solutions of chitosan in dilute aqueous acid, but there are also a number of interesting differences.

1. The solutions are smooth flowing and do not exhibit signs of the structure that is a characteristic frequently observed with solutions of chitosan in dilute organic acids. The solvent is non-degrading and no decrease in viscosity was observed in a 1% solution after standing for 15 days at 25°C. The solutions are of lower bulk viscosity than the equivalent solutions of chitosan in 0.1 M acetic acid. Also the Limiting Viscosity Number values measured in 0.05 M NaCl are lower than those obtained in 0.1 M acetic acid/0.2 M NaCl [7].

2. The solutions are stable to increases in temperature and show no change on cooling back to room temperature after being held at 95–98°C for 15 minutes.

3. The solutions, once prepared, show good tolerance for polar, water-miscible solvents such as methanol, ethanol, *iso*propanol and *tert*.butanol. For example a 1% solution can be diluted with 2.5 volumes of these alcohols without formation of a gel or precipitation of the polymer. When mixing the aqueous solution with an alcohol it is essential to add the alcohol to the solution in portions, while stirring well to ensure rapid mixing.
4. Addition of acetic anhydride to a solution diluted with methanol causes gelation in a similar manner to the formation of "Hirano-type" gels [11]. However the isolated gel is not soluble in water, suggesting that the sodium *N*-(sulphonomethyl) groups are removed during the *N*-acetylation reaction.
5. The aqueous solutions form gels on addition of glutaraldehyde, in a similar manner to that of chitosan solutions in dilute aqueous acids, and films cast from solution can be insolubilised by treatment with glutaraldehyde [12]. Presumably the reaction involves loss of the sodium *N*-(sulphonomethyl) groups from any of the amine groups involved in reaction with the aldehyde groups to form a Schiff's base structure. However, since gel formation requires only a small percentage of the amine groups to react this does not have any major effect on the "solubility" of the segments of polymer chain situated between any two crosslinking points.
6. As would be expected the solutions are compatible with anionic and nonionic surfactants but the addition of a cationic surfactant causes formation of an insoluble complex between the anionic polyelectrolyte and the surfactant cation. Addition of up to 5% (wt./vol) NaCl, Na_2SO_4 or $CaCl_2$ does not bring about precipitation.

Preparation of homogeneous chitosan/anionic polyelectrolyte blends

Chitosan has a number of interesting properties including bioactivity, biocompatibility, biodegradability and non-toxicity [13] together with bactericidal and fungicidal properties [14]. Because of these properties chitosan is increasingly being investigated for use in a range of biomedical and related applications. Other natural polyelectrolytes such as sodium alginate, carrageenan, heparin and hyaluronic acid are also known to have important medical effects, and synergistic effects would be expected from blends of chitosan with these materials. However all of these other natural polyelectrolytes are anionic in character, and on mixing a solution of chitosan in dilute aqueous acid with a solution of an anionic polyelectrolyte there is rapid formation of a precipitate through charge neutralisation. These precipitates are polyelectrolyte complexes (PECs) and although many chitosan/anionic PECs have been shown to be bioactive and bioresorbable [15], their intractable and infusible nature makes it difficult to convert them into useful forms such as fibres, films or sponges.

However chitosan is an anionic polyelectrolyte when dissolved in aqueous sodium formaldehyde bisulphite and hence is compatible in solution with other anionic polyelectrolytes. The homogeneous blend solutions can be cast into films, spun into fibres, or formed into sponges, after which the chitosan can be regenerated from the sodium *N*-(sulphonomethyl)chitosan component of the blend. At this stage the blend can be used as a chitosan/anionic polyelectrolyte blend or converted to the polyelectrolyte complex by a mild acid treatment. Furthermore

solutions of sodium *N*-(sulphonomethyl)chitosan can be readily mixed with solutions of carrageenan or pectin at temperatures above their gelation point, to give homogeneous blend gels on cooling. The physical structure of the gel can be maintained during the chitosan regeneration step, leaving a gel with chitosan in molecular form uniformly distributed throughout it.

There are two procedures reported in the literature that are claimed to be suitable for the preparation of chitosan/sodium alginate blends [16, 17]. However both procedures use chitosan in the form of a cationic polyelectrolyte, in which form it interacts rapidly with the sodium alginate to give an insoluble polyelectrolyte complex. Thus the products obtained following these prior procedures are heterogeneous blends of the two components in which there is either no continuous phase or one of the components forms the continuous phase with the second component present in the form of particles suspended in this continuous phase. A layer of polyelectrolyte complex is formed at each of the interfaces between the two components. The particle size depends on the severity of the mixing treatment, while which component forms the continuous phase depends, *inter alia*, on the relative weight proportions of each. This is in contrast to the homogeneous blends produced using the anionic polyelectrolyte derivative, sodium *N*-(sulpho nomethyl)chitosan, rather than the cationic polyelectrolyte form produced in the presence of an acid. Films cast from *N*-(sulphonomethyl)chitosan/anionic polyelectrolyte mixtures are clear and visually uniform, whereas those cast using the processes described previously in the literature [16, 17] are opaque or have a mosaic-like appearance. The difference in appearance is clearly discernible with the naked eye and is clear evidence of the non-uniform structure of the latter films.

These homogeneous blends represent a new range of materials with considerable potential for medical and pharmaceutical applications because of the likely synergistic effects arising from the intimate combination of two or more bioactive materials. The major areas in which these blend materials have potential are advanced wound care dressings, "artificial" skin for treatment of burns, medical sutures, non-thrombogenic bio-materials, tissue engineering, and drug delivery systems including micro-encapsulation for transmucal and subcutaneous systems and films for transdermal delivery. Another area of application is in the production of membranes for separation processes. Because of its excellent film-forming characteristics the use of chitosan as a membrane in separation processes has been investigated, principally by Uragami [18]. The new solvent offers a simple method for producing membranes from chitosan/anionic polyelectrolyte blends, or from the polyelectrolyte complexes formed from these blends, that may have a number of applications in separation processes .

Other applications of the solvent system

The new solvent system may be used to prepare fibres, films and sponges from chitosan alone, as well as producing them from chitosan/anionic polyelectrolyte blends. As different conditions would be required for the regeneration step it is likely that the physical structures of the products would differ from those obtained using the conventional acid solvent systems, and this should modify their physical properties.

The new solvent system also offers a possible route for chitosan production from crustacean shell that is more environmentally friendly than that currently used. Crustacean shells, from whatever species, contain large quantities of mineral salts, principally $CaCO_3$. Conventionally this is removed by dissolving in dilute acid, typically 2N HCl, leading to the evolution of large volumes of CO_2 and the production of large quantities of $CaCl_2$ solution that give rise to effluent treatment costs in addition to the cost of the HCl. There is also the risk of possible acid hydrolysis of the chitin. An alternative processing route would be to carry out the deacetylation step on the chitin/$CaCO_3$ matrix and then extract the chitosan produced with a solution of sodium formaldehyde bisulphite. Following removal of the $CaCO_3$ by filtration the solution may be used directly to form blends with anionic polyelectrolyte, as described above, or the sodium *N*-(sulphonomethyl) chitosan isolated by precipitation or spray drying, or the chitosan regenerated and isolated.

Finally, because of the quality of the solutions produced, the non-degradative nature of the solvent system, and the fact that the chitosan is present in solution as an anionic polyelectrolyte, aqueous sodium formaldehyde bisulphite offers an interesting alternative to acid-based solvents for characterisation of chitosan samples.

CONCLUSIONS

Aqueous solutions of sodium formaldehyde bisulphite may be used to dissolve chitosans having $F_A < \sim 0.45$ for heterogeneously prepared chitosans, or $F_A < \sim 0.75$ for homogeneously prepared *N*-acetylchitosans, through formation of the sodium *N*-(sulphonomethyl) derivative. The reaction between chitosan and sodium formaldehyde bisulphite is reversible so that the chitosan may be regenerated by mild acid treatment. The solutions are stable at neutral pH and may be used to produce homogeneous blend solutions with anionic polyelectrolytes such as sodium alginate, hyaluronic acid, carrageenan or sodium poly(acrylate). These blend solutions may be readily converted into fibres, films or sponges by normal techniques. Other possible applications for the new solvent system include production of fibres, films or sponges from chitosan itself, the extraction of chitosan from a chitosan/[$CaCO_3 + Ca(OH)_2$] matrix, and molecular weight determinations of chitosan.

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THEORETICAL AND PRACTICAL ASPECTS OF BIOLOGICAL ACTIVITY OF CHITIN DERIVATIVES IN AGRICULTURE

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Key words: chitin derivatives, biological activity, agriculture, effectiveness, costs, plant species, type of pathogen, properties of chitin derivatives

INTRODUCTION

Chitin derivatives such as chitin oligomers, chitosan, chitosan oligomers and its chemical modification show very wide biological activity. Wide biological activity, biocompatibility, biodegradability and harmlessness for environment are great advantages of chitin derivatives for their use in agriculture. Various biological activity of chitin derivatives in plant production can manifested by:

1. induction of plant resistance to various pathogens
2. inhibition of the growth of microorganisms
3. stimulation of seeds germination
4. stimulation of plant growth
5. increasing of plant yield and quality
6. antinematode activity
7. suppression of heavy metal plant stress
8. preservation of harvested vegetables and fruit
9. improving quality of soil and water
10. increasing number of nitrogen fixation nodules and stimulation of nitrogen fixation by nodules
11. reduction of the amounts of fertilizers

Chitin derivatives due its biocompatibility may be used in various way:

– foliar spraying
– seed coating or dressing
– soil treatment
– supplementation of the nutrient solution in hydroponic system
– fruit and vegetables coating
– seedling roots dipping

Biodegradability and harmlessness for environment predispose these natural products to application in the production of vegetables and organic agriculture. In spite of all these advantages the use of chitin derivatives in agriculture is still

limited. Efficiency of chitin derivatives very often significantly varied, especially under field condition and may lead to many disappointments. This presentation focus on the some factors influencing biological activity of chitin derivatives used in plant production.

Biological activity of chitin derivatives is influenced by many different factors and exact an recognition of them allow for effective application of chitin derivatives in agriculture.

KNOWLEDGE OF FARMERS ABOUT THE NATURE OF CHITIN DERIVATIVES

In agriculture chitin derivatives, mainly chitosan and its oligomers are used at widest scale in plant protection against pathogens.

Farmers expect that chitosan will work as a classical pesticide: quickly and totally.

Chitin derivatives opposite to classical pesticides mainly work by plant metabolism, preparing plant to response more rapidly to pathogen invasion. These substances are less effective at high concentration of pathogen and induced resistance like each resistance can be break down.

Therefore, preparation based on the chitin derivatives should be used first of all preventively, prior to pathogen attack. These natural substances do not eliminate pathogen totally rather limit their occurrence.

Chitin derivatives act on plant unspecificaly and simultaneously stimulate various profitable biological processes (stimulation of the growth, induction of the resistance and others). However, chitin derivatives can not be used uncritically for all plants and for all pathogens. Knowledge of farmers about the nature of chitin derivatives action is one of the most important factor influencing effective used of them in agriculture.

COSTS RELATED TO ELABORATION OF GEN PRODUCTS

The response of plant to the stimulator is characterized by the induction of numerous genes and synthesis their products, what is connected with using plant resources (energy), [1, 2, 3]. Gen products generated during plant stimulation have been referred to the secondary metabolism.

Possibilities of plant are not limited. Under conditions of environmental shortage the competition between primary and secondary plant metabolisms may occur, that may lead to significant decrease of plant respons to chitosan, even inhibition of the growth of plants. At the conditions of shortage of water or nutritional elements , inducer may act as an additional stressing factor.

TYPE, SPECIES AND VARIETY OF PLANTS

Numerous experiments showed that biological activities of chitin derivatives are essentially dependend on plant species and sometimes cultivar and type also. The antifungal activity is more marked in dicotyledones (vegetables) than

in monocotyledones (maize) [4]. Effectiveness of chitin derivatives as stimulators depends at a high extant on plant species [5, 6]. The spectacular example of such situation were cases of bean and tobacco, and *Tobacco mosaic virus*. We showed that chitosan completely protected bean plants against virus while tobacco ones in very limited extent [5]. It may be explain that plant species would have specific receptors for perception of chitosan. It was shown that on plasma membrane surface of the species as soybean, tomato, rice and wheat sites of high affinity to chitin derivatives occurred while in the membranes isolated from cells of tobacco (line BY-2) similar binding sites for chitin derivatives were not find [7, 8, 9]. There were also differences in a sensitivity of plant varieties to chitin derivatives [10]. It was shown that the effects of chito oligosaccharides on seeds germination also depended on plant species. The stimulation of the plant growth by chitosan applied to the soil depended on plant species and concentration of chitosan [11].

CHITIN DERIVATIVES MAY BE HARMFUL FOR PLANT (DIRECTLY AND/OR NON DIRECTLY)

Some responses of plants to chitosan such as formation of physical barriers in xylem vessels which protect plant against pathogen invasion, but this defence reaction may be harmful for plant itself by preventing the transfer of water and nutrients to aerial parts and induces wilting [12]. Resistance of plants induced by chitosan derivatives is non specific for microorganisms and there is a high probability that induced resistance can influence negatively mutualistic organisms also.

Treatments of plants with too high concentration of chitosan or chitosan derivatives are less effective or even harmful for plants [13, 14].

Choice of the optimal chitosan concentration depends on plant species, mode of application and even term of treatment. Examples of the optimal chitosan concentrations for various applications are presented below:

soil treatment	0.1%–0.5%
foliar treatment	0.02%–0.4%
root dipping	0.01%–0.05%
seed coating	0.05%–0.1%
fruit/vegetables coating	1.0%–1.5%
supplementation of nutrient solution in hydroponics system	0.01%–0.02%

For example, it was showed that, under field study, the optimal concentration of chitosan sprayed on *Brassica compestris* ssp. *perkinensis* as a crop protection was 2–4% [14] but potato against *Phytophtora infenstans* the most effectively was proctected at the concentration of 0.02–0.04% [15]. In the case of application of chitosan to the soil the most favorable concentration for rice was 0.5% while lettuce 0.1% [11]. These data showed that any case of chitosan application in plant

production should be examined individually to estimate optimal chitosan concentration.

ENVIRONMENTAL FACTORS

Unfortunately, only non numerous studies documented the influence of environmental factors on effectiveness of chitin derivatives. The biological activity of chitosan applied to plants is realized by their metabolism. It is clear, that plant growing in favorable environment, being in a good conditions would be able to response to chitin derivatives on a high level. For plant stressed by shortage, chitin derivatives my act as the additional stressing factor. Our fragmentary studies showed that temp. 40°C, often occurring in greenhouses in summer decreased effectiveness of chitosan in the induction of plant resistance in 30% [16]. Chitosan used for coating tropical fruit was more effective at 13°C than 4°C [17, 18]. Chitin derivatives added to the soil or the medium rich in organic substances may be less effective because of it absorption by these substances [19].

Similarly, water quality especially content of the substrate absorbing chitin derivatives may influenced their ability to inhibit the growth of fungi [20].

It should be also taken into consideration that in the nature at the same time various factors could stimulate plant metabolism, very often by signaling mutually exclusive pathway. Then beneficial effect of inducer treatment did not appear moreover, could negatively influence condition of plant [21].

It was shown that pH of chitosan solution and media is one of the factors influencing antimicrobial activity of chitosan.

Generally, chitosan is more active at pH 5 or lower.

TYPE OF MICROORGANISMS

Antimicrobial activity of chitin derivatives is realized in two ways: [1] indirectly, via plant metabolism (induction of resistance) and/or [2] directly, by inhibition of microorganisms development.

Both modes of chitin derivatives actions are non specific to various microorganisms and pathogens. Nevertheless, it does not mean that antibacterial activity of chitin derivatives against all microorganisms is manifested in the same extant. Generally, chitin derivatives are more effective against fungi than others type of microorganisms and pathogens [22] and foliar pathogens than ones invading plant systemically. Direct inhibition of the growth of bacteria (fruit/vegetables coated with chitosan) by chitosan is more effective against gram positive than gram negative ones [19, 23].There are known cases when phytopathogenic fungi actively oppose to the induction of plant resistance by chitin derivatives.

In natural conditions plants activate as response to pathogen attack, the synthesis of hydrolytic enzymes that liberate oligosaccharide from fungus chitin. These chitin oligomers induce plant resistance to fungi. Some of pathogenic fungi possess mechanisms which allow them in various way to inhibit induction of resistance by chitin oligomers of phytopathogenic fungi. Fungus *Puccinia graminis* f.sp. *tritici* has had chitin in its cell walls but hyphe penetrating plant cells contained chitosan. This transformation of chitin to chitosan in cell walls of

fungus could protect it against activity of plant chitinase and liberation of chitin oligomers inducing resistance [24]. Fungus *Colletotrichum lindemuthianum* has produced an enzyme chitin deacetylase, which completely deacetylates chitin oligomers liberated during first stages of infection by plant chitinase enzyme. Completely deacetylated chitin oligomers did not have any ability to stimulate of plant resistance [25].

Prophylactic treatment by chitosan prepares plants to the attack of fungi, which possess mechanisms inhibiting induction of resistance.

PROPERTIES OF CHITIN DERIVATIVES

Chitin derivatives, especially chitosan can induce a multitude of biological processes in plant tissue. In addition these polysaccharides directly inhibit the growth of microorganisms. The level of their biological activity depends in considerable extent on its properties such as: charge of molecules, type, forms, degree of polymerization, degree of deacetylation, molecular structure and so on.

Generally, for particular applications, chitin derivatives with various properties are needed.

Charge of molecules

A lot of facts have indicated, that biological activities of chitin derivatives were closely connected with positive charge of molecules.

We showed that the anionic chitosan derivatives such as sulfate-chitosan and carboxymethyl-chitosan did not inhibit the growth of phytopathogenic bacteria and did not induce plant resistance to bacteria infection [16]. Native chitosan more effectively inhibited the growth of fungi in vitro than its amphoteric derivatives such as *N*-carboxymethyl-chitosan, 5-methylpyrrolidinone-chitosan and *N*-methylidenephosphate-chitosan [26]. In others experiments *N*-carboxymethyl-chitosan showed higher antimicrobal activity in vitro than not modified but in growing plants and harvested grain and fruits native chitosan exhibited higher antimicrobial activity than *N*-carboxymethyl-chitosan.

Types and forms of chitin derivatives

Liquid chitosan as an microbal agent or stimulator of plant resistance and growth is more effective than microcrystalline form (suspension) [27]. Solution of chitosan is readily uptaken by plants and microbial cells as compared with slow uptake of the solid form [28, 4]. Powdered chitin or chitosan showed no efficacy in induction of plant resistance as compared with liquid chitosan. Microcrystalline chitosan added to the soil acted slowly but longer than solution of chitosan. The type of acid used in preparation of chitosan solution may influence its biological activity. Chitosan prepared with acetic acid exhibit higher antifungal activity than chitosan prepared with lactic acid [20]. Properties of chitosan prepared with acetic acid were more stable at room temperature than solution of chitosan in lactic acid.

Generally chitosan (polymer) showed higher antibacterial activity than chitosan oligomers [23, 16]. However, both types of chitosan derivatives induced resistance of plant to bacteria and viruses [29].

Search for modifications of such chitin derivatives that guarantee its stable biological activity on the higher level still goes on. *N*-alkyl-chitosan and *N*-aryl-chitosan showed higher activity against fungi than non-modified one [30]. Also salicyden-oligoglucosamine manifested higher antibacterial activity than non-modified chitosan oligomers [31].

Degree of polymerisation and acetylation

A lot of studies showed that such parameters of chitin derivatives as degree of deacetylation (DA) and polymerization (DP) seemed to have significant influence on their effectiveness. Gueddari and Moerschbacher [32], using chitosan oligomers and polymers with known DPs and DAs showed their elicitor activities in wheat strictly depended on DP and DA of chitosan derivatives.They concluded that success in using chitosans is crucially dependent on the use of well characterized chitosans. We have obtained the highest effectiveness of chitosan as an inducer of plant resistance and antimicrobial agent when acetylation degree of the polymer ranged from 25 to 30% and molecular weight was 50–150 KDa. For the lower and higher values effectiveness of chitosan decreased [16]

Fully deacetylated chitin and chitosan oligomers or those hydrolysed to the monomers lacked their biological activities totally [25]. In the case of the induction chitinase, chitosanase and 1,3-glucasanase enzymes was put forward a hipothesis that the stimulation synthesis of these enzymes by chitin oligomers depended on their degree of acetylation, by 5% acetylated chitosan on its molecular size and by 30% acetylated chitosan on combination both factors [33].

CONCLUSIONS

1. Numerous factors influencing biological activities of chitin derivatives undoubtedly limit their application in plant production. An exact recognition of all circumstances influencing biological activities of chitin derivatives allows their effective application.
2. Any case of chitin derivatives application in plant production should be examined individually, taking into consideration plant species, properties of chitin derivatives and various environmental conditions.
3. Chitin derivatives as organic product of wide biological activities and harmless for environmental still have a great chance for wide application in agriculture, especially organic one.

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CHAPTER II

CHARACTERIZATION AND STANDARDIZATION

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PREPARATION AND CHARACTERIZATION OF HEXANOYL CHITOSAN/POLYLACTIDE BLEND FILMS

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Abstract: In the present contribution, blend films of H-chitosan and polylactide (PLA) were prepared by the solution-casting technique. Fourier-transformed infrared spectroscopy results indicated that no significant interaction between H-chitosan and PLA molecules was observed. The thermal degradation behavior of the as-prepared blend films was found to be intermediate to those of the pure components. Only the blend film having the H-chitosan content of 20% exhibited the degradation temperature greater than those of the pure components. All of the blend films exhibited one composition-dependent glass transition temperature, suggesting that partial miscibility between H-chitosan and PLA molecules may be possible in the bulk amorphous phase. The apparent degree of crystallinity calculated from the apparent enthalpy of fusion revealed no significant variation with the blend composition.

Key words: chitosan, hexanoyl chitosan, polylactide, polymer blend

INTRODUCTION

Nowadays, natural polymeric materials have become increasingly important due to their natural abundance and low costs. Chitosan is one of chitin's derivatives, achieved by *N*-deacetylation of chitin though the reaction is never complete [1]. Due to the abundance of hydrophilic functional groups, chitosan is not soluble in most organic solvents. In order to solve this problem, some chemical modifications to introduce hydrophobic nature to chitosan such as phthaloylation, alkylation, and acylation [2] reactions can be done. Organically soluble derivatives of chitosan can be used to formulate by-designed materials for biomedical applications such as polymeric drugs and artificial organs with high specificity and wide applicability. Acylated chitosans are soluble in various organic solvents, such as chloroform, benzene, pyridine, and tetrahydrofuran (THF). *N*-acylchitosan has been fabricated as membranes, fibers, and films. Among the various aliphatic degradable polyesters, polylactide (PLA) has been considered as one of the most interesting and promising biodegradable materials and has been used in medical applications, such as surgical sutures, drug delivery systems, and bone fixtures.

In the present contribution, blend films of hexanoyl chitosan (H-chitosan) and PLA were prepared by the solution-casting technique. Chloroform was used as

the solvent. The effect of blend composition on miscibility, morphology, thermal properties, and mechanical properties was investigated. The main objective of this work was to find an economical way for improving the applicability of H-chitosan through the blending with PLA.

EXPERIMENTAL

Materials

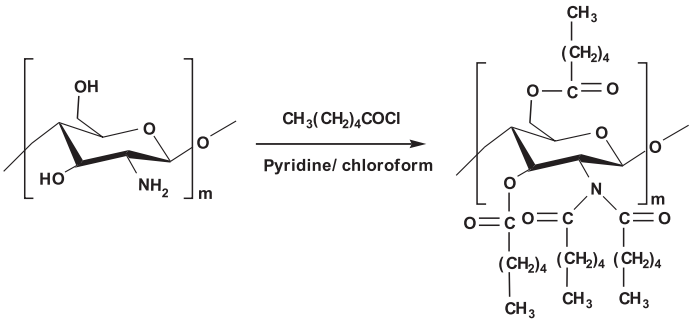
Chitosan having the degree of deacetylation of ca. 91% was prepared from shrimp shells by acid and alkali treatments. Other chemicals used were hexanoyl chloride (Fluka, Switzerland), methanol [Labscan (Asia), Thailand], pyridine (Sigma-Aldrich, USA), and chloroform (Sigma-Aldrich, USA). The other chemicals were analytical grade and were used as-received. PLA was supplied as courtesy from Daiseru Chemicals (Japan). The viscosity-average molecular weight of PLA was ca. 70,000 g·mol^{-1}.

Synthesis and characterization of hexanoyl chitosan (H-chitosan)

H-chitosan was synthesized by reacting chitosan with hexanoyl chloride in a mixture of anhydrous pyridine and chloroform, as shown in Scheme 1. The hexanoylation of chitosan was thoroughly described in an earlier work by Zong et al. [3].

Preparation of blend films

To prepare blend films of H-chitosan and PLA, solutions of H-chitosan and PLA were first separately prepared at the concentration of 1% w/w in chloroform. Slight stirring was used to expedite the dissolution and to homogenize the solutions. Blends films of different compositions (i.e. the weight ratios between H-chitosan and PLA of 100/0, 80/20, 60/40, 50/50, 40/60, 20/80, and 0/100, respectively) were then prepared by casting a mixture of the solutions in a respective weight ratio on a Teflon dish. The casting was let dry at room temperature for one day and later let dry at room temperature under vacuum for another two days.



Scheme 1. Synthesis route for perfect H-chitosan.

Characterization techniques

FT-IR spectroscopy was used to characterize chemical functional groups of as-synthesized H-chitosan and as-prepared blend films. FT-IR spectra were collected on a Bruker Instrument Equinox55 FT-IR spectrometer at a resolution of 4 cm^{-1}. Thermal properties of the blend films were analyzed by DSC and TGA. DSC thermograms were recorded on a Mettler DSC 822e/400 analyzer at a heating rate of 10°C·min^{-1} under nitrogen atmosphere TGA patterns were measured on a Perkin Elmer Pyris Diamond TG-DTA analyzer at a heating rate of 10°C·min^{-1} under nitrogen atmosphere over the temperature range of 30 to 750°C. Phase morphology of the blend films was investigated by a JEOL 520-2AE scanning electron microscope (SEM). Prior to observation under SEM, the blend films were either etched with cyclohexane or concentrated acetic acid solution in order to remove H-chitosan or PLA, respectively, for two minutes at room temperature.

RESULTS AND DISCUSSION

Characterization of H-chitosan/PLA blend films

Chemical characteristics

Blend films of H-chitosan and PLA in various blend compositions were prepared using chloroform as the common solvent. Figure 1(a) shows FT-IR spectra of films of pure H-chitosan (i.e. the topmost curve), pure PLA (i.e. the bottommost curve), and H-chitosan/PLA blends. It is apparent from Figure 1(a) that the char

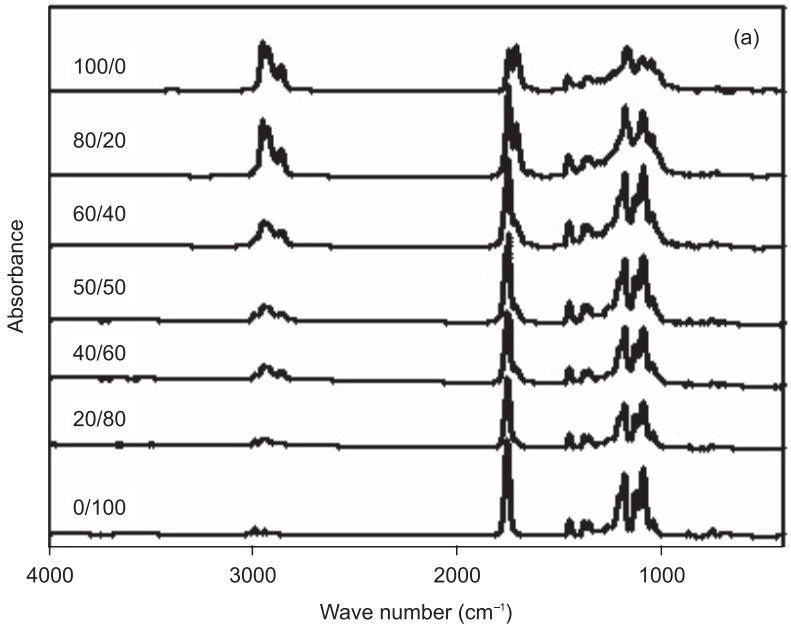


Figure 1. a) FT-IR spectra of pure H-chitosan, pure PLA, and H-chitosan/PLA blend films, and b) FT-IR spectra of pure H-chitosan, pure PLA, and H-chitosan/PLA blend films illustrating the crystalline region of PLA.

acteristic carbonyl-stretching absorption peak of H-chitosan at 1716 cm^{-1} (C=O of $N(COR)_2$) was present in all of the spectra observed (with an exception of that of the pure PLA), whereas the characteristic carbonyl-stretching absorption peak of PLA at 1768 cm^{-1} (C=O of OCOR) was present in all of the spectra observed (with an exception of that of the pure H-chitosan). The intensity of these peaks was found to be more pronounced with an increase in the content of the respective content. Since, apart from the absorption peaks specific to H-chitosan and PLA being observed in all of the blend films, no additional peaks signifying possible interaction between H-chitosan and PLA were observed, it is logical to postulate that the interaction between H-chitosan and PLA might be either non-existent or too weak to be detected by FT-IR.

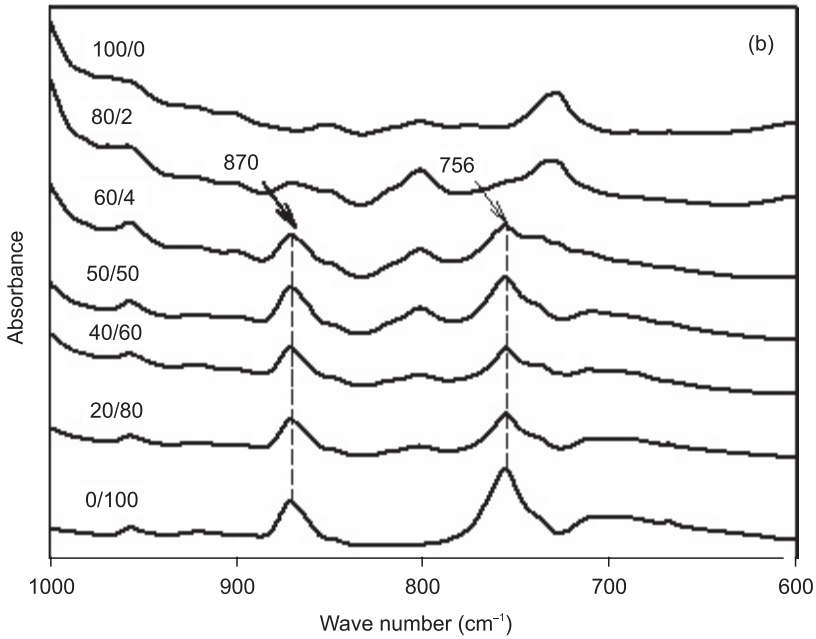


Figure 1. a) FT-IR spectra of pure H-chitosan, pure PLA, and H-chitosan/PLA blend films, and b) FT-IR spectra of pure H-chitosan, pure PLA, and H-chitosan/PLA blend films illustrating the crystalline region of PLA.

Figure 1(b) shows characteristic absorption peaks at 756 and 870 cm^{-1} which have been assigned to the crystalline and the amorphous phase of PLA [4]. Obviously, the intensity of both peaks was found to decrease with decreasing PLA content in the blends. Relatively, the decrease in the intensity of the crystalline peak was more pronounced than the decrease in the intensity of the amorphous peak. The ratio between the intensity of the crystalline peak to that of the amorphous peak for blend films was found to decrease from that of the pure PLA film, while the ratio between the intensity of the crystalline peak to that of the amorphous peak among the blend films was not found to differ much from one another. The results suggest that the apparent degree of crystallinity of the blend films may be slightly lower than that of the pure PLA films and the blend composition did not have a significant effect on the apparent degree of crystallinity of the films.

Thermal characteristics

Thermal stability of pure H-chitosan, pure PLA, and corresponding blend films was evaluated by TGA technique. According to the derivative TGA curves, pure PLA film was found to degrade at ca. 327°C, while pure H-chitosan film exhibited two degradation peaks at ca. 257 and 327°C, respectively. Apparently, the 50/50 H-chitosan/PLA blend film exhibited degradation behavior intermediate to those of the pure components, exhibiting two degradation peaks at ca. 253 and 312°C, respectively. Table 1 summarizes the observed degradation peak value(s) (denoted T_d) for all of the films investigated. For most blend films, their degradation behavior was found to be intermediate to those of the pure components. Interestingly, only 20/80 H-chitosan/PLA blend film exhibited only one degradation peak, with the observed T_d value being much greater than those of the pure components.

Table 1. Thermal decomposition temperature, glass transition temperature, low-temperature melting peak, high-temperature melting peak, apparent enthalpy of fusion, and apparent degree of crystallinity of pure H-chitosan, pure PLA, and H-chitosan/PLA blend films.

Blend composition (H-chitosan/ PLA) (w/w)	1st T_d (°C)	2nd T_d (°C)	T_g (°C)	T_{ml} (°C)	T_{mh} (°C)	ΔH_f ($J \cdot g^{-1}$)	χ_c (%)
0/100	–	327 ± 2	50 ± 1	162 ± 1	170 ± 1	49.4	53.2
20/80	–	336 ± 0	46 ± 1	158 ± 0	168 ± 0	33.6	45.1
40/60	264 ± 0	316 ± 1	43 ± 2	159 ± 1	168 ± 1	29.1	52.2
50/50	254 ± 1	312 ± 2	42 ± 1	159 ± 1	168 ± 2	20.7	44.5
60/40	255 ± 3	313 ± 1	42 ± 0	160 ± 0	169 ± 0	16.8	45.1
80/20	257 ± 2	309 ± 1	43 ± 2	–	168 ± 0	10.0	53.5
100/0	257 ± 0	327 ± 2	–	–	–	–	–

In DSC, the miscibility of two polymers in the bulk amorphous phase can be evaluated by the presence of a single, composition-dependent T_g value between those of the constituent polymers. No obvious T_g was observed in the DSC thermogram obtained for pure H-chitosan film, while a T_g of around 50°C was observed for pure PLA film (see Table 1). The value of 50°C observed for pure PLA film is in general accord with the value of 56°C observed by thermomechanical analysis [5]. For all of the blend films investigated, a single, compositional dependent T_g was clearly observed for each blend composition (see Table 1). The facts that only one composition-dependent T_g was observed in all of the blend films investigated and that the observed T_g value was found to decrease slightly with increasing H-chitosan content (with an exception of the 80/20 H-chitosan/PLA blend film) indicates partial miscibility between H-chitosan and PLA molecules in the bulk amorphous phase.

Melting behavior and apparent degree of crystallinity

For both pure PLA and corresponding blend films of various compositions, either one or two melting endotherm(s) was observed. The peak temperature of the low-temperature melting endotherm denotes T_{ml}, while that of the high-temperature one denotes T_{mh}. For pure PLA film, the observed T_{ml} and T_{mh} values were found to locate at 162 and 170°C. With increasing H-chitosan content, both the T_{ml} and the T_{mh} values were found to slightly decrease from that of the pure PLA, and no significant dependency of these values on H-chitosan content was observed. Another important information which can be deduced from the melting thermograms (not shown) is the apparent degree of crystallinity that was present in each of the as-casted films. Qualitatively, the area under the melting endotherm, directly relating to the amount of the crystals present within the film sample, was found to decrease with increase H-chitosan content, with the area under the melting endotherm for pure PLA film being the greatest. The apparent enthalpy of fusion ΔH_f associated with the area under the melting endotherm for each film sample is summarized in Table 1. According to the ΔH_f values listed, the apparent degree of crystallinity χ_c can be approximated according to the following equation:

$$\chi_c(\%) = \frac{\Delta H_f}{\Delta H_f^0 \cdot w_{PLA}} \times 100 \quad (1)$$

where ΔH_f^0 is the equilibrium enthalpy of fusion for PLA (i.e. 93 $J.g^{-1}$ [6]) and w_{PLA} is the weight fraction of PLA in the as-casted film. The calculated value of χ_c for each film prepared is listed in Table 1. Apparently, the χ_c values observed were not much different and no significant variation with H-chitosan content was observed.

Morphological characteristics

In order to clearly observe the phase morphology of H-chitosan and PLA in the as-casted blend films, each blend film was either etched in cyclohexane or conc. acetic acid solution in order to remove either H-chitosan or PLA which was perceived as the minor component from the blend film prepared. Figure 2 shows SEM micrographs illustrated the surface morphology of the as-etched blend films. Figure 2a–c shows SEM micrographs of the blend films having the weight fraction of H-chitosan of 20, 40, and 50, respectively. The voids present in these micrographs were H-chitosan particles which were dissolved away after the blend films were immersed in cyclohexane for two minutes. Figure 2d–f shows SEM micrographs of the blend films having the weight fraction of PLA of 50, 40, and 20, respectively. Similarly, the voids present in these micrographs were PLA particles which were dissolved away after the blend films were immersed in conc. acetic acid solution for two minutes. From these micrographs, H-chitosan and PLA were found to phase-separated during the evaporation of the solvent. When H-chitosan was the minor phase, the H-chitosan particles were found to distribute quite regularly throughout the PLA matrix and the size of the particles became smaller, while the number of the particles was found to increase, with increasing amount of H-chitosan from 20 to 50%. On the other hand, when PLA was the minor phase, the PLA particles were found to distribute very regularly

throughout the H-chitosan matrix and the size of the particles was found to increase, while the number of the particles was found to decrease, with increasing amount of PLA from 20 to 50%. Comparatively, PLA minor phase was found to distribute much evenly in the matrix than that of the H-chitosan minor phase.

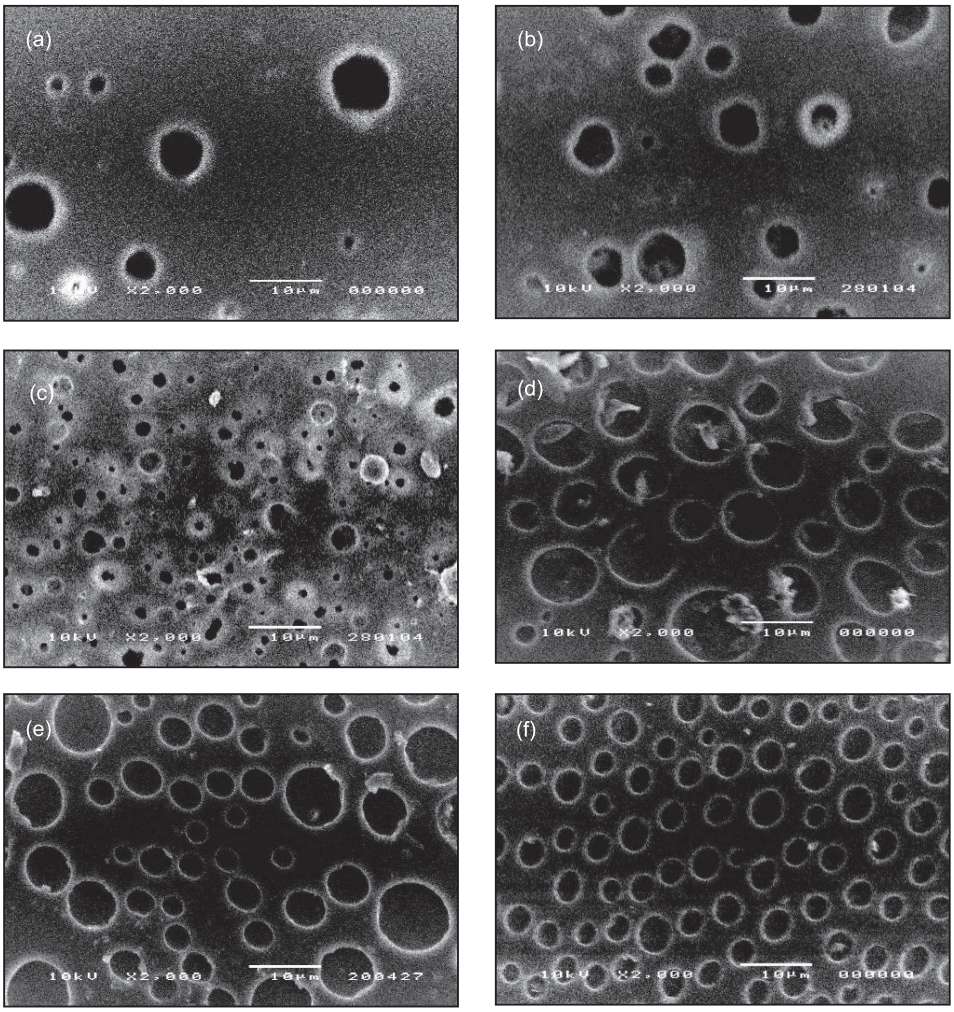


Figure 2. Scanning electron micrographs for as-etched H-chitosan/PLA blend films having the weight-based H-chitosan/PLA compositions of a) 20/80, b) 40/60, c) 50/50, d) 50/50, e) 60/40, and f) 80/20, respectively. Figures a–c were etched by cyclohexane, while figures d–f were etched by concentrated acetic acid solution

CONCLUSIONS

Blend films of H-chitosan and polylactide (PLA) were prepared by the solution-casting technique. Fourier-transformed infrared spectroscopy results indicated that no significant interaction between H-chitosan and PLA molecules

was observed. The thermal degradation behavior of the as-prepared blend films was found to be intermediate to those of the pure components. Only the blend film having the H-chitosan content of 20% exhibited the degradation temperature greater than those of the pure components. All of the blend films exhibited one composition-dependent glass transition temperature, suggesting that partial miscibility between H-chitosan and PLA molecules may be possible in the bulk amorphous phase. The melting behavior of the as-casted blend films exhibited double endothermic peaks, with both of the peak values being slightly lower than those of the pure PLA and no significant dependency on the blend composition being observed. The apparent degree of crystallinity calculated from the apparent enthalpy of fusion revealed no significant variation with the blend composition.

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EFFECT OF TRANSGLUTAMINASE ON THE SOLUBILITY OF CHITOSAN-GELATIN FILMS

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Abstract: Chitosan can be used as a valuable component of edible and biodegradable films. However, the application of chitosan films for packaging of some foods is limited due to the high solubility of chitosan in aqueous, acidic media, especially when heated up to 100°C. It has been assumed that the solubility could be limited by combining chitosan with some proteins and by cross-linking with transglutaminase. This enzyme catalyses the formation of the covalent bonds between γ-carboxyamide groups of peptide-bound glutamine residues and primary amino groups of a number of components. Chitosans with different deacetylation degree, obtained from krill chitin, and pork gelatin (300 Bloom) or gelatin obtained from Baltic cod skins were used for preparation of the films. The solubility of films in buffers was determined at pH 3 and 6 after 24 hours of gentle shaking of the samples at room temperature, or after one hour heating at 100°C. At room temperature and pH 3, the solubility of the chitosan films decreased with the increase in deacetylation degree of the polymer. Films obtained from chitosan with deacetylation degree of 96% (chitosan-96) and 73% (chitosan-73) were soluble in about 70% and 95%, respectively. However, at 100°C and pH 3, both films were almost completely dissolved. The film prepared from chitosan-96 was dissolved only in about 5% after one hour of heating at 100°C and pH 6, while from chitosan-73 in 45%. The solubility of films prepared from chitosan-96 and pork gelatin at a ratio of 1:1 (w/w) was decreased at room temperature to 13% at pH 3, and, at a ratio of 1:4 to about 2%. The solubility of these films was still high at 100°C and was not decreased even when the polymers were modified with transglutaminase at concentrations 0.1 and 0.2 mg/ml of film forming solution. Transglutaminase was also used for cross-linking chitosan-96 and fish gelatin (1:1). Enzymatic modification decreased the solubility of the films only at pH 6 at room temperature. The solubility of the films at pH 3 and room temperature, and pH 3 and 6 at 100°C was similar and did not depend on the transglutaminase concentration. Enzymatically unmodified films obtained from chitosan-96 and fish gelatin in proportion 1:4 were soluble at room temperature in about 86% and 64% at pH 3 and pH 6, respectively. The solubility of films decreased to 19% and 23% at pH 3 and pH 6, respectively, after cross-linking of the components with transglutaminase at a concentration 0.2 mg/ml. Enzymatic treatment of the mixtures of chitosan-96 with pork or fish gelatin did not limit the solubility at pH 3 and 6 at 100°C.

Key words: chitosan, gelatin, edible films, transglutaminase, solubility

INTRODUCTION

Packaging films made from proteins, polysaccharides, lipids or mixtures of these compounds are of special interest due to their biodegradability. However, some of their properties are inferior to those of films made of synthetic polymers. Particularly, high sensitivity to moisture and poor water vapour barrier properties can limit the applications of the protein and polysaccharide films as packaging materials. Therefore, new mixtures of components [1–3], and new chemical, enzymatic or physical modifications of films components have been investigated [4–6]. The aim of this effort is to improve the mechanical, barrier and other properties of the films that are important for some packaging applications.

A valuable component of natural packaging films is chitosan. It is generally obtained from natural chitin after its *N*-deacetylation by an alkaline treatment. Chitosan is a biodegradable and non-toxic polymer. Due to its biological activity it can potentially serve as a bactericidal agent in food packaging materials [6, 7]. Furthermore, to the antimicrobial films based on the chitosan matrix, additional bacterial inhibitors could be incorporated [8].

The degree of deacetylation in chitosan chain (as well as its distribution) has large impact on the chitosan properties, among other things, on its solubility. The solubility of chitosan in aqueous, acidic medium is an asset at the stage of preparing film-forming solution. However, because of the solubility, the chitosan films are not suitable for packaging acidic food. This problem arises when packaging material should be resistant to solubilization during heating at elevated temperatures.

It has been assumed that the solubility of chitosan films could be limited by combining chitosan with some proteins and by cross-linking with transglutaminase (TG). TG catalyses the formation of the covalent bonds between γ-carboxyamide groups of peptide-bound glutamine residues and primary amino groups of a number of components. As a donor of γ-carboxyamide groups gelatins of different origin could be used. Gelatin from fish is presently of great interest due to religious or health reasons.

The objective of this study was to determine the possibility of decreasing the water solubility of films made of chitosan and pork gelatin or chitosan and fish gelatin by cross-linking with TG.

MATERIALS AND METHODS

Materials

Chitosans of different deacetylation degrees (DD) were obtained from krill chitin according to Kołodziejska *et al.* [9]. The viscosity-average molecular weight (Table 1) was evaluated according to the procedure of Roberts and Domszy [10]. The source of protein were pork gelatin (300 Bloom) purchased from Sigma Chemical Co. and gelatin obtained from Baltic cod skins as described by Kołodziejska *et al.* [11]. TG was a commercial product (Ajinomoto co's Transglutaminase Activa ® -WM, Tokyo, Japan). The preparation contained 99% of maltodextrin and 1% of TG. The dry preparation was stored at –20°C. Before use, 6 g of preparation was mixed with 20 ml of water at 0°C for 15 min and centrifuged at 6,000 x g for 15

min at 0°C. The protein content in the enzyme solution was determined according to Lowry *et al.* [12].

Table 1. The properties of chitosans used in the experiments

DD [%][1]	Viscosity-average molecular weight [kDa]
73	1 408
84	1 318
90	1 682
96	1 674

[1] evaluated as described in Kołodziejska *et al.* [9]

Film formation

The chitosan films were prepared from 1% (w/w) solutions of polymers of different DD (pH 5 adjusted with 0.5 M HCl).

For preparation of gelatin films the pork and fish gelatins were dissolved in water, respectively, at about 80–90°C or room temperature, to a final concentration of 5% (w/w).

Chitosan-gelatin films (1:1, w/w) were obtained from the mixture prepared by dissolving 0.5 g of pork gelatin in 70 ml hot distilled water (in case of fish gelatin at room temperature), adding 0.5 g of dry chitosan with DD of 96% (chitosan-96) and 0.5 M $HCl_{aq.}$ to bring the solution to pH 5. After adding water to final mass of 100 g, the solution was centrifuged at 2,000 x g and 20°C for 15 min.

Chitosan-gelatin films (1:4 w/w) were obtained by mixing 2% solution of chitosan-96 (pH 5 adjusted with 0.5 M HCl) with 25% solution of pork gelatin or fish gelatin. The resulting mixture was occasionally stirred during 2 h of incubation at 50°C and centrifuged at 2,000 x g and 20°C for 15 min.

Enzymatic modification of films was conducted before their formation. To film forming solutions TG was added in concentrations 0.1 and 0.2 mg/ml. In case of film forming solutions containing pork gelatin TG was added at 50°C. Enzymatic modification of chitosan-96-fish gelatin solutions were conducted at room temperature.

In all experiments, 40 g of solutions were cast on a square area (255 cm^2) of a polyester surface and spread manually to the outside borders. The films were dried first during 12 h in 65–70% relative humidity and room temperature and later in ambient conditions.

Solubility measurements

The solubility of the films was determined at the pH 3 and 6, after 24 h incubation of the samples at room temperature or after one hour of heating at 100°C. About 50 mg of the dry film was immersed in 20 ml of 0.2 M Mc'Ilvaine buffer of appropriate pH and temperature. The insoluble material was separated on a funnel with cotton wool and the Kjeldahl method was used for the determination of nitrogen in the insoluble pieces of films. The solubility was expressed as the percent of nitrogen that migrated from the film to the buffer medium.

RESULTS AND DISCUSSION

The solubility of chitosan, especially at pH above 6, depends on the DD of the polymer [13, 14]. In the conditions applied by these authors the chitosans with the lower DD values are more soluble than polymers with the higher DD. According to Wojtasz-Pająk and Ulański [14] the lower solubility of chitosans with smaller number of acetyl groups is caused by difficulties in attaching protons to amino groups that are in close neighbourhood of already protonated amino groups, due to electrostatic repulsive forces.

Similar relationship was observed in case of solubility of chitosan films, even at pH 3. The solubility of the films at room temperature in buffer at pH 3 decreased with the increase in the DD of the polymer (Fig. 1).

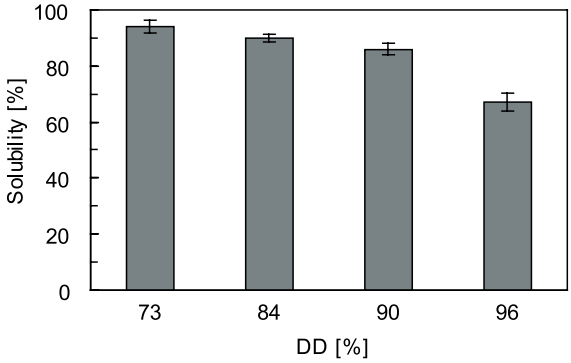

Figure 1. Effect of the DD of chitosan on the solubility of films at pH 3 (room temperature, 24 h)

In such conditions the solubility of films obtained from chitosan with deacetylation degree of 73% was 94%, while that of films from chitosan-96 was only 67%. However, both films were almost completely dissolved in buffer at pH 3 and 100°C (Table 2). Although both chitosan-films were almost insoluble at pH 6 and room temperature, at 100°C the films cast of chitosan-96 were more resistant to solubilization than films obtained from chitosan with the lower DD (Table 2). Some applications of packaging materials require materials that are resistant to higher temperatures. That is why chitosan with the higher DD was selected for preparing chitosan-gelatin films.

The properties of gelatins depend on the origins of the raw material. Also the films obtained from different gelatins show diverse properties. Depending on the origin of the gelatin, the solubility of pure gelatin films differed very much (Table 3). Fish gelatin films were completely soluble even at room temperature at pH 6. In such conditions films made from pork gelatin with the Bloom value of 300 were soluble only in about 7%, and showed the same level of solubility at pH 3 at room temperature. As could be expected, both kinds of gelatin films were completely not resistant to heating at 100°C in buffers at pH 3 and 6 (Table 3).

Table 2. Effect of the DD of chitosan on the solubility of films (room temperature, 24 h; 100°C, 1 h)

DD	Solubility [%]			
	pH 3		pH 6	
	Room temperature	100 °C	Room temperature	100 °C
73	93.5 ± 2.3	97.7 ± 2.8	3.5 ± 0.9	45.0 ± 4.0
96	66.9 ± 3.2	97.3 ± 3.4	2.5 ± 0.8	4.8 ± 0.6

Table 3. Effect of the origin of gelatin on the solubility of films (room temperature, 24 h; 100°C, 1 h)

Gelatin	Solubility [%]			
	pH 3		pH 6	
	Room temperature	100°C	Room temperature	100°C
Pork	8.5 ± 0.6	97.5 ± 0.6	6.5 ± 0.5	98.5 ± 0.5
Fish	98.7 ± 0.5	97.9 ± 0.1	97.5 ± 0.9	99.5 ± 0.3

At room temperature, the solubility of films prepared from the mixture of chitosan-96 and pork gelatin at a ratio of 1:1 (w/w) was 13% at pH 3. The films were completely insoluble at pH 6 (Table 4a). However, after one hour of heating at 100°C they were totally dissolved at pH 3 and their solubility at pH 6 was 68% (Table 4a). The modification of film-forming components with TG at concentrations 0.1 and 0.2 mg/ml of film forming solution did not decrease the solubility of the films at 100°C. Furthermore, enzymatic treatment of the polymers increased the solubility of films at room temperature at pH 3 (Table 4a).

Table 4. Effect of the enzymatic modification on the solubility of chitosan-pork gelatin films (room temperature, 24 h; 100°C, 1 h)

a) chitosan-96 : pork gelatin (1:1)

TG concentration [mg/ml]	Solubility [%]			
	pH 3		pH 6	
	Room temperature	100°C	Room temperature	100°C
0	13.1 ± 2.5	99.2 ± 0.6	2.1 ± 3.7	67.8 ± 2.5
0.1	25.9 ± 3.3	97.5 ± 1.6	0.0 ± 0.0	67.7 ± 0.8
0.2	36.6 ± 2.7	97.4 ± 0.7	2.7 ± 3.1	65.9 ± 6.1

b) chitosan-96 : pork gelatin (1:4)

TG concentration [mg/ml]	Solubility [%]			
	pH 3		pH 6	
	Room temperature	100°C	Room temperature	100°C
0	1.4 ± 1.0	98.7 ± 0.3	n.d.	83.5 ± 1.6
0.1	0.2 ± 0.5	98.9 ± 0.5	n.d.	84.6 ± 1.7
0.2	7.6 ± 1.1	99.2 ± 0.2	n.d.	85.1 ± 1.5

n.d. – not determined

When mixing chitosan-96 and pork gelatin solutions at a ratio of 1:4 (w/w) at room temperature an enormous growth in viscosity was observed. It caused difficulties in preparing of films. For this reason the mixture of chitosan and gelatin was incubated at 50°C before film forming. On the other hand, such mixture could be used as a good thickener in food application. Films obtained from chitosan-pork gelatin at the ratio (1:4) were elastic, smooth and transparent. Their solubility at room temperature and pH 3 (Table 4b) was smaller than that of films made from single components (Table 2 and 3). However, the solubility of films at 100°C and pH 3 and 6 was still high and enzymatic modification did not decrease it.

TG was also used for modification of chitosan-96 and fish gelatin mixture prepared at the ratio (1:1). Although it was not possible to form films from 1% fish gelatin solution, transparent, elastic and smooth films were obtained after mixing it with chitosan. Decrease in solubility of films modified with TG was observed only at pH 6 at room temperature. At the concentration of enzyme 0.2 mg/ml of film forming mixture the solubility of films was reduced twice as compared to that of unmodified films. The solubility in the other variants of experiments was similar and did not depend on TG concentration (Table 5a).

Table 5. Effect of the enzymatic modification on the solubility of chitosan-fish gelatin films (room temperature, 24 h; 100°C, 1 h)

a) chitosan-96 : fish gelatin (1:1)

TG concentration [mg/ml]	Solubility [%]			
	pH 3		pH 6	
	Room temperature	100°C	Room temperature	100°C
0	68.8 ± 4.4	98.3 ± 0.2	39.9 ± 3.2	69.0 ± 1.2
0.1	61.8 ± 5.5	97.1 ± 0.7	26.6 ± 4.0	67.8 ± 2.3
0.2	68.3 ± 4.9	95.3 ± 1.1	18.7 ± 2.9	75.5 ± 2.1

Enzymatically unmodified films obtained from chitosan-96 and fish gelatin in proportion 1:4 were soluble at room temperature in about 86% and 64% at pH 3 and pH 6, respectively (Table 5b). After cross-linking of the components with TG at a concentration 0.2 mg/ml the solubility of films at room temperature decreased to 19% and 23%, respectively, at pH 3 and pH 6 (Table 5b). According

to Kołodziejska *et al.* (2004), the concentration of substrate, apart from the concentration of enzyme, is also very important during modification of fish gelatin. It probably explained why no effect of modification was observed in case of films at the ratio (1:1).

Table 5. Effect of the enzymatic modification on the solubility of chitosan-fish gelatin films (room temperature, 24 h; 100°C, 1 h)

b) chitosan-96 : fish gelatin (1:4)

TG concentration [mg/ml]	Solubility [%]			
	pH 3		pH 6	
	Room temperature	100°C	Room temperature	100°C
0	85.6 ± 1.1	97.9 ± 0.6	64.3 ± 3.0	88.1 ± 1.0
0.1	58.9 ± 5.3	98.1 ± 0.3	23.7 ± 4.1	92.1 ± 4.1
0.2	19.1 ± 4.1	94.5 ± 1.1	23.2 ± 5.7	90.7 ± 0.7

Although enzymatic treatment of the mixtures' components limited the solubility at room temperature, it had no influence on the solubility of films at pH 3 and 6 at 100°C (Table 5b).

CONCLUSIONS

TG did not exert any influence on the solubility of films prepared from chitosan-96 and pork or fish gelatin during prolonged heating at 100°C at pH 3 and 6. However, the solubility of chitosan-fish gelatin films can be limited at room temperature, even at pH 3. Chitosan with high DD and pork gelatin with the Bloom value of 300 can be used for manufacturing films insoluble at acidic pH at room temperature without enzymatic modification.

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COMPLEXATION AND BIOLOGICAL PROPERTIES OF *N*-(2-CARBOXYETHYL)CHITOSANS

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Key words: chitosan, carboxyethylation, metal chelation, antioxidant, antimutagen

INTRODUCTION

In the last decades substantial research and efforts have been made to development of suitable procedures for preparation of water-soluble derivatives of chitin and chitosan. *N*-(2-carboxyethyl)chitosan (CE-chitosan) is a novel water-soluble aminopolysaccharide that could be used in a broad range of applications. Several methods for preparing CE-chitosan are available: (a) by alkaline hydrolysis of the acrylamide, methyl- or ethylacrylate adducts of chitosan [1, 2]; (b) by direct addition of acrylic acid [3]; and (c) by reaction with 3-halopropionic acids [4, 5]. It has been found that CE-chitosan appears suitable for mediating transport of hydrophilic drugs such as vitamin B6 through the skin [5] or as a nitric oxide carrier for use in a variety of medical applications [6]. This paper is summarizing our recent research on physicochemical and biological properties of a novel CE-chitosan.

EXPERIMENTAL

Preparation of CE-chitosans

Chitosan (MW = 150000, Aldrich) was treated with 3-chloropropionic acid in water in the presence of $NaHCO_3$ as described in [4]. The reaction conditions and some characteristics of CE-chitosan samples are summarized in Table 1.

Table 1. Reaction conditions[a] and some parameters of CE-chitosan samples

Sample	Reagents ratio[b]	Reaction time, hours	DF[c]				DS[d]
			DA	x	y	z	
1	1	72	0.09	0.76	0.15	0.0	0.17
2	5	12	0.07	0.68	0.25	0.0	0.27
3	2	72	0.07	0.55	0.39	0.0	0.42
4	2×2	72×2	0.10	0.36	0.54	0.0	0.60
5	2×3	72×3	0.10	0.28	0.62	0.0	0.69
6	3×3	72×3	0.10	0.11	0.79	0.0	0.88
7	5	72	0.07	0.16	0.69	0.08	0.92
8	5×2	72×2	0.07	0.0	0.35	0.57	1.61

[a] T=60°C, pH=8–9 ($NaHCO_3$)
[b] 3-chloropropionic acid/Glc-NH_2 molar ratio
[c] DF – degree of functionalization was determined from ^1H NMR spectra
[d] DS was calculated per Glc-NH_2 residue of chitosan DS=(y+2z)/(x+y+z)

Euglena gracilis mutagenicity assay

E. gracilis strain Z was a generous gift of Dr. S. H. Hutner from Haskins Laboratory, Pace University, NY, USA. The cells were cultivated and the bleached mutants were evaluated as described earlier [7]. *E. gracilis* cells were co-treated with 5 μg/ml of acrydine orange (AO) or 15 μg/ml of ofloxacin with or without addition of the CE-chitosan samples at 27°C and a constant light of 2000 lx. Following a 24-hour co-treatment, the cells were washed out, diluted and spread on agar plates with Cramer-Myers medium (1.2% agar). Normal green and mutant white colonies were analyzed after 10–14 days of cultivation. Solutions of CE-chitosan samples were used as control samples without addition of AO or ofloxacin. Three plates were used in each cultivation experiment for each concentration of CE-chitosan. The experiments were repeated in three independent series.

Determination of antioxidant activity

The antioxidant activity of CE-chitosans and was assessed by a modified trolox equivalent antioxidant capacity method according to [8]. The decolorization assay involved initial formation of the blue/green $ABTS^+$ chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants led to reduction of $ABTS^+$ radical and therefore to elimination of color. This effect was dependent on the concentration and antioxidant activity of the individual CE-chitosans, and on the duration of the reaction. Trolox was used in our assay as a standard and antioxidant activity of tested compounds was compared with the antioxidant activity of trolox.

General methods

The ^1H NMR spectra of the D_2O/DCl solutions of the CE-chitosan samples at a concentration of 10 mg·mL^{-1} were performed using BRUKER DRX 400 spectro-

photometer at T=70°C. The differential scanning calorimetry (DSC) was carried out using a Netzsch DSC 204 linked to a TASC 414/3 controller furnished with a software Netzsch DSC 204 Measurement v 3.4. The equipment was calibrated as for the standard procedures, using a set of standard materials from Netzsch. For the analysis, accurately weighed material was placed into aluminium cup with a pierced lid. An empty cup was used as a reference. The sample was heated between 25–500°C with a gradient 10°C/min under continuous flow of dry nitrogen (20 mL/min). pH-titration were performed as described in [4].

RESULTS AND DISCUSSION

Antioxidant activity

of the CE-chitosans was assessed using the procedure of quantitative evaluation of quenching of color produced by $ABTS^{+}$ chromophore. In this assay the activity of the three CE-chitosan samples were compared with that of the reference antioxidant, trolox that is a derivative of α-tocopherol (vitamin E) and is used as a standard antioxidant [9]. All prepared CE-chitosan samples revealed high concentration-dependent antioxidant activity, which was expressed as a corresponding amount of trolox exhibiting similar antioxidant activity. Fig. 1 demonstrates linear concentration dependence of the antioxidant activity of CE-chitosan samples. The results obtained indicate that the prepared CE-chitosans are 100–300 times more potent than trolox.

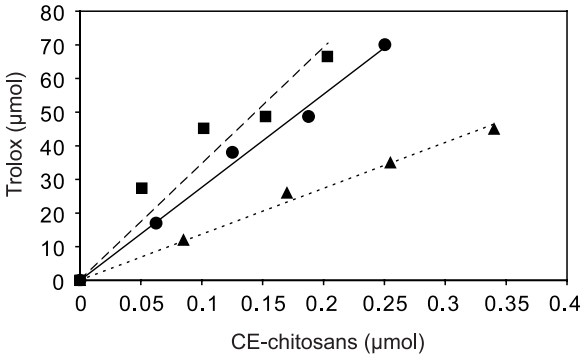


Figure 1. Antioxidant activity of CE-chitosans in concentration dependence (x) for samples 2 (▲, dotted line), 7 (●, solid line), and 8 (■, dashed line) expressed in μmol of trolox

Antimutagenic activity

of the synthesized CE-chitosans was assayed in the experiments involving application of genotoxic agents AO and ofloxacin to *E. gracilis*, which resulted in formation of mutant white colonies. In the control plates, no incidence of white colonies was observed. Also, application of CE-chitosan samples 2, 7, and 8 at any tested concentrations did not cause formation of mutant colonies of *E. gracilis*. No change in cells viability was observed. On the other hand, application of mutagens led to marked incidence of mutations. The viability of *Euglena* cells after ofloxacin treatment did not change when compared to negative control, whereas viability of *Euglena* cells after AO treatment decreased from 100 ± 11% to

80 ± 19% ($p < 0.01$). Fig. 2 shows that the genotoxic action of both mutagens was significantly suppressed by all CE-chitosan samples. The possible mechanisms of protection will be discussed in detail in our future publication [10].

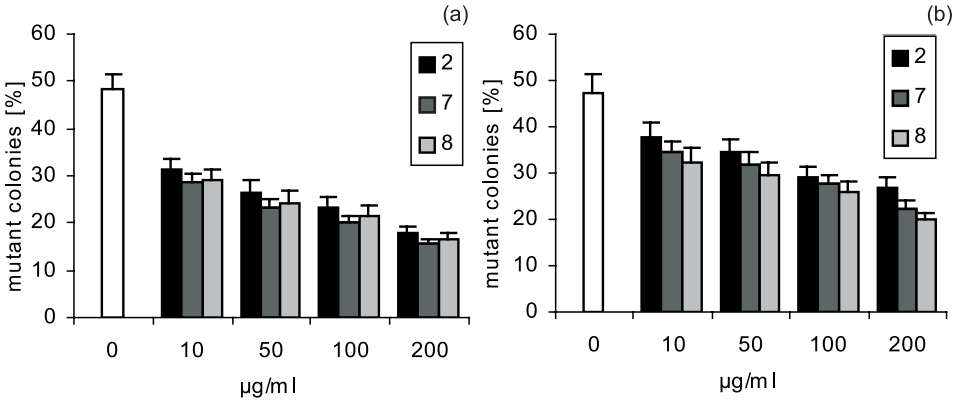
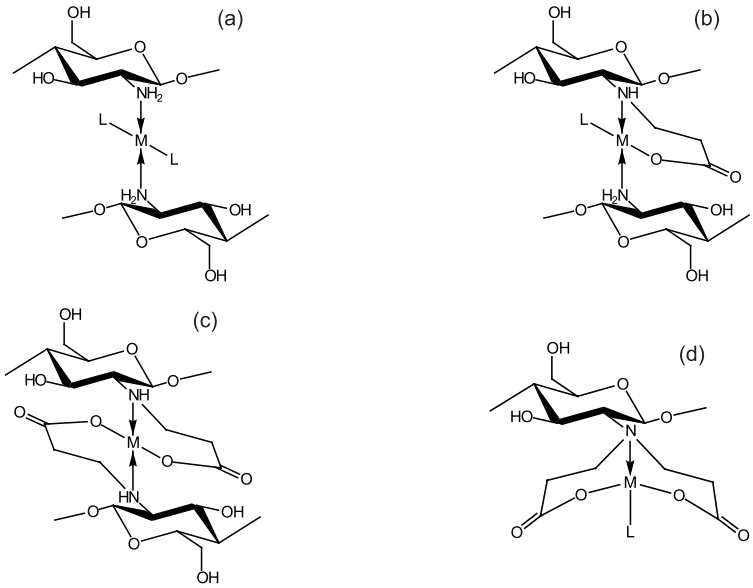


Figure 2. Effect of CE-chitosan samples 2, 7 and 8 on genotoxicity in *Euglena gracilis*, ofloxacine 15 µg/ml (a), acrydine orange 5 µg/ml (b)

Complexation of CE-chitosan with transition metal ions (M^{2+}=Cu, Ni, Zn, Cd) were studied in aqueous solution by pH-potentiometry in the pH range 3-7. Table 2 lists the most probable complex species together with the correspondent complex formation constants. The results allow us to propose a "bridge model", which corresponds to $[M(Glc\text{-}NR_2)_2]$ complexes, as a predominant coordination mode of CE-chitosans with low degree of carboxyethylation (samples 3,7; DS<0.9) and a $[M(Glc\text{-}NR_2)]$ 'pendant model' as a main species for high substituted CE-chitosan 8. Both models are presented in Scheme 1 (a–c) and (d), respectively. The observed selectivity series for CE-chitosans was Zn<Cu>Ni>Cd, which allowed to selectively separate Cu^{2+} ions from the first row transition metal ions.

Table 2. Complex formation constants $\beta_{pqr}=[M_pL_q(OH)_r]/[M]^p[L]^q[OH]^r$; 25°C; 0.1 M KNO_3

Sample	Complex	$\log\beta_{pqr}$			
		Cu^{2+}	Ni^{2+}	Cd^{2+}	Zn^{2+}
3	$[M(Glc\text{-}NR_2)_2]$	10.06 ± 0.02	7.03 ± 0.14	6.8 ± 0.3	6.60 ± 0.08
7	$[M(Glc\text{-}NR_2)_2]$	11.6 ± 0.2	–	–	–
8	$[M(Glc\text{-}NR_2)]$ $[M(Glc\text{-}NR_2)(OH)]$	6.41 ± 0.11	4.70 ± 0.10	3.55 ± 0.10	3.80 ± 0.15 12.8 ± 0.2



Scheme 1. Metal coordination modes of different residues of CE-chitosan; L = H_2O, Cl^- etc

Thermal stability of CE-chitosans

The thermograms of CE-chitosans with different DS are shown in Fig. 3 together with that of initial chitosan. Values for the temperatures events and their associated enthalpies are given in Table 3. The first thermal event registered in all samples was a wide endothermic peak centred between 67°C–95°C, which was related to the evaporation of adsorbed water presented in samples. The area of this peak does not directly correlate with DS of CE-chitosan because the amount of hydration water depends not only on hydrophilic character of the polymer, but also on polymer preparation and conditioning.

Table 3. DSC data for chitosan and CE-chitosans with different DS

Sample	T_{max}/°C	ΔH/J g^{-1}	T_{max}/°C	ΔH/J g^{-1}	T_{max}/°C	ΔH/J g^{-1}
Chitosan	107.94	288.21			306	–134.62
1	87.06	324.55	217	50.1	266	–38.30
3	84.87	280.11	215	77.6	256	–36.23
4	85.85	289.78	214	146.8	295	–45.31
5	100.12	118.13	217	140.1	295	–74.30
6	96.01	218.56	213	170.2	295	–72.30

For CE-chitosans, the thermograms reveal the second endothermic event centered between 214-218°C. A good correlation between DS and the enthalpy for this transition (ΔH=180.7·DS+17.2, r=0.96) was observed. We assume that this peak corresponds to the endothermic decarboxyethylation of CE-chitosan resulting in formation of acrylic acid [11]. Accurate assigning of this thermal transition

is a subject of debate. The last thermal event was a broad exothermic peak that arose between 256°C and 296°C and resulted due to the thermodegradation of the chitosan backbone. The temperature onset of the thermodegradation depends on the samples morphology (1, 3 are thin films, 4–6 represent powders) and it is higher for powder samples indicating their superior thermostability.

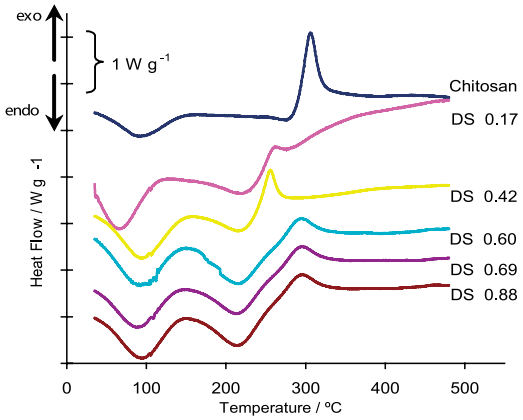


Figure 3. DSC curves of chitosan and CE-chitosan samples with different DS

ACKNOWLEDGMENTS

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ISOTHERMS OF WATER SORPTION IN BIOMATERIALS BASED ON CHITOSAN

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Abstract: Water sorption isotherms of chitosans and chitosan blends with PVAL and HPC in the form of films determined by gravimetric and spectroscopic methods are well fitted to GAB equation. X_m values reflected monomolecular layer amount of adsorbed water increase with deacetylation degree of chitosan and weight fraction of chitosan in the blends. We have predicted that the strong effect of deacetylation degree on ability of water sorption by chitosan resulted from a restriction of steric packing of molecular chains due to large size of acetamide groups. Modification of chitosan steric structure by blending with polyvinyl alcohol and hydroxypropylcellulose causes lowering its sorptive properties depending on water activity and blend composition.

Key words: sorption kinetics, water isotherm, chitosan blends

INTRODUCTION

Due to the modification of the structure of polymer material after water sorption, the physical properties of hydrophilic polymers can vary significantly. In hydrophilic polymers below a water content threshold (for PVAL equal to 25% [1] water molecules are so strongly bound to specific polar sites by hydrogen bonds that they cannot crystallise – it is nonfreezable water.

The sensitivity of dried products to moisture can be determinted on the basis of sorption isotherms. The sorption isotherms of polysaccharides show often a sigmoid curve whose shape can be explained by the model based on multilayer adsorption theory (BET, GAB) [2–4]. For the first layer the water molecules are adsorbed by the polar groups in the polymer chains and the next n-th layer corresponds to the water molecules successively condensed on the first layer.

In this study the sorption isotherms of water vapour by chitosans with various deacetylation degrees and modified chitosan blended with polyvinyl alcohol and hydroxypropylcellulose were measured. The GAB adsorption theory was applied to the biomaterials based on chitosan. The effects of chitosan deacetylation degree and the blends composition on the isotherms shape were discussed examining the X_m, C and k parameters, which represent moisture content corresponding to the monomolecular layer (proportional to the total number of ad-

sorption sites), interaction energy between water and the adsorption sites and the number of adsorbed layers, respectively.

EXPERIMENTAL

Materials

Chitosan (CH) is partly deacetylated chitin. Chitosan under study was produced in Sea Fishery Institute (Gdynia) and had various deacetylation degrees (DD) – see Table 1.

Polyvinyl alcohol (PVAL) of M=72000 was purchased in the Polish Chemical Reagents (Gliwice).

Hydroxypropylcellulose (HPC) is water soluble derivative of cellulose. Molecules of HPC can create stiff structures due to intermolecular hydrogen bonds.

Weak acetic acid (M=60.05 g/mol) of 1% concentration was used as a solvent for chitosan.

Table 1. Sample characterisation:
a) chitosan of various deacetylation degrees (DD)
b) chitosan blends with polyvinyl alcohol and hydroxypropylcellulose of various weight fractions of chitosan (wf)
Xm,C, k – parameters of the GAB equation.

a)

No.	Sample	DD 59	X_m	C	k
1	CH59	59	0.16	13.25	0.62
2	CH67	67	0.15	11.68	0.61
3	CH73	73	0.11	12.31	0.67
4	CH78	78	0.09	10.01	0.70
5	CH86	86	0.07	9.84	0.65

b)

No	w_f	Sample – Oznaczenie próbki							
			X_m	C	k		X_m	C	k
1	1	CH86	0.07	9.84	0.65	CH78	0.09	10.01	0.70
2	0,8	CH/PVAL/0,8	0.06	15.69	0.62	CH/HPC/0,8	0.09	9.02	0.65
3	0,6	CH/PVAL/0,6	0.05	12.53	0.59	CH/HPC/0,6	0.06	8.00	0.72
4	0,4	CH/PVAL/0,4	0.04	11.25	0.56	CH/HPC/0,4	0.04	7.53	0.79
5	0,2	CH/PVAL/0,2	0.03	10.94	0.65	CH/HPC/0,2	0.03	7.04	0.75
6	0	PVAL	0.02	5.68	0.68	HPC	0.02	5.69	0.82

Film preparation

Chitosans were dissolved in weak acetic acid but PVAL and HPC in distilled water at 24°C for 48 h. To obtain blends of proper compositions the solutions were mixed in chosen ratios. The films 15 μm thick were prepared by casting on glass plates. Then, they were dried at room temperature for 24 h and at 90°C for 1 h to remove residual acid. Chitosan films were etched with methyl alcohol for 24 h additionally.

Methods

Gravimetric and spectroscopic studies of sorption kinetics of water by chitosan and its blends at 25°C were used. Various water activities in 9 desiccators were obtained by using saturated solutions of various salts (Table 2). Before measurements the samples were dried at 90°C to constant mass to remove the sorbed water. Equilibrium was reached after about 3–4 hours.

Table 2. Water activity of saturated solutions of various salts

Salt	Water activity (a_w) WODY(a_w)
CH_3COOK	0.234
$MgCl_2$	0.329
K_2CO_3	0.443
$Mg(NO_3)_2$	0.536
$NaNO_2$	0.654
NaCl	0.765
KCl	0.846
$BaCl_2$	0.904
H_2O	1.000

RESULTS AND DISCUSSION

The sorption isotherms can be divided into three parts [3] as it is illustrated in Fig. 1a, analysing the type of moisture bonds with the material and the character of the interactions. In region I ($a_w < 0.2$) moisture molecules adsorbed are on polar components of the material – monomolecular adsorption. Range II is characterised by a polymolecular adsorption. In region III moisture fills up the pores in the material structure – capillary condensation.

Fig. 1. a–c presents the sorption isotherm obtained for a) chitosan of various deacetylation degree (DD), b) CH86, CH/PVAL and PVAL blends, c) CH78, CH/HPC and HPC blends. For each film at every water activity the equilibrium moisture content X was estimated and drawn versus water activity a_w.

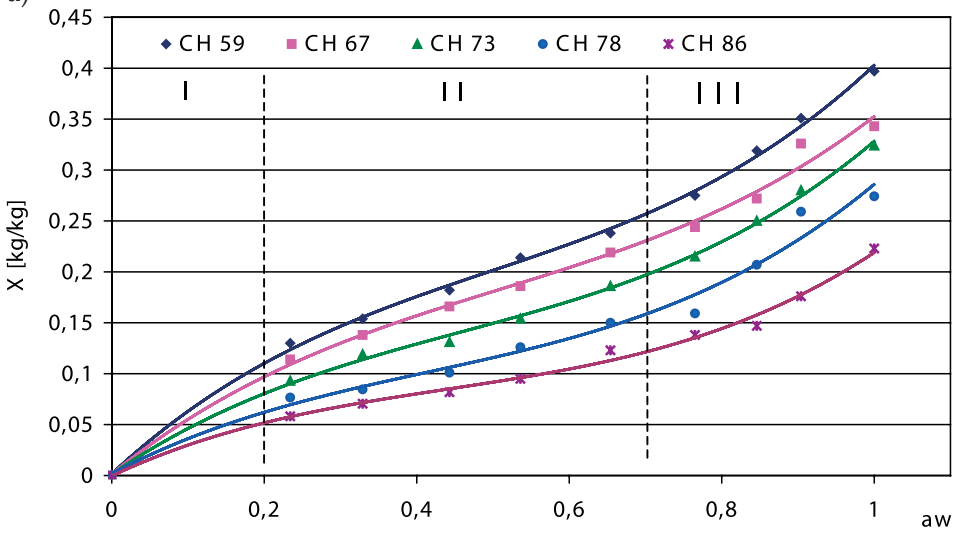
Analysing the medium hygroscopic shape (type II of isotherm shape) of isotherm curves one observes well fitting to GAB equation:

$$\frac{X}{X_m} = \frac{Cka_w}{(1-ka_w)(1-ka_w+Cka_w)}$$

where: X_m, C and k – parameters of the GAB equation.

The GAB parameters X_m, C and k were computed from linear regression of 1/X versus a_w. Estimated GAB parameters X_m, C and k are introduced in Table 1. The values of GAB parameters C and X_m change with DD of chitosan and weight fraction w_f of chitosan in blends. C and X_m increase with lowering value of DD. In the case of CH/PVAL and CH/HPC blends X_m and C value increase with rising weight fraction of chitosan.

a)



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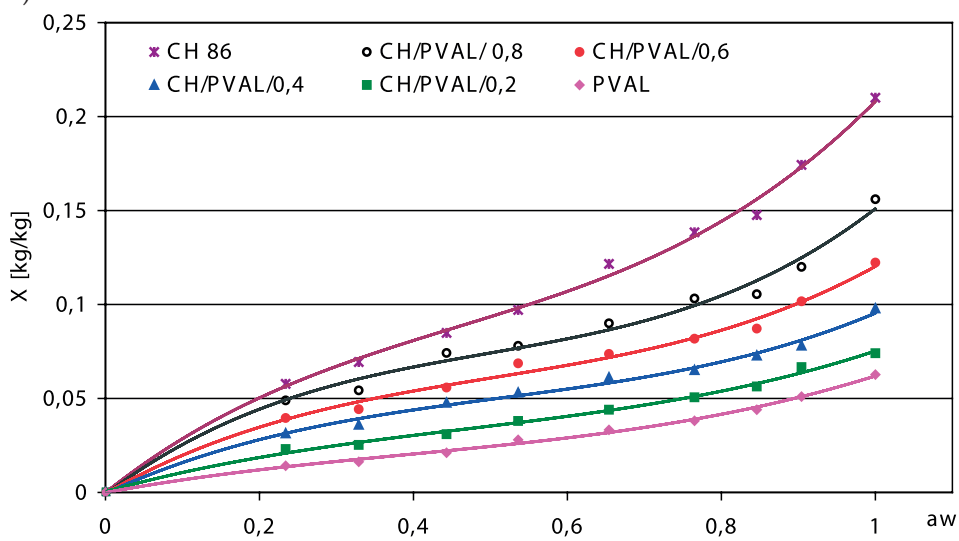

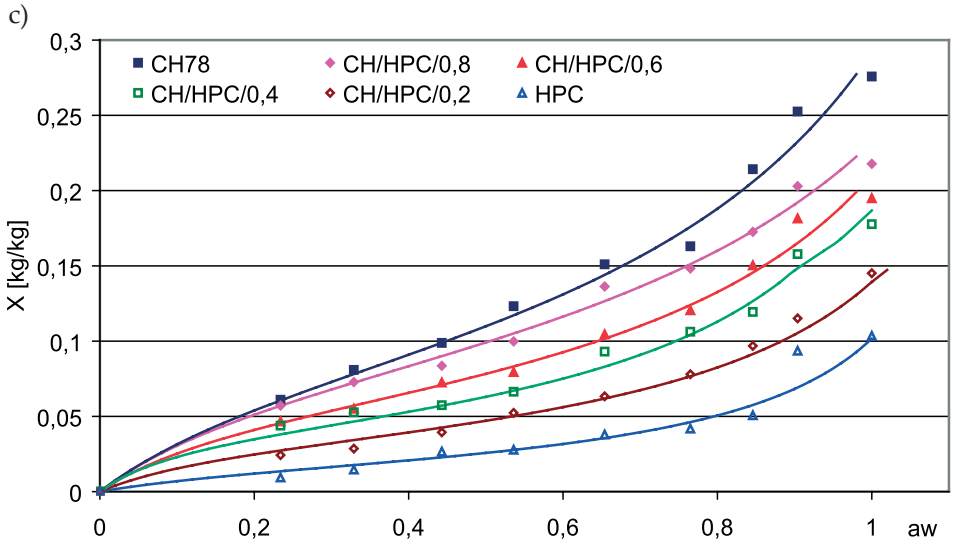

Figure 1. Sorption isotherms obtained for a) chitosan of various deacetylation degree (DD), b) CH (86), CH/PVAL and PVAL blends, c) CH (78), CH/HPC and HPC blends, (point – experiment, curve –GAB equation)

CONCLUSIONS

Water sorption isotherms of chitosans and chitosan blends with PVAL and HPC in the form of films determined by gravimetric and spectroscopic methods are well fitted to GAB equation. X_m values reflected monomolecular layer amount of adsorbed water increase with deacetylation degree of chitosan and weight fraction of chitosan in the blends. We have predicted that the strong effect of deacetylation degree on ability of water sorption by chitosan resulted not from molecular weight difference [5] of the samples but from a restriction of steric packing of molecular chains due to large size of acetamide groups (large free volume) affecting perhaps also various crystallinity degrees of the chitosans [6–8].

Modification of chitosan steric structure by blending with polyvinyl alcohol and hydroxypropylcellulose causes lowering its sorptive properties depending on water activity and blend composition.

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CHAPTER III

CHITO-OLIGOSACCHARIDES AND ENZYMATIC MODIFICATION

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A PALINDROMIC DNA SEQUENCE INVOLVED IN THE REGULATION OF CHITOSANASE GENE EXPRESSION IN ACTINOMYCETES

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Abstract: *Kitasatospora* sp. N106 (formerly *Nocardioides* sp.) is a potent producer of an extracellular chitosanase. We characterized DNA-protein interactions potentially involved in the regulation of chitosanase gene expression. Two DNA-binding complexes, N106BC1 ("slow") and N106BC2 ("fast") were detected by gel retardation experiments. DNAse I footprinting assigned the N106BC1 binding site to the palindromic sequence AGGAAANTTTCCT which is also found in the promoter areas of several genes from other actinomycete species. These genes encode chitosanases but also other proteins possibly related to chitosan metabolism. The protein involved in N106BC1 complex formation was partly purified by hydroxyapatite and used in binding studies with oligonucleotides. The dissociation constant K_d was 0.15 nM. Equilibrium competition assays with mutated oligonucleotides revealed a hierarchy of affinities in the various nucleotide components of the palindromic sequence.

INTRODUCTION

Chitosan is a versatile biopolymer of growing importance in biotechnology with many emerging applications in medicine, pharmacology, agriculture and environment protection. The molecular weight of chitosan is, besides the deacetylation degree, the most important parameter determining its biological properties (recently reviewed by Blanchard *et al*, 2003). Enzymatic hydrolysis with chitosanase is a convenient way of reducing and controlling the molecular weight of chitosan. A variety of enzymes catalyzing the hydrolysis of chitosan have been found in many microorganisms, bacteria or fungi. The enzymes so far characterized at the primary sequence level belong to five families of glycoside hydrolases: the families 5, 8, 46, 75 and 80 [2–6]. However, very few studies have been dedicated to the regulation of chitosanase gene expression. Such studies are essential to improve the strain productivity and decrease the cost of enzymatic hydrolysis of chitosan.

In our previous research (Dubeau *et al*., to be submitted), we characterized the DNA-protein interactions in the regulatory segment of the chitosanase gene from *Kitasatospora* sp. N106 (formerly *Nocardioides* sp. N106), a potent chitosanase producer [7]. Two complexes, N106BC1 and N106BC2 were identified by gel retardation experiments. The interaction of the N106BC1 complex-forming protein with the DNA segment upstream from the translation initiation codon of the chitosanase coding sequence was analyzed in detail. DNAse footprinting experiment revealed that this complex-forming protein interacted with the sequence CAATCTAGTTAGGAAACTTTCCTAACTCTCC spanning over 31 base pairs, from –10 to +21 positions relatively to the transcription start point and including a palindromic sequence (underlined).

In this work we further analyze the interaction of N106BC1 complex-forming protein with the palindromic sequence using equilibrium competition assays with mutated oligonucleotide segments.

MATERIALS AND METHODS

Protein extract was prepared from strain N106 mycelium growing for 10 h at 37°C in salt medium with low molecular weight chitosan prepared by enzymatic hydrolysis and lyophilisation (Dubeau *et al*., to be submitted). The bacterial culture was centrifuged 5 min at 3 000 g at 4°C, washed with cold extraction buffer (50 mM Tris, 60 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, pH 8.0) and suspended in 2–3 pellet volumes of extraction buffer containing a protease inhibitor cocktail. The bacterial cells were then disrupted by two passages in a French press at 18 000 psi. The extract was centrifuged at 3 000 g for 10 min at 4°C. The proteins were precipitated with ammonium sulfate at 25% – 50% saturation. The precipitate was suspended in two volumes of 1 mM phosphate buffer (pH 6.8) with 5% glycerol (buffer A) and the sample was then applied on a hydroxyapatite column (12 x 2.6 cm) (Bio-Gel HTP, Bio-Rad) equilibrated with buffer A. The column was then washed with one column volume of buffer A, then volumes of 40 mM phosphate buffer (pH 6.8) with 5% glycerol and the protein was eluted with 270 mM phosphate buffer (pH 6.8) with 5% glycerol (buffer B). Protein concentration was estimated using the BioRad Protein Assay method.

The formation of DNA-protein complex was measured with the gel mobility shift assay. Labeled probe and competitor DNAs were prepared from double stranded (d-s) oligonucleotides. The probe MP12F was obtained by annealing the oligonucleotide 5′-CAATCTAGTTAGGAAACTTTCCTAACTCTCC-3′ with its complement. The probe corresponded to the entire segment protected from DNAse in the footprinting assay for N106BC1 complex. This d-s oligonucleotide probe was end-labeled with T4 polynucleotide kinase and purified on a G-25 size-exclusion mini-column. The *mutants* used as competitors were also annealed oligonucleotides corresponding to the segment covered by the MP12F probe but each included a different point mutation in the palindromic part (see Fig. 2). The *heterologous motifs* competitors were annealed oligonucleotides corresponding to palindromic sequences found upstream of the genes SCO0677 and SCO2657 of *Streptomyces coelicolor* A3(2) and the chitosanase gene of *Streptomyces* sp. N174 [8]. We tried also two *candidates motifs* found by bioinformatic search for other similar

motifs in the genome of *Streptomyces coelicolor* A3(2) [9]. All the *heterologous* or *candidate* competitors are of the same length (31pb) as MP12F. Finally, we tried the sequence that binds the complex N106BC2 as a negative control. This oligonucleotide is named MP34 and corresponds to the 5′-AGGGCCTTGCGCGGTG-GTGGGCGTGAACGCTTCAATC-3′ sequence found in the regulatory part of the chitosanase gene from *Kitasatospora* sp. N106 but upstream from the sequence covered by the MP12F oligonucleotide.

DNA binding reactions (24 µl) contained 10 mM HEPES (pH 7.9), 10% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.25 mM DTT, 1 µg poly(dI-dC), 150 mM KCl, 0.1nM of labeled MP12F probe, 2 µg of partially purified N106BC1 and different concentrations of competitor DNA (0.05 nM to 1000 nM). For K_d determination, different concentrations of labeled MP12F probe (0.08 nM to 2.0 nM) and a fixed amount of partially purified N106BC1 were used. The reaction mixtures were incubated at room temperature for 25 min and then subjected to electrophoresis in a pre-run gel (15 min) of 6% polyacrylamide (10 mM Tris, 80 mM glycine, 0.4 mM EDTA, pH 8.3). The electrophoresis of the samples was carried out for 60 min at 120 V. The gels were dried and the band intensity was estimated with a Phosphorimager (Molecular Dynamics). K_d for MP12F and IC_{50} calculations were done with GraFit 4 software [10].

DETERMINATION OF DISSOCIATION CONSTANT

The partly purified N106BC1 complex-forming protein was used in gel mobility shift assay in the presence of increasing concentrations of labeled oligonucleotide MP12F as ligand. The bound and free portion of the ligand were determined by densitometry. Non linear regression analysis of the plot of bound v/s free ligand (Fig. 1) resulted in a dissociation constant K_d value of 0.15 nM. This oligonucleotide concentration was then adopted for all the subsequent equilibrium competition tests.

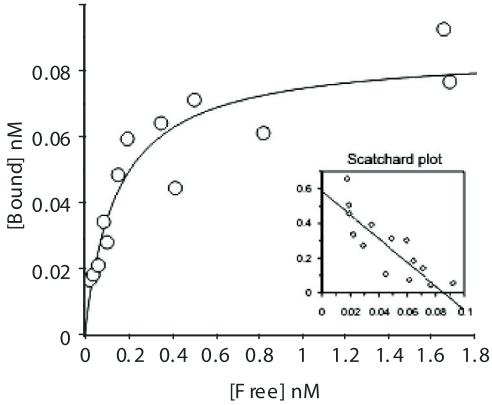

Figure 1. Determination of dissociation constant

COMPETITION TESTS WITH MUTATED OLIGONUCLEOTIDES

To determine the value of various base-pairs inside the palindromic sequence for the affinity to the N106BC1 complex-forming protein, we synthesized a series of oligonucleotides, each with one base mutated compared with the wild type palindrome. The mutations were all of transversion type, such that, for instance, a purinic nucleotide forming two hydrogen bonds when paired in double-stranded DNA was replaced by a pyrimidylic nucleotide formed three hydrogen bonds (A to C mutation). The mutations are shown on Fig. 2. On this figure, we show also the numbering of the nucleotides in the oligonucleotide MP12f as well as the nomenclature adopted for the various mutated oligonucleotides: for instance an oligonucleotide having a T (instead of a G) in position 12 was named G12T, while a nucleotide having a G (instead of a T) in position 19 was named T19G, and so on.

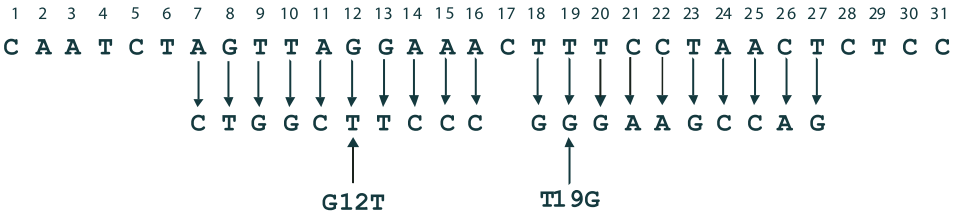

Figure 2. Sequence of the oligonucleotide MP12F and a complete listing of mutations used in this study together with examples of nomenclature of mutations

Example of equilibrium competition experiment are shown on Fig. 3. The labeled wild type oligonucleotide probe and the unlabeled competitor were mixed at the beginning of the experiment and combined with the buffer and the protein extract. After an appropriate incubation time the samples were loaded on an electrophoresis gel.

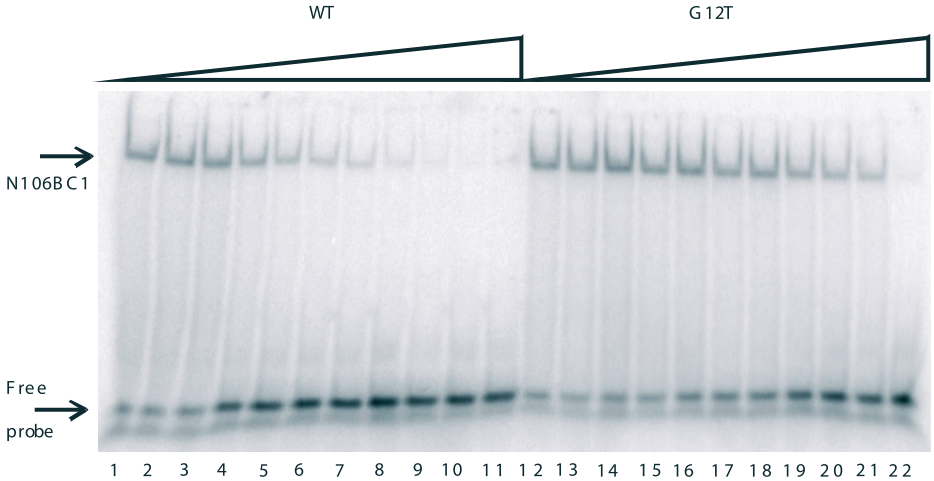

Figure 3. Equilibrium competition experiments. The labeled wild-type MP12F oligonucleotide was tested against increasing amounts of two unlabeled competitors: the homologous, wild type oligonucleotide MP12F (lanes 1–11) and the mutated G12T oligonucleotide (lanes 12–22)

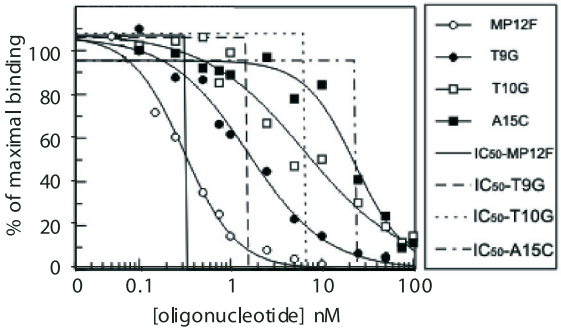

Figure 4. Equilibrium competition curves for the wild type competitor MP12F and three mutant competitor oligonucleotides. IC_{50} values were determined using the GRAPHIT software

Table 1. IC_{50} values for various oligonucleotides derived from the equilibrium competition experiments

Oligonucleotide	IC_{50} (nM)	Std. Error	K_i (nM)	$IC_{50mutant}/IC_{50wt}$
wild type				
MP12F	0.31	0.04	0.18	1.00
mutant				
A7C	0.34	0.05	0.20	1.08
G8T	1.03	0.19	0.61	3.32
T9G	1.56	0.24	0.93	5.02
T10G	6.48	1.69	3.85	20.79
A11C	6.15	0.62	3.65	19.73
G12T	3.50	0.95	2.08	11.22
G13T	0.22	0.04	0.13	0.72
A14C	1.68	0.15	1.00	5.39
A15C	23.32	2.74	13.83	74.80
A16C	0.99	0.10	0.59	3.19
T18G	1.23	0.08	0.73	3.94
T19G	32.15	4.49	19.07	103.11
T20G	1.51	0.47	0.90	4.84
C21A	0.20	0.02	0.12	0.65
C22A	2.07	0.45	1.23	6.64
T23G	5.47	0.63	3.24	17.54
A24C	4.52	0.62	2.68	14.49
A25C	1.28	0.16	0.76	4.10
C26A	1.44	0.21	0.85	4.62
T27G	0.31	0.03	0.18	0.99
heterologous motifs				
Sco0677	0.40	0.05	0.24	1.29
Sco2657	0.20	0.01	0.12	0.66
N174	0.20	0.01	0.12	0.63
binding site for N106BC2				
MP34	>1000	N.D.	>590	>3200
candidate motifs				
Sco6552-1	>50	N.D.	>30	>160
Sco6552-2	>50	N.D.	>30	>160
Sco5931	>50	N.D.	>30	>160

Twenty mutated oligonucleotides, corresponding to mutations from nt 7 to nt 27 in the sequence of MP12F nucleotide were tested in equilibrium competition experiments. The mutations covered the whole length of the palindromic sequence such as present upstream from the N106 chitosanase coding sequence (except the central nucleotide). For each mutant oligonucleotide, an IC_{50} value has been calculated and compared with the IC_{50} obtained for the homologous, wild type sequence. A similarity between the two IC_{50} values indicated that the mutated nucleotide had no influence on oligonucleotide-protein binding. Such a position in the palindrome is thus not expected to play an essential role in binding to the N106BC1 complex-forming protein. Inversely, mutations at positions important for binding are expected to have an important effect on the IC_{50} value. Examples of curves obtained with four different competitors and determination of their IC_{50} values are given on Fig. 4. The complete results for IC_{50} determinations are given in Table 1.

An elevated ratio of $IC_{50mutant}/IC_{50wt}$ indicates that the mutated oligonucleotide has lost most of its affinity for the N106BC1 complex-forming protein. An idea of how would react a non-specific sequence is given by the use of the oligonucleotide MP34 as competitor for which the ratio could not be determined (ratio > 3200). Inversely, a ratio close to 1 indicates that the mutation had no effect on DNA-protein binding (the mutated oligonucleotide behaves as the wild type sequence).

The effect of various mutations is also summarized on Figure 5 which better visualizes the symmetrical effect of most mutations on binding.

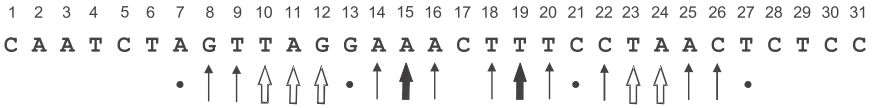

Figure 5. Summary of effects of point mutations on DNA-protein binding affinity. Bold arrows: $IC_{50mutant}/IC_{50wt}$ > 50; empty arrows, 10<ratio<20; thin arrows, 2<ratio<5; dots, ratio<2; void, not tested

We further tested DNA sequences found in published genomic sequences that could include a putative motif recognized by the N106BC1 complex-forming protein. A BLAST search with the query sequence AGGAAANTTTCCT allowed to find seven such sequences and three of them were tested: the upstream regions of the chitosanase gene from *Streptomyces* sp. N174, the putative chitosanase gene SCO0677 from *S. coelicolor* A3(2) and a putative ROK protein gene SCO2657 from *S. coelicolor* A3(2). The equilibrium competitions tests revealed that the corresponding nucleotides had a very high affinity for the N106BC1 complex-forming protein (Table1): two of them (SCO2657 and N174) had even a higher affinity that the wild type N106 sequence, as the respective $IC_{50mutant}/IC_{50wt}$ rations were lower than 1. These sequences correspond to the following mutations: SCO0677 is equivalent to an A7T + T9G + A25T + T27A mutant, SCO2657 to an A7C + G8A + T9A +, T10A+ T27C mutant and N174 to an A7G mutant.

To identify further motifs in published genomic sequences, we adopted a bioinformatic approach. We used all the known binding site sequences to generate two log-odds matrices, representing the sequences that are likely to be recognized by this transcriptional regulator (A. Gervais, unpublished). A different

matrix was created for each genome involved, since log-odds matrices take into account prior nucleotide frequencies. Searching for the best matches for these matrices yielded many putative binding sites. This algorithm identified first all of the motifs already found by BLAST as high-score sequences but also several other sequences with lower scores. We tested three of these candidate sequences in the genome of *Streptomyces coelicolor* A3(2): two were localized upstream from the long-chain-fatty-acid-CoA-ligase gene SCO6552, and one upstream from the xylanase A gene SCO5931. However, in equilibrium competitions tests, none of the corresponding oligonucleotides seemed to have a detectable affinity to the N106BC1 complex-forming protein (Table 1). These sequences corresponded to the following mutations: SCO6552-1 is equivalent to an A7C + T10C + A16T + T19G + T23G + A25C + T27A mutant, SCO6552-2 to an A7G + A11G + G12A + A14T + C22G +T23G + A24T + A25G + T27C mutant and SCO5931 to an A7G + G8A + A11T + G12C + C22G + T23C + A25C + C26A + T27A mutant.

As compared with the wild type N106 sequence, each of these low-score sequences includes changes in several critical positions such as 10, 11, 12, 15, 19, 23, 24. The cumulative effect of such mutations results in a complete loss of affinity between them and the N106BC1 complex-forming protein.

CONCLUSIONS

We identified a new palindromic motif involved in the regulation of chitosanase gene expression in *Kitasatospora sp*. N106. The motif is also found upstream from chitosanase genes in other actinomycetes such as *Streptomyces* sp. N174, *Streptomyces coelicolor* A3(2) (this work) and also *Streptomyces avermitilis* and *Streptomyces lividans* (not shown). By equilibrium competition experiments, we derived the following consensus motif: gtTAGnaAantTtncTAac. The motif could be an essential element of a regulatory mechanism for chitosanase gene expression (and, possibly, other stages of complex aminosugar metabolism) shared by a whole array of actinomycete species.

ACKNOWLEDGMENTS

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CHITOSAN AND ITS OLIGOSACCHARIDES AS *β*-SECRETASE INHIBITOR

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Key words: Chitosan, Chitooligosaccharides, *β*-secretase, molecular weight distribution

INTRODUCTION

Chitosan is a deacetylated polymer of *N*-acetyl glucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. It has shown to possess a hypocholesterolemic effect, an immunomodulating function, and a hypoglycemic effect [1]. However, recent studies on chitosan have attracted interest for converting chitosan to its oligosaccharides, because the oligosaccharides are not only water soluble but also reported to have special functional properties such as antitumor activity, immunostimulating effect, antimicrobial activity, radical scavenging activity, and angiotensin I converting enzyme inhibitory activity [2]. In the present study, β-secretase inhibitory activity of hetero-chitooligosaccharides (hetero-COSs) prepared from partially different deacetylated chitosans was investigated, and the inhibition pattern was also determined by using Dixon plots.

EXPERIMENTAL

Preparation of hetero-COSs and synthesis of sulfated hetero-COSs

Three kinds of partially deacetylated chitosans, 90%, 75% and 50% deacetylated chitosan, were prepared from crab chitin by *N*-deactylation with 40% (w/v) sodium hydroxide solution for different durations based on the previously method, and hetero-COSs, which are COSs prepared from 90%, 75%, and 50% deacetylated chitosans, were prepared by hydrolysis of heterochitosans in an UF membrane reactor system. The sulfated COSs were synthesized using general method.

Assay for *β*-secretase inhibitory activity

The inhibition assay of *β*-secretase was performed by the method of Ermolieff et al [3]. Assay for *β*-secretase inhibitory activity was performed in 0.1 M sodium acetate buffer (pH 4.0) at 37°C with 10% dimethyl sulfoxide (DMSO) using substrate FS-1. The increase of fluorescence intensity produced during substrate hydrolysis was studied in a continuous assay using Fluorescence spectrophotometer (Perkin-Elimer LS50B, Beaconsfield Bucks, UK). An excitation wavelength of 350 nm and an emission wavelength of 490 nm were used to monitor the hydrolysis of substrate FS-1. In addition, inhibition constants (*Ki*) of *β*-secretase inhibitors were calculated by Dixon plots.

RESULTS AND DISCUSSION

Different degree of deacetylated chitosan (90%, 75%, and 50%) were prepared according to the previously method. The *β*-secretase inhibitory activity of different degree of deacetylated chitosan showed that 90% deacetylated chitosan revealed the higher inhibitory activity than that of other. However, chitosan have high molecular weights and high viscosity may restrict the used in *in vivo*. Therefore, nine different kinds of hetero-COSs were prepared by an UF membrane reactor system to investigate a biological activity based on their degree of deacetylation and molecular weights. Fig. 1 depicted that COS II with 90% deacetylated showed the highest inhibitory activity on *β*-secretase. In additoin, 90-COS sulfates were synthesized from 90-COSs that exhibited the highest inhibitory activity, to investigate the effect of functional group of COSs on *β*-secretase inhibitory activity. The 90-COS sulfates showed a lower inhibitory activity compared with that of 90-COSs. Also 90-COS II were fractionated using UF membrane with 3 kDa, and investigated *β*-secretase inhibitory activity. 90-COS having 3–5 kDa molecular weights showed the highest inhibitory activity.

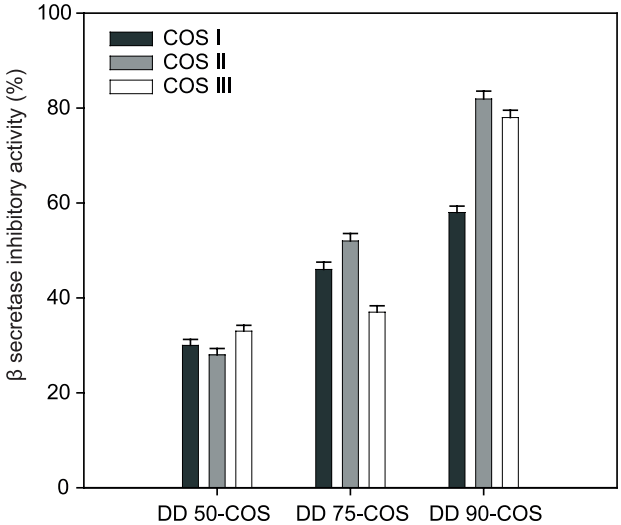

Figure 1. *β*-secretase inhibitory activity of nine kinds of hetero-COS. COS I: 5–10 kDa, COS II: 1–5 kDa, COS III: below 1 kDa

Beta-secretase inhibition pattern was found to be non-competitive at the active site of β-secretase determined using Dixon plot (Fig. 2). Thus, it strongly suggests that 90-COS having 3–5 kDa molecular weights might be bind to either another regulatory site or subsite of β-secretase. The inhibition constant (K_i) of the 90-COS having 3–5 kDa molecular weights was about 3.87~6.47 μM calculated by Dixon plot.

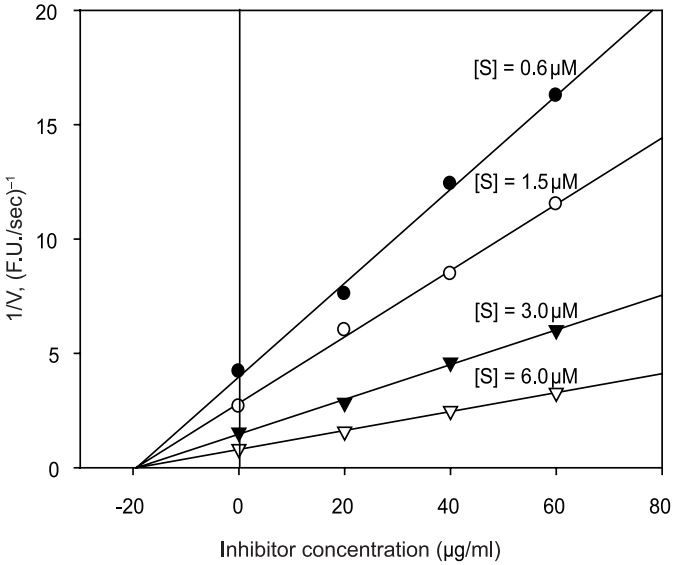

Figure 2. Dixon plot for determining inhibitor constants of 90-COS having 3–5 kDa molecular weights against β-secretase with substrate concentration

In conclusion, β-secretase inhibitory activity of COSs was dependent to relatively high degree of deacetylation, and the 90-COS having 3–5 kDa molecular weights showed the higher β-secretase inhibitory activity. Therefore, COSs is a good candidate target molecules as a β-secretase inhibitor.

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STRUCTURE, FUNCTION, AND APPLICATION OF *PAENIBACILLUS* GLYCOSYL HYDROLASE HAVING CHITOSANASE ACTIVITY AND DISCOIDIN DOMAIN

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INTRODUCTION

A chitosanase-producing bacterium is isolated in our laboratory and this bacterium is named "*Paenbacillus fukuinensis*" D-2 by the analysis of a 16s ribosome RNA gene. Cells of the *P. fukuinensis* D-2 produced chitosanase into surrounding medium, in the presence of colloidal chitosan or glucosamine.

In this study, we described the gene structure of this bactrerium-producing chitosanase, the over-production of the chitosanase by recombinant DNA in *Bacillus brevis*, and the application as a antifungal agent of the D-2 chitosanase to plants.

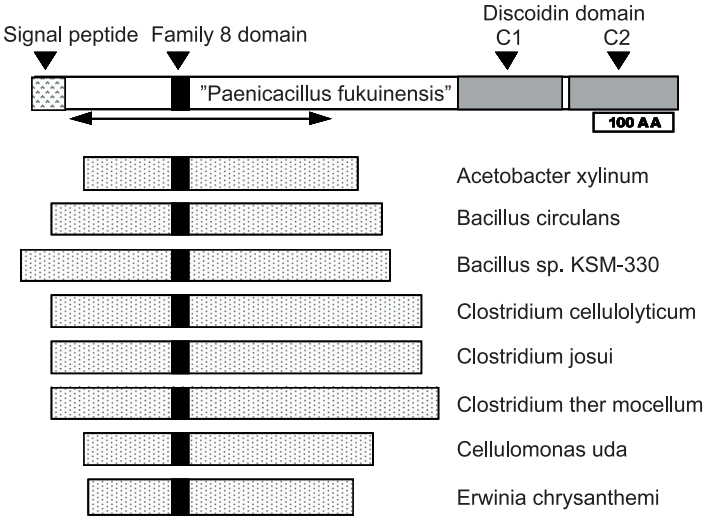

Figure 1. Gene structures of family 8 hydrolases

The gene of this enzyme was cloned, sequenced, and subjected to site-directed mutation and deletion analyses [1, 3]. The nucleotide sequence indicated that the chitosanase was composed of 797 amino acids and its molecular weight was 85610. Unlike conventional family 46, the enzyme has family 8 domain glycosyl hydrolase. The molecular weight of the D-2 chitosanase is the highest among these family 8 group (Fig. 1).

Uniquely the *P. fukuinsis* chitosanase has discoidin domain at the carboxyl-terminal region with family 8 glycosyl haydeolase catalytic domain at the amino-terminal region. The discoidin domain has been found to function as the blood coagulation factors in eukaryote [1]. Interestingly prokaryotic proteins having discoidin domain are generally pathogenic factors. We first found the discoidin domain in chitosanases.

EXPERIMENTAL

The strain D-2 of *Paenibacillus fukuinensis* was used as a sourse of DNA for gene cloning. The discoidin domains were transformed into *Echerichia coli* BL-21 using expression vector, pGEX-4T-1 containing fusion protein, glutation-s-transferase (GST). The recombinant plasmids containing inserted fragments, which contain chitosanase genes with various DNA sizes amplified by PCR, are constructed and introdued into *Bacillus brevis* HPD31-M3 and plasmid pNY301 using plasmid pNY301 as a expression-secretion vector. The nucleotide sequences of cDNA were determined with an Applied Biosystems 373A sequencer.

RESULTS AND DISCUSSION

Gene suructure of a D-2 chitosanase [1–3]

It is very intereting to clarify the disocoidin domain in the D-2 chitosanase gene. We investigated the chitosanase activity and chitosan binding ability of this domain. Three discoidin domains with different base pair sizes were amplified by PCR and named two discoidin domain, DD, one discoidin domain to the amino-terminal side, DD-N, and one discoidin domain to the carboxy-terminal side, DD-C (Fig. 2)

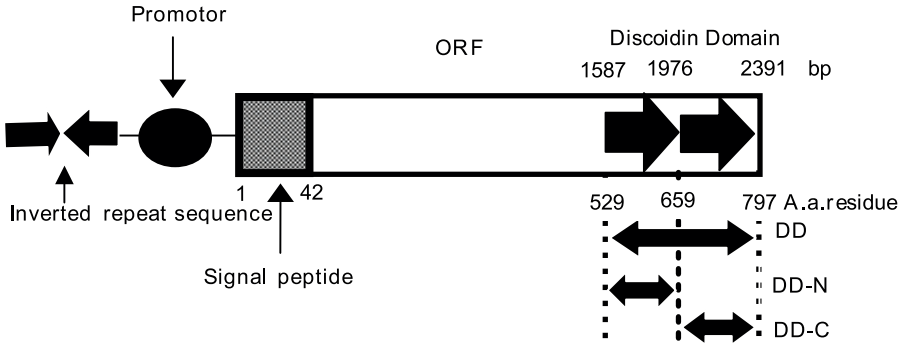

Figure 2. The expression of discoidin proteins in *Esherichia coli* BL-21 using expressin vector for fusion protein consisted of glutation-s-taransferase (GST)

These discoidin domains were transformed into *Echerichia coli* BL-21 using expression vector. The expression vector, pGEX-4T-1 produces fusion protein, glutation-s-transferase (GST). The chotosanase activity was not found in the cytoplasm of transformants. Consequently, three differet disocoidin domains showed no chitosnase activity. This indicates that the discoidin domain is not conserned in the activity. We investigated if the discoidin domain has the binding ability to chitosan. The DD-N to the amino-terminal side has been shown to function as a chitosan binding domain. Unluckily DD and DD-C proteins could not have been purified, informing inclusion body in *Echerichia coli.* The binding assay of these domain proteins are currently under investigation.

We found inverted repeat sequences consisting of 21 bp in the upstream of the promoter region. We support that the inverted repeat sequences might be negatively controlled in transcription level.

Over-prodauction of D-2 chitosanase by recombinant DNA [3]

We tried to over-produce the D-2 chitosanase by recombinant DNA. Two genes, *csnA* and *csnB* with different molecular sizes were amplified by PCR. The *csnA* gene encodes 756 amino acid except for a signal peptide composed of 41 amino acids, and the *csnB* gene encodes 384 amino acids by deleting 372 amino acids to C-terminal side. In order to over-produce the chitosanase in the medium, two genes were constructed by using *Bacillus brevis* HPD31-M3 and plasmid pNY301 as high expression vector. The plasmid pNY301 has five cell surface proteins as a expression promotor.

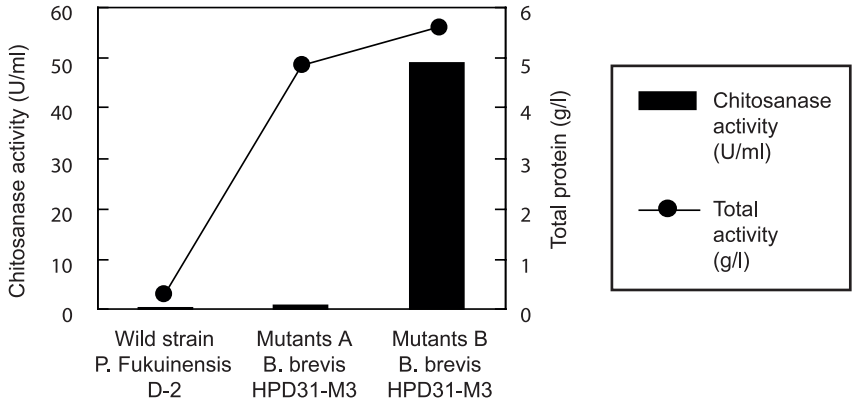

Figure 3. Activity of chitosanases produced by *Bacillus brevis* mutants

Fig. 3 shows the activity of the chitosanase from mutants. The chitosanase activity from mutant B carrying a short *csnB* gene was 48.7 U/ml in culture. The activities of wild strain and mutant A carrying *csnA* were 0.3 and 0.6 U/ml, respectively. That is, the chitosanase activity of *csnB* increased 162 fold over that of the control wild strain. From the results, the chitosanase was over-produced by using *Bacillus brevis* mutant B. Total protein contents of mutants A and B increased 15 fold over that of the control wild strain D-2.

Table 1. Biochemical properties of three chitosanases with different molecular weights produced by *B. brevis* mutants

	Chitosanase		
	41 kDa	61 Da	68 Da
Specific activity (U/mg)	723.8	3.8	12.7
Optimal temp. (°C)	55	50	50
Optimal pH	5.5	6.0	6.0
Isoelectric point	7.1	6.7	6.5
Thermal stability (°C)	20	30	40
Metal iron inhibition	Ag+, Hg++, Fe++	Ag+, Hg++, Fe++	Ag+, Hg++, Fe++
Sustrate specificity	Chitosan, CMC Lichnan	Chitosan, CMC Lichnan	Chitosan, CMC Lichnan
Hydorlysis pattern	End-type	End-type	End-type

Table 1 shows biochemical properties of three chitosanases, 41, 61, and 68 kDa produced by mutants. The two chitosanases, 61kDa and 68 kDa were purified from mutant A. The 41kDa chitosanase was purified from mutant B. The specific activities of the 41, 61 and 68 kDa chitosanases were 723.8, 3.8, and 12.7 U/mg protein, respectively. Thus, the 41 kDa chitosanase had high activity, compared with those of two chitosanases of 61 kDa and 68 kDa. The Optimal temperature, the Optimal pH, the isoelectric point, and thermal stablility were slightly shifted with the molecular size of these enzymes (Table 1). This indicates that the molecular size of the enzyme affects these biochemical characterizartion.

Application for antifugal agents of D-2 chitosanase to plants

We investigated whether the D-2 Chitosanase can be used as a biochemical agent by spraying the enzyme onto pathogenic fungi infecting vegetable leaves and strawberries. The D-2 chitosanase consequently inhibited the resistance to pathogenic fungi infecting the plants. Using the 41 kDa chitosanase, the effect of the chitosanase on a fungous *Botrytis cinerea* infecting cabbages was investigated. The cabbages discolored to yellow by infecting the fungi. By spraying the chitosanase enzyme of 10.6 U and 106 U per one strain, the coloration of cabbages were not found and the spots of a foungous *Botrytis cinerea* infected on the surface of the cabbage leaves were found (data not shown). These spots were not observed by the chitosanase treatment.

We also investigated the effect of the D-2 chitosanase on a pathogen, *Fusarium solani* infecting strwaberries. The growth of the mycelia of *Fusarium solani* was observed on the surface of strawberries after the incubation of 24 to 48 hr at 25°C. The mycelia was not formed by spraying the chitosanase solution. These results indicate that the D-2 chitosanase enzyme may be useful for a biocontrol agent.

CONCLUSIONS

Cells of a new species, "*Paenibacillus fukuinensis*" D-2 produced chitosanase into medium, in the presence of colloidal chitosan. The enzyme has family 8 glycosyl hadrolase catalytic domain at the amino-terminal region, inverted repeat sequence at the beside of promotor region, and discoidin domain at the carboxy-terminal side. The discoidin domain has been shown to function as the chitosan binding domain. The *P. fukuinensis* D-2 chitosanase was over-produced by *Bacillus brevis* HPD-M3 into medium using an expression-secretion vector, plasmid pNY 301. The D-2 chitosanase might be useful for biodegradable biocontrol agent having antifungal activity, instead of conventional fungicides.

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THE GENERAL RELATIONSHIPS OF CHITIN AND CHITOSAN CHEMICAL HYDROLYSIS

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INTRODUCTION

The chemical hydrolysis is the basic industrial method of chitin and chitosan processing in great volumes. Main its advantages are high yields of products, fast speeds of transformation, low cost of expenses for processing. Its basic lacks are formation of difficultly utilizable waste products, difficulty of monitoring of product properties.

The improvement of chemical methods of processing demands detailed studying of mechanisms of the hydrolysis in acid and alkaline condition, an establishment of the main relationships of chemical transformations.

By the consideration of a chemical hydrolysis of chitin and chitosan we have made attempt to connect in uniform pattern the reactions in alkaline and acid medium, to establish the common laws and the main distinctions of these processes.

On the establishment of the comparison of these two processes we wanted to find out the basic mechanisms of chemical hydrolysis. In the present report we have tried to touch the key moments in the study of hydrolysis of glycosidic and amidic linkages in chitin and chitosan.

OBJECTS AND METHODS

As the objects of researches chitin, chitosan, D-glucosamine hydrochloride (GlcNHCl) and *N*-acetylglucosamine (GlcNAc), obtained from the shell of Northen shrimp and Antarctic krill by common methods [1] were used.

The contents of amino groups, and also degree of deacetylation (*DD*) of chitosan and concentration of GlcNHCl were determined by potentiometric and conductometric titration of solutions in 0.1 M HCl by 0.1 M NaOH [2].

Number-averaged molecular mass (*MM*) of chitosan was determined by a viscosity method (0.334 M CH_3COOH and 0.3 M NaCl, 21°C) and calculated from the Mark-Houwink-Sakurada equation [3].

Molecular-mass distribution of products, concentrations of GlcNAc and acetic acid (AcOH) were estimated by SEC HPLC on LC-10Avp (Shimadzu Corp., Japan) with column TSK-gel Alpha-2500 and Alpha-4000 (30 x 0.78 cm) (Tosoh, Japan) and detected on optical absorption at 210 nm. Eluent was 0.3 M NaCl acidulated up to pH 3.0 HCl.

DISCUSSION

The chemical hydrolysis of chitin and chitosan consists in breakage of amidic and glycosidic chemical linkages. The deacetylation and depolymerization took place in the first and second cases accordingly. The deacetylation is a basic process in alkaline hydrolysis. Acid hydrolysis of CN and CS breaks off both glycosidic and amidic linkages.

Preliminary results of studying of deacetylation kinetics of chitin from various sources (the shrimp and krill) have shown, that the kinetics practically does not depend on particle size (from 0.05 up to 5.0 mm) and a source of chitin (the shrimp or krill) [4].

We have assumed, that such results are caused by the form of chitin particles of the shrimp and krill, representing thin flakes which intrinsic areas are easily accessible from the side of planes.

Deacetylation

At the deacetylation of chitin in alkaline condition it was found, that it is not possible to prepare completely deacetylated product by continuous processing in alkali. Only after washing of intermediate product it is possible to increase easily the degree of deacetylation practically up to 100% [4, 5].

Kinetic curves of continuous alkaline deacetylation in the homogeneous and heterogenous conditions had a similar kind and have consisted of the area of fast increase of the degree of deacetylation and the flat area, not achieving the full deacetylation [4, 6].

It is common view that the total rate of deacetylation consists of rates of amidic linkages hydrolysis in amorphous and crystalline areas of chitin [6]. The fast deacetylation is observed in amorphous areas, the slow – in crystalline ones. After full deacetylation of the amorphous areas the hydrolysis rate falls up to the rate in the stayed crystalline regions. It determines the characteristic form of the kinetic curves. After washing, the part of the crystalline regions gets the amorphous form because of increase of the degree of deacetylation. During the following step of processing the kinetics of deacetylation repeats the kinetics of the previous step.

It is known, that the portion of crystalline structure in chitin decreases with increase of degree of deacetylation and then it is disappears. At high values of degree of deacetylation (about 95%) the crystalline structure of chitosan starts to form. It is necessary to expect, that the kinetics of alkaline deacetylation would be differ for samples with various initial degree of deacetylation.

We have shown experimentally, that the form of the kinetic curves of deacetylation of samples with DD from 15 up to 93% is identical with high accuracy [4, 7]. It was made the conclusion that the mechanism of deacetylation is identical

to anyone initial degree of deacetylation. This result contradicts the theory about the dependence of deacetylation kinetics from inhomogeneous crystallinity of chitin samples.

We also have carried out the comparison of the deacetylation kinetics of chitosan samples with the initial and destroyed structure (re-precipitated). For all samples we have obtained practically full coincidence of kinetics of DD change and molecular mass.

If the influence of crystalline structure on the deacetylation kinetics is not observed, what is the cause of observable deceleration of deacetylation process?

We have made the assumption of existence of two simultaneous reactions. The first reaction leads to the deacetylation, the second – to the formation of low-activity product (for example, a complex with OH^-) which is destroyed at washings.

Performance of Invariant I has been shown [4, 7]. Hence, these two reactions are reactions of the first or pseudo-first order [8]. Rates of these both reactions are proportional to concentration of acetylated units or degree of acetylation (DA).

We have obtained the performance of the following expression:

$$\Delta DD(t) = \frac{\Delta DD(t, DA_{i0})}{DA_{i0}}$$

During the studying of the acid deacetylation of chitin and chitosan we have obtained the same interrelation for the acetic acid formation [9]:

$$C_{AcOH}(t) = \frac{C_{AcOH}(t, DA_{i0})}{DA_{i0}}$$

Total deacetylation achieves 100% in this case.

The obtained dependences have allowed to make an assumption that the deacetylation can be considered as the homogeneous reaction of the interaction of acetylated units with hydroxyls or protons. Mathematical treatment of results has allowed to calculate the rate constants of the basic reactions in alkaline [4] and acid deacetylation [10] and to fit calculated kinetic curves, with high adequacy with experimental results (Fig. 1).

Alkaline hydrolysis:

$A_n + OH^- \rightarrow D_n + AcO^-$; $k = 0.064\ min^{-1}$
$A_n + OH^- \rightarrow [A_n . OH^-_m]$; $k = 0.033\ min^{-1}$

Acid hydrolysis:

$A_n + H^+ \rightarrow D_n + AcOH$; $k = 0.029\ min^{-1}$

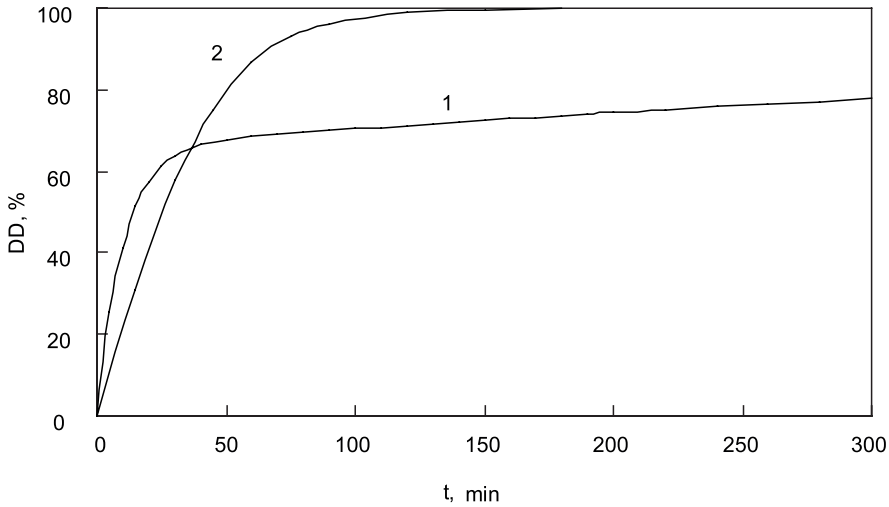

Figure 1. Deacetylation of chitin in alkaline and acid conditions. (1) 50% NaOH, 95°C; (2) 35.8% HCl, 80°C

Depolymerization

Alkaline and acid depolymerizations are differing.

Acid depolymerization depends from initial degree of acetylation of chitin. As known, it explains by considerably more high rate of acid hydrolysis of glycosidic linkages at the redicung ends of acetylated monomer units (A) in comparison with those at deacetylated ones (D) [9, 11, 12]. Because of irregular distribution of A and D units along the polymeric chain the formation of the following products will be observed: from the reducing end of the molecule – A, D, DA, DDA, DDDA, etc.; from non-reducing end – A, AA, AAA, etc., and also the same partly deacetylated oligomers.

A-|-A-|-D--D--D-A-|-D--D--A-|-A-|-D--A-|-D

Non-reducing end	Reducing end
A, D-A, D-D-A, D-D-D-A …	A, D, D-A, D-D-A, D-D-D-A …

As a result, the growth of DD of chitin and chitosan leads to the increasing of the amount of deacetylated oligomers because in acid conditions its are essentially more stably then acetylated oligomers. The yield of monomers A and D will decrease. The experimental results submitted by SEC HPLC of hydrolysis products [9].

Taking into account, that simultaneously the deacetylation takes place during the acid hydrolysis, all products, including GlcNAc, will be deacetylated and in a polymeric chain the number of single or located successively D units will increase. It will lead to increase of yields of deacetylated di- and higher oligomers and to decrease of the yield of monomer GlcNHCl.

Thus, we cannot obtained 100% yield of GlcNHCl even at acid hydrolysis of the completely acetylated chitin. It will be less on magnitude of the yield of deacetylated chitin oligomers.

It is impossible to exclude completely a possibility of decomposition of glycosidic linkages in deacetylated parts of the chitin molecule. The rate of this reaction is very low and, probably, brings the contribution to the slow increase of GlcNHCl yield during long acid hydrolysis. But in this case the process of GlcNHCl destruction (deamination) starts to affect. It leads to the decrease of the content of amino groups in hydrolysate [10].

It is quite possible, that oxidation decomposition takes place during the acid hydrolysis.

During the alkaline hydrolysis only slow decrease of the molecular mass.is observed. Usually (or exclusively) only insoluble products (a polymer part of all products) are considered in various investigations of alkaline hydrolysis of chitin. The information about low molecular weight soluble oligomers and monomers is not present in the literature practically. This fact is due to the extremely nonresistant of these substances in strong alkaline solutions.

The investigations of chemical hydrolysis are continued in our laboratory and new results would able to add greatly to our knowledge of reaction mechanism.

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EFFECTS OF PHOSPHORYLATED CHITOSAN OLIGOSACCHARIDES ON CALCIUM ABSORPTION IN THE OVARIECTOMIZED RAT

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Key words: phosphorylated chitosan oligosaccharides, calcium absorption, ovariectomized rat

INTRODUCTION

Calcium (Ca) is known to be an essential element required for numerous functions in our body including strengthening of teeth and bones, nerve function, and enzymatic interactions. Generally, Ca is supplied from various foods in the diet. Dairy products, such as milk and cheese, are known as the most effective and trusted Ca sources because these products have not only a large amount of Ca but also a significant Ca bioavailability [1]. However, many Asians do not easily take dairy products like milk because of lactose indigestion and intolerance which make them allergic to milk. Thus, for increasing Ca bioavailability, various materials has been studied as alternatives for dairy foods, and Ca-casein phosphopeptides (Ca-CPPs) were elucidated as a representative Ca-fortifier due to its ability to maintain solubility even at neutral pH in the ileum. In this paper, we demonstrated that phosphorylated chitosan oligosaccharides (P-COSs) have a potent Ca-bioavailability by *in vitro* Ca-solubility test and *in vivo* Ca-absorption test using an ovariectomized rat.

EXPERIMENTALS

Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). Chitosanase (35,000 U/g protein) derived from *Bacillus* sp. was purchased from Amicosen Co. (Jinju, Korea). Utrafiltration membrane reactor system for production of hetero-COSs was from Millipore Co. (Bedford, USA).

Methods

Preparation of P-COSs

Phosphorated chitosan oligosaccharides (P-COSs) were prepared from 90% deacetylated COSs with various ranges of molecular weights (>10 kDa, 10–5 kDa,

5–1 kDa and <1 kDa) according to the method by Nishi et al [2]. To the mixture of COSs (2.0 g) in methanesulphonic acid (14 ml) was added phosphorus pentoxide (0.5–4.0 mol. equiv. to the residue) and the mixture was stirred at 0–5°C for 3 h under moisture-free condition. The products were precipitated with ether and centrifuged. The residue was washed with ether (X5), acetone (X3), methanol (X3) and ether (X1), and then dried.

Ca solubility test

Various concentrations up to 500 mg/l of samples were mixed with 5 mM CaCl2 and 20 mM sodium phosphate buffer, pH 7.8 [3]. The mixture was stirred at 22°C for 30 min, and the pH was maintained at 7.8 with a pH meter. After removal of insoluble calcium phosphate salts and filtration using a 0.45 μm membrane, calcium contents of the supernatant were determined by the flame atomic absorption spectroscopy (FAAS). The experiments was performed in triplicate, values were expressed as mean ± SD.

Design of in vivo test

Sprague-Dawley rats (6 weeks old, female, body weight between 180–200 g, 8 rats per 1 group) were ovariectomized, and fed a free-calcium diet (AIN-76 purified free-Ca diet, ICN pharmaceuticals Inc.) for 4–6 weeks in order to induce osteoporosis-modeling rats. After examination of bone density, the rats were classified into 4 groups; free-Ca diet, CaCO3 diet, CPP + CaCO3, and P-COS + CaCO3. After 4 week diets, the rats were sacrificed and the following in vivo tests were performed; the calcium concentration in serum, urine and feces, bone strength and density in femur.

RESULTS AND DISCUSSION

In order to fortify calcium absorption acceleration activity of chitosan oligosaccharides (COSs), the COSs separated into various ranges of molecular weights (>10 kDa, 10–5 kDa, 5–1 kDa and <1 kDa) were phosphorated by reaction with sodium trimetaphosphate (Fig. 1)

Phosphorated COS (P-COS) with the range of molecular weight (MW), 5–1 kDa had the highest bioavailability *in vitro* (antiprecipitation activity in phosphate solution) than that of P-COSs with other molecular weights, and then the P-COS with MW 5–1 kDa was applied on *in vivo* test (Fig. 2).

Six-week-old female Sprague-Dawley rats were ovariectomized and fed experimental diets containing a low-calcium diet (0.5% Ca, 0.4% P) for 6 weeks in order to investigate effects on calcium and bone metabolism. The final calcium concentration in serum and urine was significantly higher than that of basal (fed $CaCO_3$ as calcium source) and COS diet group and the concentration in feces was lower than both. Moreover, bone strength and density in femur is significantly higher than both. These results demonstrated that P-COS had a potent calcium-absorbable material like casein phosphopeptide and definitely involved in calcium metabolism *in vivo* (Fig. 3).

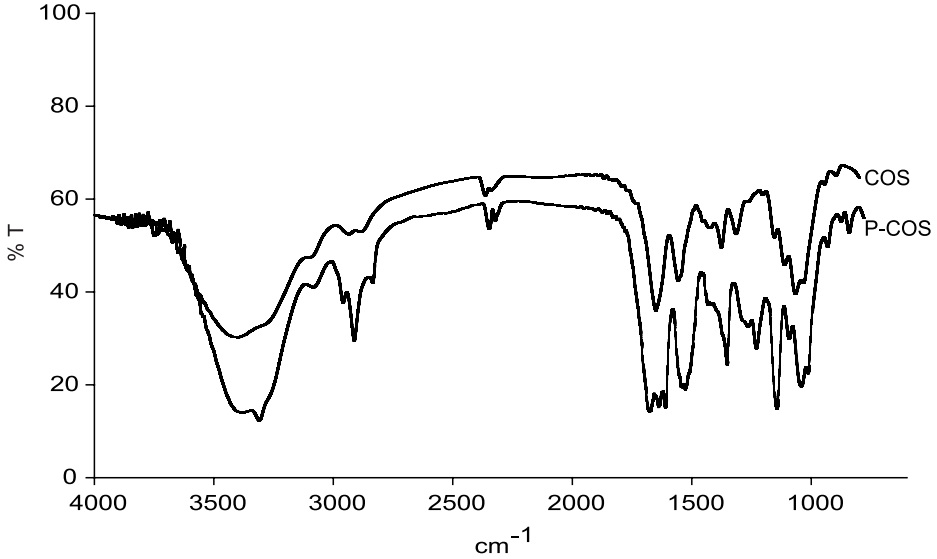

Figure 1. FT-IR spectra of COS & P-COS

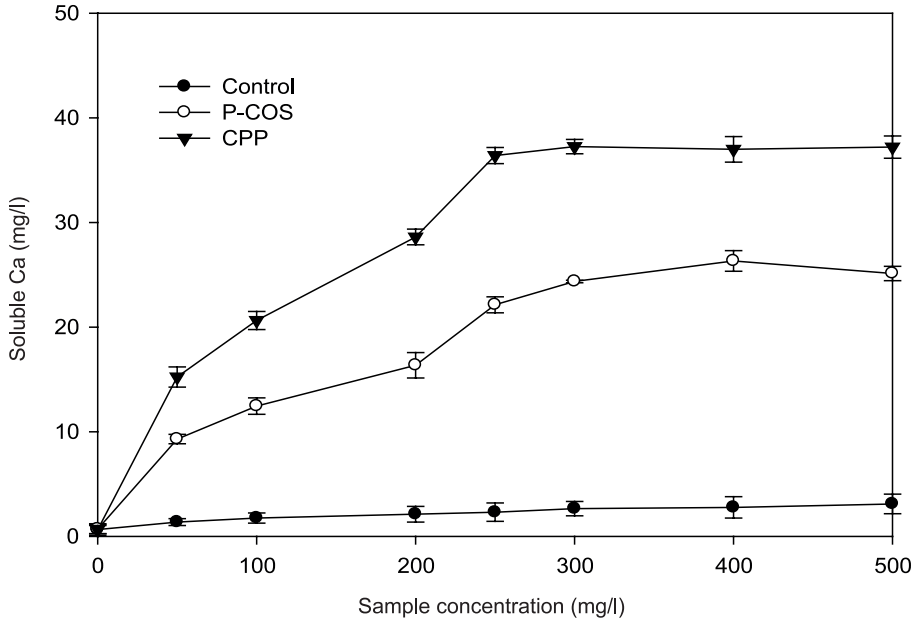

Figure 2. *In vitro* Ca-Solubility of P-COS

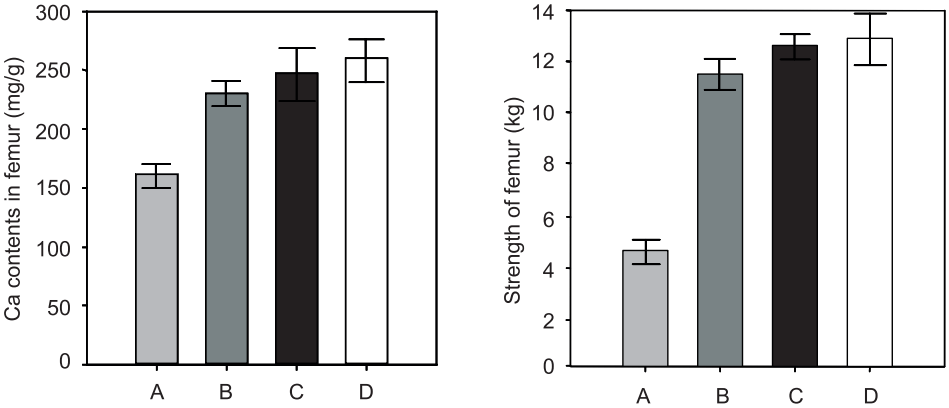

Figure 3. Measurements of Ca contents and strengths in femur after 4 week diets A, lack of Ca; B, CaCO3 ; C, CPP + CaCO3; D, P-COS + CaCO3 group (Mean ±SEM of 8 rats per group; $p<0.05$, ANOVA followed by Duncan's multiple range test)

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β-N-ACETYLHEXOSAMINIDASES FROM KOREAN GINSENG (PANAX GINSENG C MEYER) ROOTS

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Key words: *β-N*-acetylhexosaminidases, Ginseng roots, isoformes

INTRODUCTION

Panax ginseng is renowned for its beneficial effects. The root of this plant induces cell-proliferation [1], stimulates immune responses [2], DNA and protein synthesis [3], and inhibits human platelets [4]. It may also be useful for its hypoglycemic [5] and anti-lipolytic effects [6].

Plant *β-N*-acetylhexosaminidases(Hex) are much less studied than those of animal and bacterial origin. Plant Hexes have been characterized in kidney bean (*Phaseolus vulgaris*) [7] and in jack bean (*Canavalia ensiformis*) [8]. In particular, most investigations were carried out in seeds, young seedlings or leaves [9–11]. In the course of studying chitinases from Korean ginseng roots [12], a high activity of Hex was also observed in the extract of one-year old roots.

We have purified a Hex devoid of chitinases using ion exchange chromatography, hydrophobic interaction chromatography (HIC) and gel filtration. Apparent molecular weight, optimal temperature and pH, Vmax and Km of the purified enzyme were studied.

EXPERIMENTAL

Extraction and purification of a Hex from Ginseng roots

The whole roots including rhizomes, main and lateral roots were minced with Ace-homogenizer. *β-N*-acetylhexosaminidases were extracted as previously described [12]. The protein extracts were centrifuged at 3,000 g for 10 min and then at 100,000 g for 60 min. The freeze-dried extracts were re-dissolved in 0.02 M Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer before the anion exchanger chromatography. Source-Q anion exchange column was eluted with

a linear gradient of NaCl from 0.0M to 1.0M QF2 fraction was freeze-dried and dissolved in the buffer for HIC. The QF2 fraction was applied on Phenyl-Sepharose HP column and eluted with a linear gradient of $(NH_4)_2SO_4$ from 2.0 M to 0.0 M. After dissolving the freeze-dried HF2 fraction in 0.04M citrate phosphate buffer (pH 4.0), the fraction was loaded on a Source-S column previously equilibrated with the same buffer. The column was amply washed with the same buffer and eluted with a linear gradient of NaCl from 0.0M to 1.0M. The active fraction QF1 from Source-S was loaded on a Superdex 75 column. The active fraction GF from gel filtration was collected and freeze- dried until use.

Enzyme assays

Chitinase activity was measured using [^{3}H]-chitin suspended in the extraction buffer (1 mg/ml, w/v), and 100 μl were taken under continuous agitation. Four hundred and eighty eight dpm were equivalent to 5 nmol of dry chitin. Hex activity was determined with 4-methylumbelliferyl-*β-N*-acetylglucosaminide dissolved in McIlvaine buffer (pH 4.0, 0.1 M). After incubation in 98-well microtitre plate, the reaction was terminated by addition of 100 μl of 1 M glycine/NaOH (pH 10.6). Fluorescence was monitored (Excitation 355 nm, Emission 460 nm) using a microtitre plate fluorometer (Fluoroskan II). One microunit (μU) of the enzyme activity was defined as 1.0 n mole of 4-MU realeased per minute at 50°C.

RESULTS AND DISCUSSION

Purification of Hex

As seen in Fig. 1A, three fractions QF1, QF2 and QF3 showed both chitinase and Hex activity. Since QF2 fraction was most abundant in Hex activity, this fraction was chosen for further purification. HIC of QF2 fraction was showed in Fig. 1B. HF2 fraction of HIC had the highest specific activity. Although two active fraction (SF1 and SF2) were obtained from HF2 fraction, the unbound fraction (SF1) of the cation exchanger chromatography had a higher specific activity (Fig. 1C). This SF1 fraction was further purified on the gel filtration chromatograph. As seen in Fig.1D, a single Hex activity was detected in GF fraction. Its molecular weight was calculated to be 71kDa.The GF fraction was purified 1,137 folds with a yield of 3.95%. These results were summarized in Table 1.

Table 1. Purification of the Hex from one-year old Ginseng roots

Steps	Total protein (mg)	Total activity (μ Unit)	Specific activity (μ Unit/mg)	Yield (%)	Purification (fold)
Total Homogenate	1,150	11,485	9.98	100	1
QF-2	95	10,814	113	94	11.3
HF-2	1.6	7,966	4,978	69	498
SF-1	0.08	844	10,550	7.3	1057
GF	0.04	454	11,350	3.95	1,137

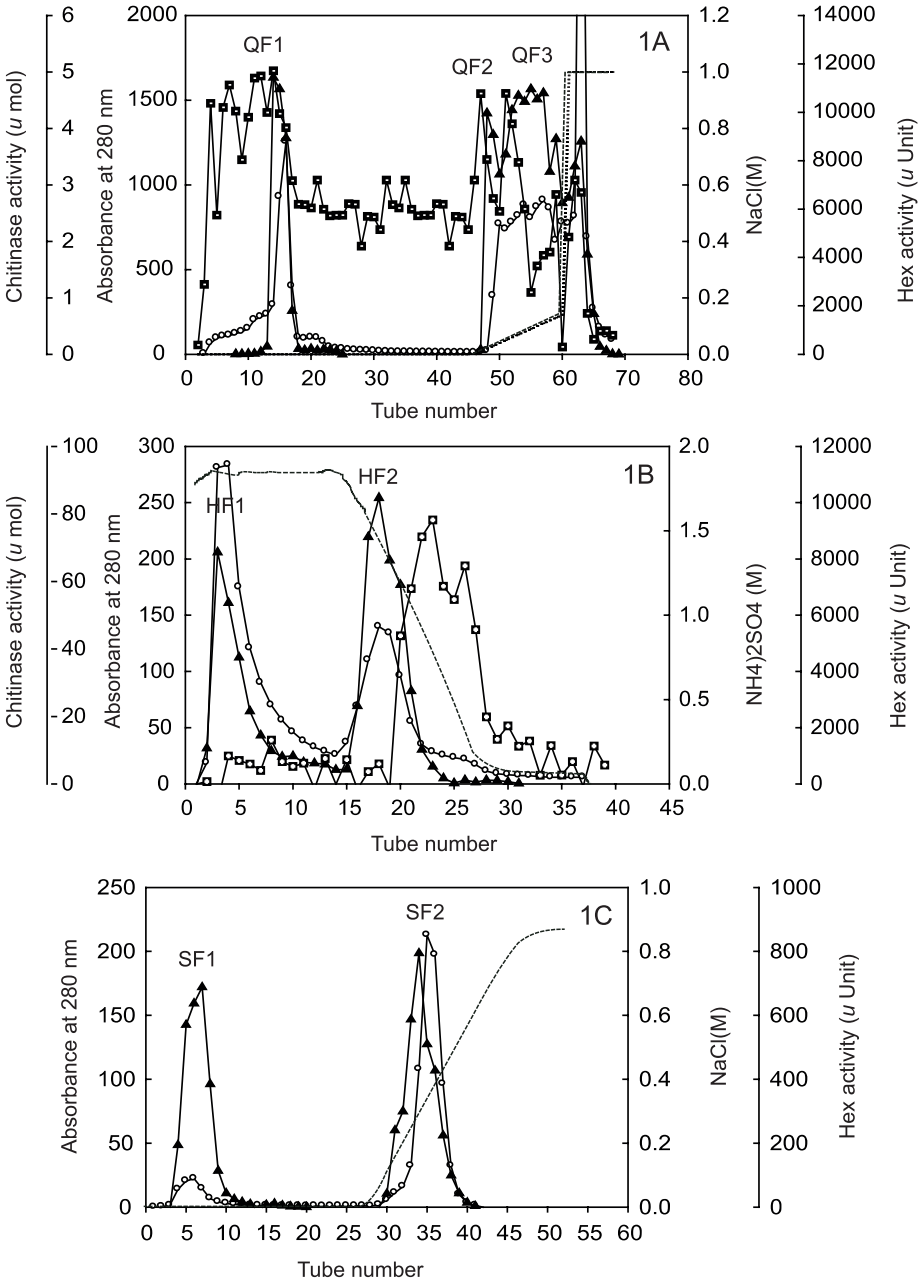

Figure 1. Chromatographic separation of the Hex

1A: Source-Q chromatogram, 1B: Hydrophobic interaction chromatogram
1C: Source-S chromatogram, 1D: Gel filtration chromatogram
—▲— : Hex activity, —○— : UV Absorbance at 280 nm (mAU),
—◘— : Chitinase activity, _ … _ : NaCl concentration

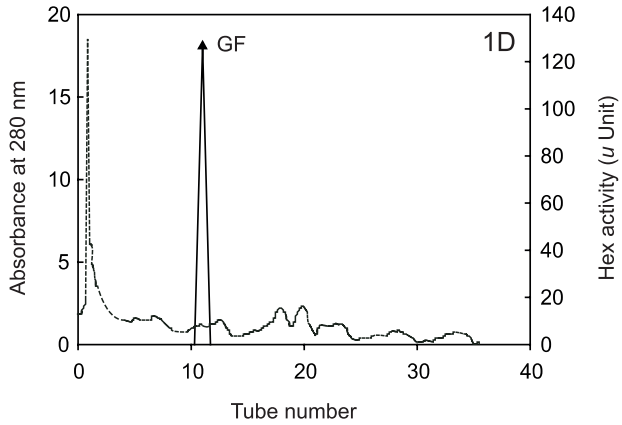

Figure 1. Chromatographic separation of the Hex

1A: Source-Q chromatogram, 1B: Hydrophobic interaction chromatogram
1C: Source-S chromatogram, 1D: Gel filtration chromatogram
—▲— : Hex activity, —○— : UV Absorbance at 280nm (mAU),
—◘— : Chitinase activity, —…— : NaCl concentration

Enzymatic properties of the purified Hex

The optimal pH of the purified Hex was 5.0 (Fig. 2A). However the optimal temperature ranged from 40 to 60°C. This wide range of optimal temperature is quite unique compared with other plant Hex enzymes. K_m and V_{max} of the Ginseng Hex were 0.326 mM and 0.569μmoles/mg, respectively.

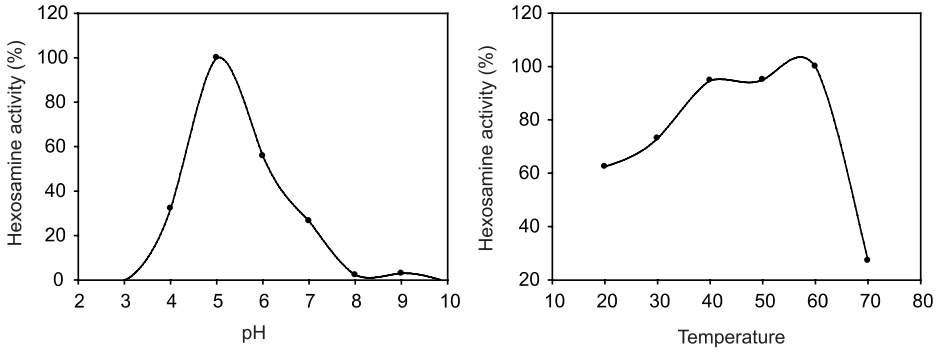

Figure 2. Optimal pH and temperature of the purified Hex 2A. Optimal pH, 2B: Optimal temperature,

CONCLUSIONS

We purified and characterized a *β-N*-acetylhexosaminidase from 1-year old Korean Ginseng roots. The molecular weight of the Hex was 71kDa on gel filtration chromatography. The optimal pH and temperature of the enzyme were 5.0 and 40–60°, respectively. The Km and Vmax against 4-MU-NAG were 0.326 mM and 0.569 μmol/mg protein-hr, respectively.

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DISTILLATION OF ACETIC ACID FROM CHITOSAN SOLUTIONS UNDER LOWER PRESSURE

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INTRODUCTION

Chitin is next to cellulose the most abundant polymer. Chitosan, derivative of chitin, has found many applications in medicine, plant protection, environment protection or waste water treatment [1].

Chitosan is produced on industrial scale by treating chitin with concentrated NaOH solution at elevated temperature. It is difficult to produce chitosan having reproducible characteristics in the chemical process. Another disadvantage of the chemical method are highly concentrated wastes [2]. The enzymatic deacetylation can be an interesting alternative to the chemical process. Using an enzyme, chitin deacetylase found in fungi belonging to Zygomycetes [2, 3], it is possible to reduce acetylation degree while avoiding strong pollution. Chitin deacetylase is an enzyme hydrolyzing the bond between amine group and acetyl group in the *N*-acetylglucosamine mers of chitin and chitosan. Chitosan with lower acetylation degree and acetic acid are obtained as a result of action of this enzyme [4].

The enzymatic deacetylation of chitin or chitosan was earlier investigated in the HCl solution. At these conditions a slow chemical deacetylation was also observed (data not published). Due to inhibitory effect of acetic acid on enzymatic deacetylation (AcOH was reported as a strong inhibitor for chitin deacetylase [5, 6]) it is necessary to remove it from the reaction solution prior to enzymatic process.

Among several possible methods the evaporation of acetic acid under lowered pressure can be applied. At pressure lower than 350 Torr the boiling point of acetic acid is lower than the boiling point of water which increases the efficiency of evaporation.

The aim of the present work was to investigate the distillation process of acetic acid from chitosan solution under conditions of lowered pressure. The correlation between pressure and the change of the amount of acetic acid the in reaction mixture was also evaluated.

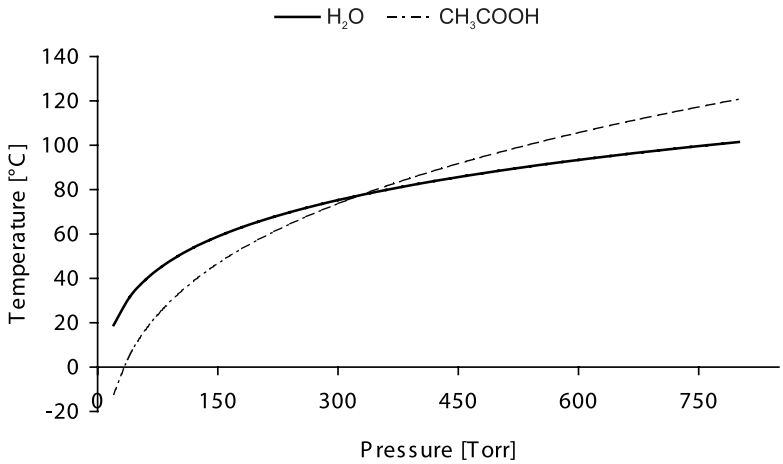

Figure 1. Dependence of boiling points on pressure for water and acetic acid

MATERIALS AND METHODS

Materials

Chitosan with acetylation degree of 27% was kindly donated by Gilett-Manhtani-Chitosan (FRANCE, INDIA). Acetic acid (0.1 M) was supplied by Sigma-Aldrich, other chemicals were supplied by Polish Chemical Reagents (POCh).

All chemicals were of the analytical grade.

Experimental set-up

All experiments were carried out in a termostated, stirred reactor with a volume of 800 mL containing 500 mL of chitosan solution (1 mg/mL) in HCl solution (pH 4.0 – optimal pH for chitin deacetylase). A desired amount of 0.1 M acetic acid was added into reaction mixture. Experiments were carried out at 50°C (temperature optimal for chitin deacetylase [7, 8]), and at pressure 240–420 Torr.

Every 10 minutes the reaction mixture was sampled and acetic acid was determined.

Analytical methods

Concentration of acetic acid was determined using Waters HPLC system with 410 Differential Refractometr. A Shodex KC-811 Column (7mm, 8x300mm, 40°C) with 0.1% H_3PO_4 solution was used (1 mL/min) as an eluent.

RESULTS

The rate of evaporation of acetic acid from chitosan solution under lowered pressure was investigated. Chitosan solutions were termostated at three different pressures: 240, 300, and 400 Torr. 0.1M acetic acid (8.4 ml) was added into investigated mixture. The changes of the amount of acetic acid in the reaction mixture are presented at figure 2.

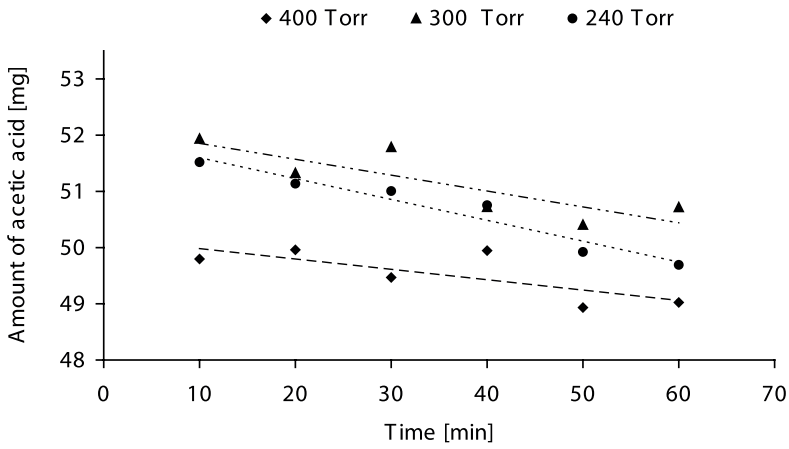

Figure 2. Changes of the amount of acetic acid in the reactor mixture during process

The amount of acetic acid in the reaction mixture decreases linearly during distillation process. The rate of evaporation of acetic acid from the reaction mixture increases linearly with decreasing the pressure (Fig. 3).

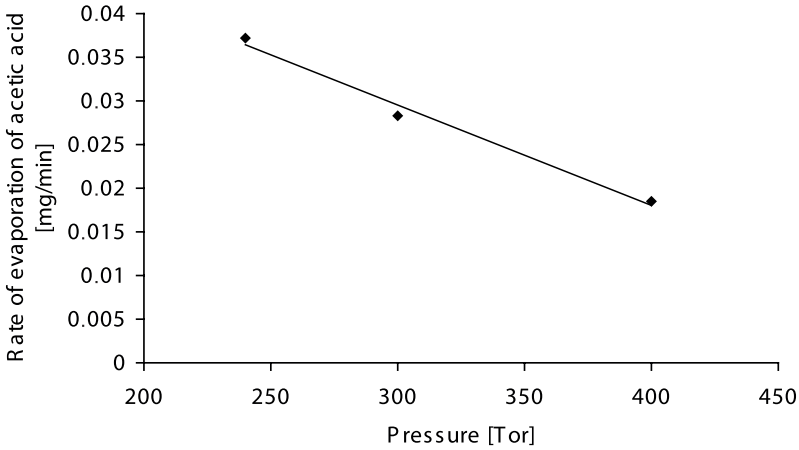

Figure 3. Dependence of the rate of evaporation of acetic acid on the pressure

In the next series of experiments the pressure was kept at constant level (240 Torr) and different amounts of 0.1 M acetic acid solutions were added to reaction mixture (8.4, 4.2, 2.1 ml respectively) (Fig. 4).

The highest rate of evaporation of acetic acid was observed for the mixture with the highest initial amount of acetic acid. The linear correlation between the rate of evaporation of acetic acid and the initial concentration of acetic acid was observed (Fig. 5).

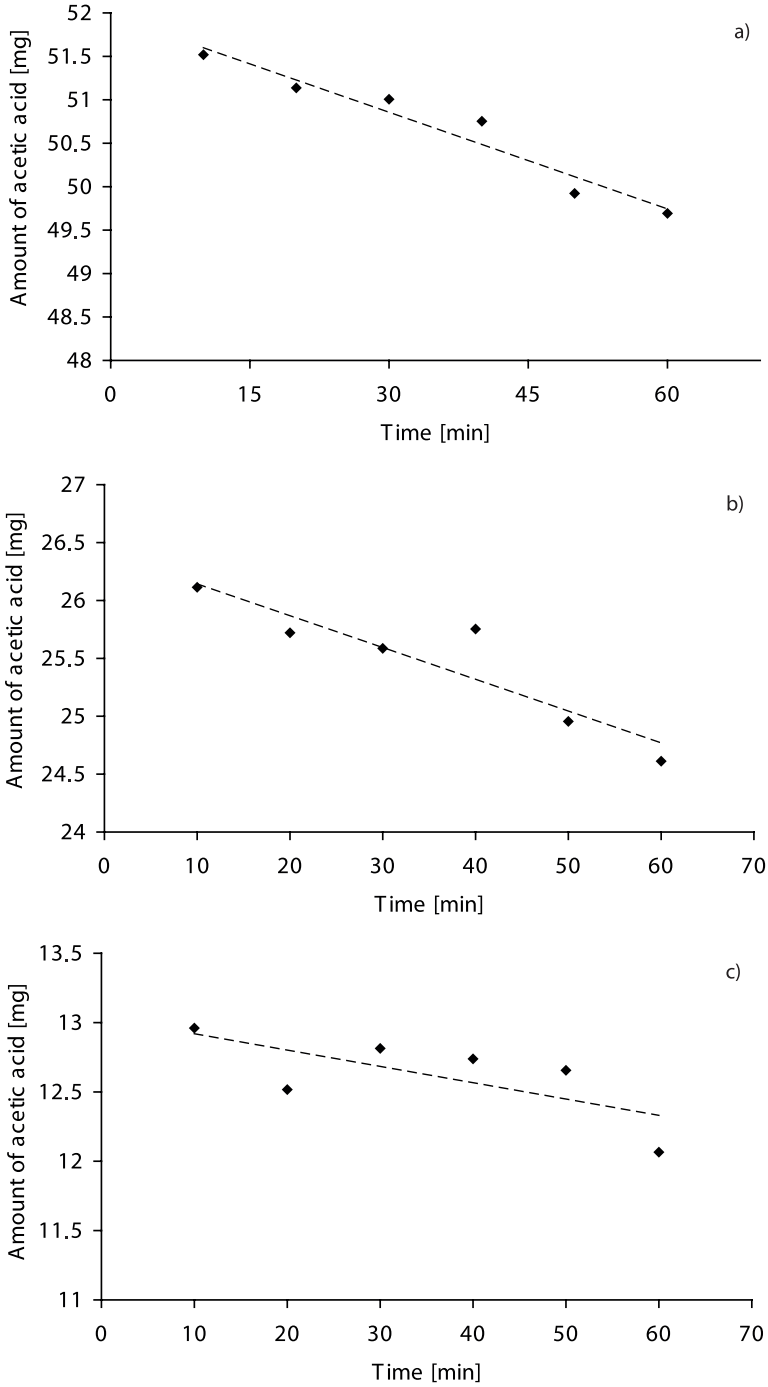

Figure 4. Changes of the amount of acetic acid in solution during distillation process. Pressure – 240 Torr; temperature 50°C; initial concentration of acetic acid: a) 100 mg/L; b) 50 mg/L; c) 25 mg/L

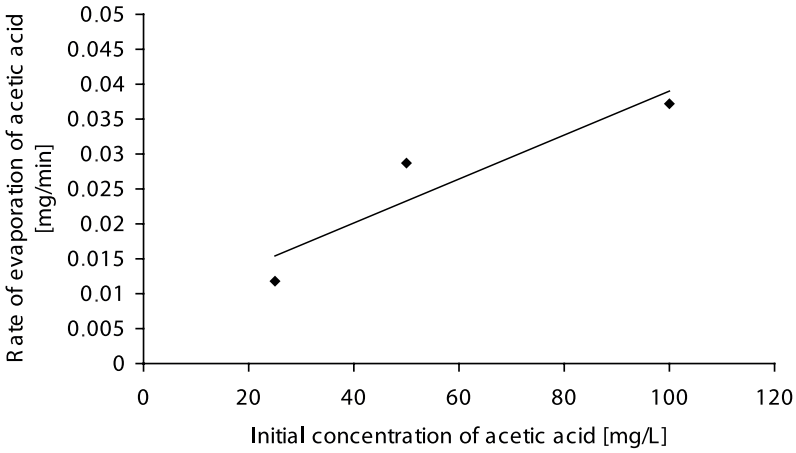

Figure 5. Dependence of the rate of evaporation of acetic acid on initial concentration of acetic acid

CONCLUSIONS

Enzymatic deacetylation of chitin and chitosan can be an alternative to chemical methods. Since acetic acid strongly inhibits chitin deacetylase it is necessary to remove it from the reaction mixture. Distillation under lowered pressure is one of the possible methods. The aim of the experiments was to investigate the dependence of the rate of distillation of acetic acid on pressure and initial concentration of acetic acid in the reaction mixture.

A linear increase in the rate of evaporation of acetic acid with decreasing pressure was observed. A linear relationship was also observed for the dependence of the rate of evaporation of acetic acid from chitosan solutions on the initial concentration.

Further research will be focused at the determination the best conditions of enzymatic deacetylation process under lowered pressure.

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SELECTED PROPERTIES OF OLIGO – AND POLYAMINOSACCHARIDES OBTAINED BY ENZYMATIC DEGRADATION OF CHITOSAN

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INTRODUCTION

The bioactivity of chitosan largely depends upon the susceptibility of the polymer to hydrolytic and enzymatic degradation and the possibility of obtaininig oligo-and polyaminosaccharides as degradation products [1]. Oligoaminosaccharides reveal valuable biomedical activity [2–6]:

– bind excessive lipides and decrease their absorption from the alimentary tract
– reduce LDL cholesterol density
– show anticarcinogenic and antibacterial activity
– stimulate immunity
– lower the blood-glucase and uric acid level
– prevent cardiopathy

In this work the performance was explored of enzymatic degradation of microcrystalline chitosan characterised by varying physico-chemical properties of: average molecular weight (179–600 kD).

Two enzymatic agents were used in the research: *neutral cellulase* from AB Enzymes Oy, Finland and *xylanase* marked *HA* from Novo Industri A/S Denmark. The process performance, quality and quantity of obtained products are closely related to the kind of used enzymes, form of the applied chitosan and process conditions.

It has been found that under selected conditions, the *neutral cellulase* causes chitosan degradation and as a result, except of the significantly decreased average molecular weight of chitosan, some amount of dissolved oligoaminosaccharides is observed.

Chitosan degradation products selected in the course of the research will be used in agriculture to elaborate, amongst other, growth stimulators and agents for plant care and protection, and in medicine to elaborate a new generation of preparation for the healing of difficult wounds.

EXPERIMENTAL

Materials

Microcrystalline chitosan being a useful form of the polymer was applied in the works conducted within the grant project. Microcrystalline chitosan was obtained by aggregation of macromolecules from its salt solution, according to the method developed in the Institute of Chemical Fibres [7]. Some properties of microcrystalline chitosan (MCCh) used are presented in Table 1.

Table 1. Some properties of microcrystalline chitosan (MCCh)

Type of MCCh	$\overline{M}_v$ kD	DD %	WRV %
MCCh/I	179.0	78.3	674
MCCh/II	375.0	75.8	1030
MCCh/III	600.0	76.4	915

Characteristics of used enzymes are presented in Table 2

Table 2. Characteristics of used enzymes

Enzyme type	Producer	Enzyme activity Unit/cm^3				Reducing sugar content mg/cm^3
		Endo-1,4-β glucanase	Filter paper (FPA)	β-glucosidase	Endo-1,4-β xylanase	
Neutral cellulase	AB Enzymes Oy, Finland	186.0	42.8	20.7	–	17.8
Xylanase (HA)	Novo Industri A/S Denmark	893.0	71.7	9.0	1914	9.5

ENZYMATIC HYDROLYSIS OF MCCh

The enzymatic hydrolysis of MCCh was investigated under dynamic conditions in a water bath at 20°C during 7 hours. Following enzymatic complexes were used:

– *Neutral cellulase* with the module E/MCCh = 186 – 4650 U CMC/g
– *Xylanase* marked HA with the module E/MCCh = 107 – 2163 U/g

ANALYTICAL METHODS

The average molecular weight $\overline{M}_v$ of chitosan was determined according to the viscometric method using an aq. solution of 0.1 M sodium chloride, 0.2 M acetic acid and 4.0 M of urea, calculating $\overline{M}_v$ according to the Mark-Houwink's equation with K=8.93 x 10^{-4} and a = 0.71 [8] The GPC method [9] was employed to calculate the average molecular weights and to draw the distribution curve of molecular weights.

Content of aminosugars was assessed by colorymetric method using 3.5 dinitrosalycylic acid (DNS) [10]

Deacetylation Degree (DD) of chitosan was assessed with the use of potentiometric titration method [11]. Water Retention Value (WRV) was estimated according to the Standard Procedure GLP [12]

RESULTS AND DISCUSSION

The efficiency of the enzymatic degradation was assed basing on the change of the chitosan average molecular weight, and amount of water-soluble oligoaminosaccharides formed in the reaction. The impact of the E/MCCh module on the change of the average molecular weight of chitosan, and saccharification degree is presented in Figures 1–4.

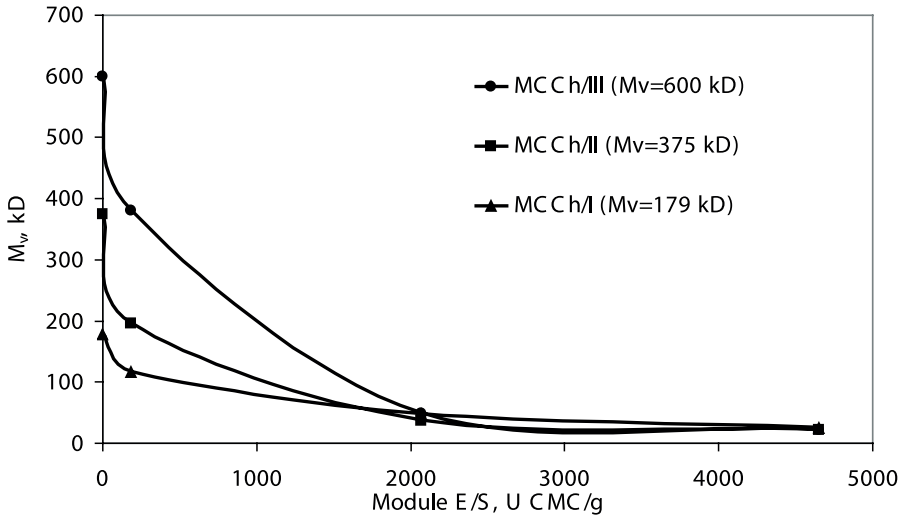

Figure 1. The impact of E/S module on $\overline{M}_v$ of MCCh with average molecular weight (*neutral cellulase*)

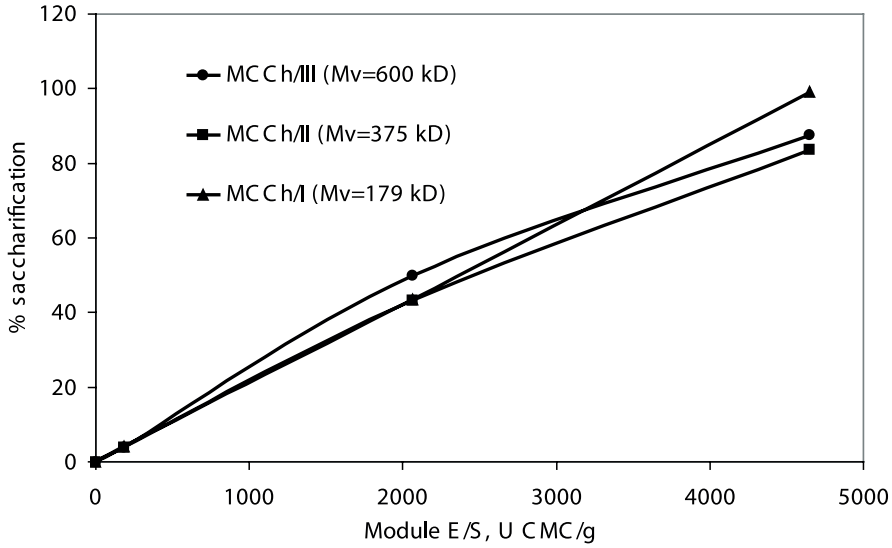

Figure 2. The impact of E/S module on saccharification degree different of MCCh with different average molecular weight (*neutral cellulase*)

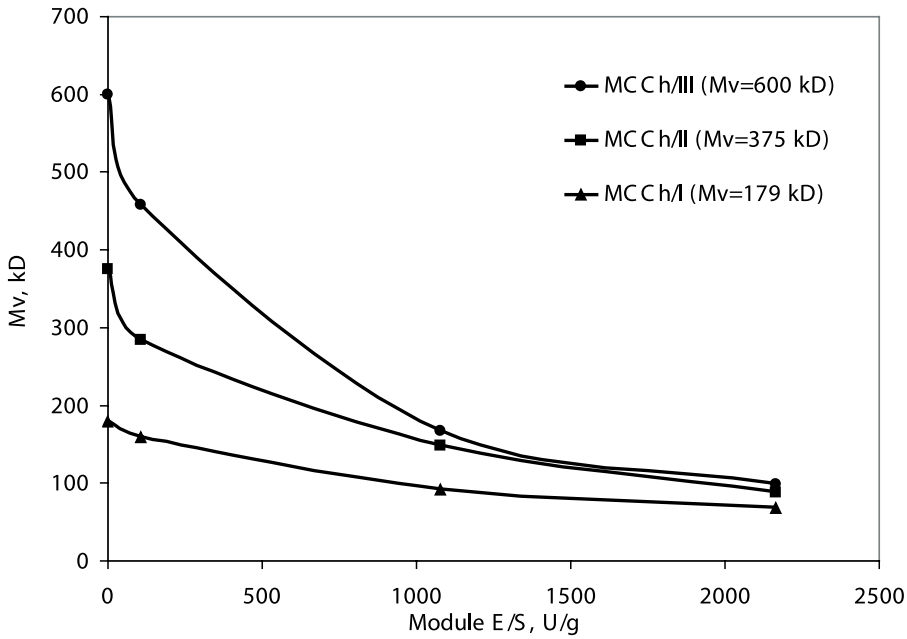

Figure 3. The impact of E/S module on $\overline{M}_v$ of MCCh with different weight (*xylanase HA*)

Basing on the conducted researd it has been found that, when *neutral cellulase* was used, the degradation process was more intensive with a distinct decrease of the average molecular weight with considerable amount of delivered dissolving oligoaminosaccharides.

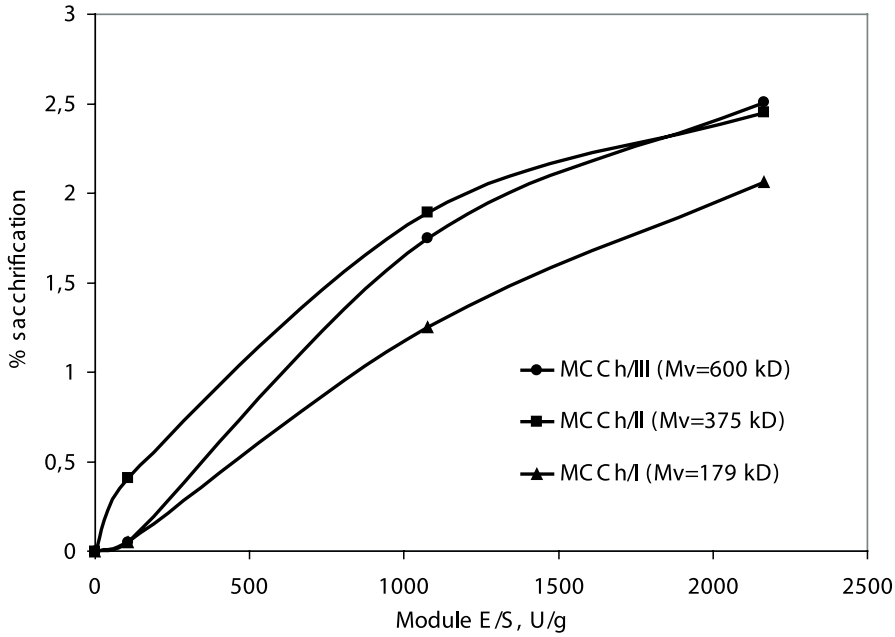

Figure 4. The impact of E/S module on saccharification degree of MCCh with different average molecular weight (*xylanase HA*)

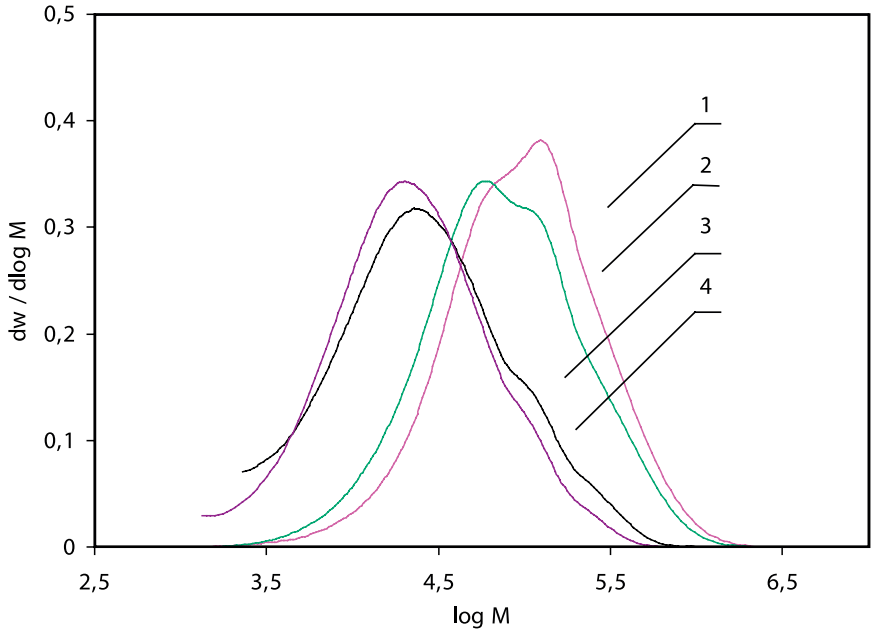

Figure 5. Molecular weight distribution of enzymatically degraded MCCh/II at different values of E/S 1 – initial chitosan, 2 – at E/S = 186 UCMC/g, 3 – at E/S = 2066 UCMC/g, 4 – at E/S = 4650 UCMC/g

In case of applied *xylanase* marked HA the degradation process has a smoother run with a statistical characteristic. An insignificant amount of dissolving polyaminosaccharides was formed during the degradation and only a slight decrease occurred of the average molecular weight of MCCh. For *neutral cellulase* the GPC investigation confirmed a distinct shift of the MCCh distribution curves towards lower fractions.

CONCLUSIONS

The increase of *neutral cellulase*/substrate module (in the range of 186–4650 UCMC/g) intensifies the MCCh degradation process

Neutral cellulase with E/S module = 4650 UCMC/g after 7 hours at temp. of 20°C cause a nearly complete saccharification (99%) of MCCh with the lowest average molecular weight 179 kD

Xylanase marked HA with the E/S module in the range of 107–2163 U/g cause MCCh degradation to the same level regardless the initial average molecular weight of the polymer. Degradation is accompanied by a low MCCh saccharification (max. 2–2.5%)

The increase of E/S module results in the shifting of molecular weight distribution towards fractions with lower molecular weight

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EFFECTIVE PREPARATION OF CHITOSAN OLIGOSACCHARIDES USING GEL MATRIX

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Abstract: Effective preparation of chitosan oligosaccharide (ChO) with high degree of polymerization (DP) was studied using poly(acrylamide) gel matrix. To a buffer solution (pH 5.6) of enzyme (cellulase), was added chitosan (3 g x 6 times) with every 3 h in stepwise (total 18 g) in the presence of gel matrix. After 4 days, 13.1 g of ChO mixture was obtained (73% of recovery) by enzymatic hydrolysis. The main component of the ChO were DP=6–8 (5.61 g), especially for DP=7 in 2.41 g of recovery. The presence of gel matrix bearing suitable molecular size is an effective for the production of high DP (6–8) of ChO in the enzymatic hydrolysis system.

Key words: Chitosan oligosaccharide, gel matrix, poly(acrylamide), enzymatic hydrolysis, cellulose

INTRODUCTION

Chitosan oligosaccharide (ChO) and its derivatives are attractive materials owing to show some biological properties such as blood coagulation control activity [1, 2], elicitor activity [3–5], Nod factor activity [6], complement activation [7] etc. To clarify the correlation between chemical structure and the above biological functions, the chemical modification of ChO is an important field. For this purpose, effective preparation of ChO with narrow distribution of degree of polymerization (DP) is a desirable technology, especially for higher DP. However, it has been difficult to prepare high DP of ChO (DP=6–8) in good yield by simple enzymatic hydrolysis.

In the present study, we successfully prepared high DP of ChOs from chitosan by enzymatic hydrolysis using poly(acrylamide) gel matrix. Gel was a good matrix to control the molecular size in desirable DP during enzymatic hydrolysis of polymeric chitosan.

EXPERIMENTAL

Preparation of gel

Acrylamide monomer (6.84 g) and *N,N*-methylene-bisacrylamide (0.36 g) were dissolved in water (100 mL). To this solution potassium persulfate (60 mg) and 3-dimethylaminopropionitrile (0.06 mL) as initiaters of polymerization was

added and inserted into 15 mm ID of glass tube. After standing rt for 18 h, the gel formed was separated from glass tube and cut into 15 mm of length.

Absorption of ChO into gel

A 3% (w/v) aqueous solution (3 mL) of ChOs (component: DP=8, 10%; DP=9, 76%; and DP=10, 14%) was immersed into a gel (2.65 mL, procedure 1). After prescribed time intervals, the concentration of ChOs was measured by indol-HCl method [8]. The absorption of glucosamine HCl (GlcN) to gel was also performed as above.

Release of ChO and GlcN from absorbed gel

A piece of ChO and GlcN absorbed gel (after 5 h of procedure 1) was immersed in water (3 mL). After prescribed time, the concentration of ChO and GlcN was measured by indol-HCl method.

Enzymatic hydrolysis

Three portion of flask (A, B, and C) including 33 piece of poly(acrylamide) gel (87 mL) was prepared. On the other hand, six portion of swelled chitosan (3 g) in 1M AcOH (30 mL) was prepared. Enzymatic hydrolysis of chitosan was performed in 3 steps as follows. 1: The enzyme solution (70 mL, pH 5.6) including meicellase (0.6 g) and sodium bicarbonate was added to the swelled chitosan (30 mL) in flask A and stored at 50 C for 3 h. 2: The reaction mixture was poured into flask B (include 33 piece of gel), swelled chitosan (3g/30 mL) was added, and stored at 50C for 3h. 3: Then the reaction mixture was poured into flask C (include gel) and stored at 0 C overnight.

This procedure (1–3:) was repeated more 2 times (total amount of chitosan=18 g) during 2 days. At 4th day, the reaction was performed as above, except for no addition of chitosan. During the reaction, the amount of reaction mixture was kept 100 mL and pH 5.6. Finally, the hydrolyzed product (ChO) absorbed in poly(acrylamide) gel was eluted with water (100 mL x 3 times).

Separation of ChO by column chromatography

The Biogel P-2 and P-4 packing chloumns (30 x 500 mm) were used for the separation of ChO. The aqueous solution of CO was concentrated to 140 mL, and the concd. ChO solution (20 mL 7 times) was charged with Biogel P-4 column. ChO was eluted with distilled water and eluted ChO was separated into 3 fractions (Fr-1, 2, and 3). Fr-3 was concentrated to 80 mL and re-charged with Biogel P-2 column (20 mL x 4 times). Eluted ChO was separated into 2 fractions (Fr-3-1 and 3-2). Furthermore, Fr-3-2 was extracted with MeOH and separated into MeOH soluble (Fr-3-2-1) and insoluble fraction (Fr-3-2-2).

Analysis of CO

Each fraction of ChO was analyzed by HPLC using Tosoh TSK gel amide 80 column. The content of ChO in each DP was estimated from the peak area of HPLC chromatogram.

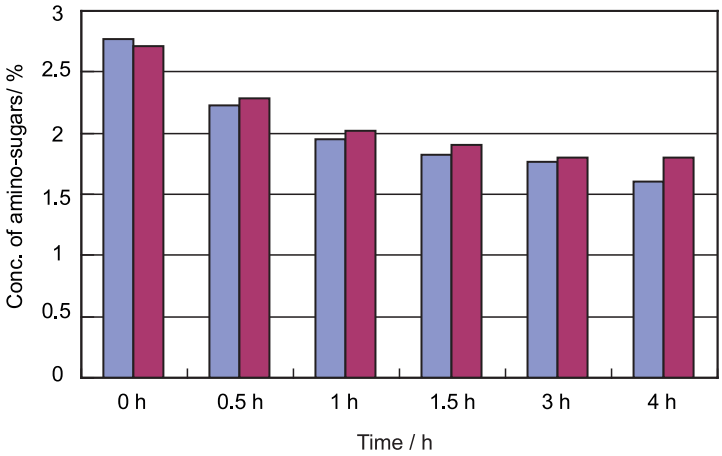

Figure 1. Time-courses of the concentration decrease of amino sugars by absorption into poly(acrylamide) gel. Blue bar, GlcN; Red bar, ChO

RESULTS AND DISCUSSION

Absorption and release of amino sugars from poly(acrylamide) gel

Fig. 1 shows the time-courses of the concentration decrease of amino sugars (ChO and GlcN) by absorption into poly(acrylamide) gel. The decreasing rate of GlcN was slightly higher than that of ChO. In any cases, the decreasing rate was proportional to time-course within 1 h. After then, the decreasing rate was gradually slow. The releasing rate of amino sugars from gel was shown in Fig. 2. GlcN released faster than ChO. The releasing of amino sugars was much fast within 0.5 h, and then these released slowly from gel. The rate of absorption and release of amino sugars was much depended on the surface area and volume of gel.

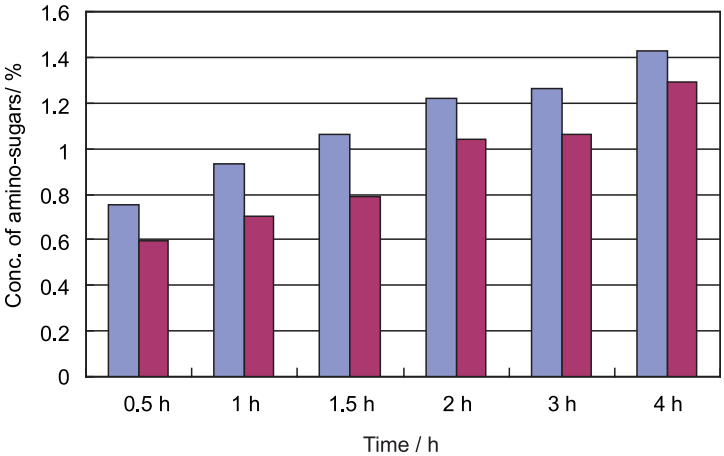

Figure 2. The releasing rate of amino sugars from poly(acrylamide) gel. Blue bar, GlcN; Red bar, ChO

The content of ChO in the faction

The optimum size of gel providing suitable adsorption and release of amino sugars was 15 × 15 mmID from the enzymatic hydrolysis (data not shown). In the presence of gel matrix, about 13 g of ChO, estimated by indol-HCl method, could be obtained from 18 g of chitosan (72% of recovery). Table 1 shows the recovery (g) and DP of ChO in each fractions. The highest content of ChO was observed in Fr-2 (6.51g, 50% of total ChO) and the main degree of polymerization (DP) was 6–8. The DP of Fr-1 was estimated to DP>9 owing to the broad peak over DP=9 fraction by HPLC analysis. The Fr 3-1 was low content and low DP (3–6) than Fr-2, Fr-3-2-1 was DP 3 and 2, and Fr-3-2-2 was obtained only GlcN monomer. Based on the results in Table 1, the total component of ChO produced by enzymatic hydrolysis with gel matrix was calculated (Fig. 3). Noteworthy fact is that the effective preparation of high DP (6–8) of ChO could be achieved in high recovery (43% of total ChO). Especially, DP=7 of ChO showed the highest recovery.

In the previous study [9], the distribution of DP of ChO obtained enzymatic hydrolysis was too wide-range in the absence of gel matrix. In general, the hydrolytic mechanism of chitosan by enzyme is a random manner, so that the wide distribution and low recovery of higher DP of ChO cannot avoid. In contrast, the quite narrow distribution and high recovery of DP of ChO could be achieved owing to use poly(acrylamide) gel matrix. The effect of gel matrix is presumed the following two mechanisms. 1) Owing to absorb ChO produced in gel matrix, the ChO bearing suitable DP could be extracted into gel matrix from the hydrolytic reaction medium. 2) The extracted ChO was protected from enzymatic hydrolysis by gel matrix. The content and DP distribution of ChO would be subjected and controlled by the degree of crosslinking or water content of poly(acrylamide) gel matrix and these factors will be studied in near future.

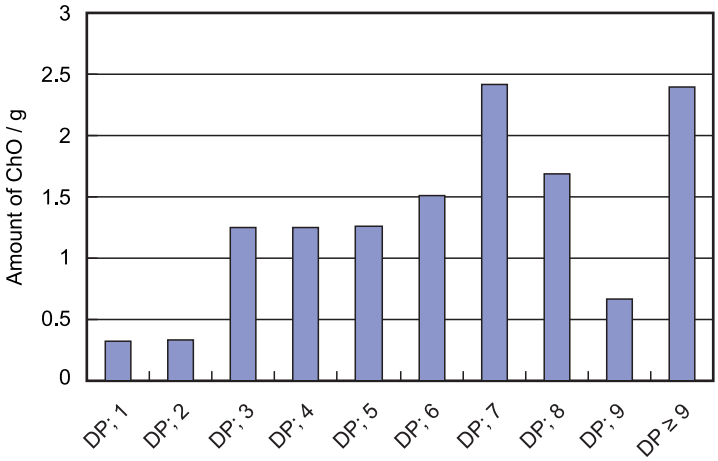

Figure 3. Total component of ChO produced by enzymatic hydrolysis in the presence of gel matrix

Table 1. Recovery and DP of ChO in each fraction from 18 g of chitosan

Recovery (g) of ChO						
DP	Fr-1	Fr-2	Fr-3-1	Fr-3-2-1	Fr-3-2-2	Total
Over 9	2.40				2.40	
9		0.66				0.66
8		1.69				1.69
7		2.41				2.41
6		1.34	0.17			1.51
5		0.40	0.56			0.96
4			1.25			1.25
3			0.25	1.00		1.25
2				0.35		0.35
1					0.32	0.32
Sub-total	2.40	6.51	2.53	1.35	0.32	13.11

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INCORPORATION OF GLUCOSE INTO CHITOSAN BY *ACETOBACTER XYLINUM*

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Abstract: Modified bacterial cellulose was biosynthesised by *Acetobacter xylinum* in a liquid medium of 2.0 wt.% glucose, 0.5 wt.% peptone, 0.5 wt.% yeast extract, 0.27 wt.% disodium phosphate, 0.115 wt.% citric acid monohydrate and 0.5 wt.% chitosan acetate (DD=84.1%, $\overline{M}_v$=98 kD) at 30°C for 7 days. The polysaccharide pellicle was digested with cellulase from *Trichoderma reesei*. Fractionation on Biogel P4 afforded, as the main products, low molecular weight oligosaccharide fractions which, as shown by MALDI-TOF mass spectrometry, were oligosaccharides of hexoses (Glc, Man and Fru) of DP ≤ 3 as well as chito-oligomers (DP ≤ 9), composed of GlcNAc and GlcN. Most interestingly, the mass spectra indicated the presence of several minor oligosaccharides containing GlcNAc, GlcN, and hexoses (Hex). Sequence analysis by MALDI-TOF MSMS of the oligosaccharides after reductive amination with 2-aminoacridone revealed, that the hexose containing products were linear hetero-oligosaccharides, where one hexose residue was randomly distributed within the sequences of chito-oligosaccharides. The hexose residue was more often positioned at the reducing end and rarely at the non-reducing end.

INTRODUCTION

Bacterial cellulose is one of the most promising materials in modern technology. It possesses a variety of unique features, such as biocompatibility, biodegradability and chemical purity, and can be easily modified during synthesis. Modified bacterial cellulose (e.g. by polyaminosaccharides) is composed of ultrafine fibrils. Some outstanding mechanical parameters are attributed to this structure, such as a very high Young modulus, tensile strength and sonic velocity [1].

Acetobacter xylinum is unique in its prolific synthesis of cellulose. Rows of pores characteristically secrete mini-crystals of glucan chains, which then coalesce into microfibres. Clusters of microfibrils result in a compound structure known as the ribbon. The ribbon can be observed directly using light microscopy, and the time-lapse studies show *Acetobacter* cells generating cellulose.

In the present study, bacterial cellulose biosynthesised by Acetobacter xylinum was modified with chitosan. The aim of the work was to examine the molecular structure and composition of the modified bacterial cellulose applying different hydrolytic, chromatographic and mass spectrometric techniques. Whereas the mass spectrometric sequence analysis of chito-oligosaccharides is established [3], the sequence analysis of hetero-oligosaccharides of glucosamine, *N*-acetylglucosamine and hexoses is challenging, as the difference of molecular masses between hexose and glucosamine is 1 Da.

MATERIALS AND METHODS

Preparation of modified bacterial cellulose:

The cellulose producing bacterium *Acetobacter xylinum* was cultured in a liquid medium (2.0 wt.% glucose, 0.5 wt.% peptone, 0.5 wt.% yeast extract, 0.27 wt.% disodium phosphate, 0.115 wt.% citric acid monohydrate) containing 0.5 wt.% chitosan acetate (DD = 84.1%, Mv = 98 kD) at 30°C for 7 days. In order to separate acid soluble and insoluble fractions, biosynthesised polysaccharide pellicle was isolated from the culture medium (pH = 4.5) by filtration, washed with distilled water, sterilised in the autoclave at 121°C for 15 min, thoroughly washed with distilled water and dried.

Monosaccharide analysis

The dry polysaccharide pellicle was milled and suspended in 7.6mL of 72% (w/w) H_2SO_4. The mixture was stirred at 40°C for 1h, diluted with distilled water to a final concentration of 5% (w/w) H_2SO_4 and stirred at 120°C for another 1.5h. After neutralisation with barium hydroxide, the solution was filtrated and lyophilised. 150 ng of the total hydrolysate were objected together with 11 monosaccharide standards (each 150 ng) to ion chromatography (HPAEC-PAD DX 600 system equipped with a CarboPac™-PA 1 column, Dionex, Idstein, Germany). For separation, the column was flushed with 200 mM NaOH for 10 min and afterwards equilibrated with 18 mM NaOH for 15 min. Analytes were eluted isocratically with 18 mM NaOH using a flow rate of 1 ml × min^{-1} and detected by pulsed amperometric detection.

MALDI-TOF MS of the cellulase digest

The polysaccharide pellicle was digested with cellulase Econase CE (AB Enzymes Oy, Finland). The lyophilisate was objected in batches of 50 mg to GPC: stationary phase, Biogel P4, fine grade (BioRad, München, Germany), column dimension, 2.5 cm i. d. × 200 cm; mobile phase, 0.05 M ammonium acetate buffer, adjusted with 0.23 M acetic acid to pH 4.2; flow rate, 25 mL × h^{-1}; detector, Shimadzu RID 6A. Fractions of 8 mL were collected, approximately combined and lyophilised. For MALDI-TOF MS of oligosaccharides, 10 μg of each fraction from GPC were redissolved in 50 μL of methanol/water (v/v 1:1). An aliquot of the solution (0.5 μL) was mixed on the target with 0.5 μL of the matrix, a 0.1 M solution of DHB in 30% aqueous ethanol, and the drop was dried under a gentle stream of air. Crystallization of the matrix occurred usually spontaneously. Mass spectra were recorded on a Bruker Reflex II (Bruker Daltonik, Bremen, Germany) in the

positive ion mode. For ionisation, a nitrogen laser (337 nm, 3 ns pulse width, 3 Hz) was used. For optimisation of the mass spectra, the laser was aimed at the outmost edge of the crystal rim. All spectra were measured in the reflector mode using external calibration (2-point calibration with α-cyano-4-hydroxycinnamic acid $[3M+H]^+$, 568.14 amu and angiotensine II $[M+H]^+$, 1046.54 amu.

Sequence analysis of hetero-oligosaccharides

10 µg of each GPC fraction were redissolved in 5 µL of a 0.1 M solution of 2-aminoacridone in acetic acid/DMSO (v/v 3:17) and agitated manually for 30 s, followed by addition of 5 µL of a 1 M solution of sodium cyanoborhydride in water and further agitation for 30 s. The mixture was heated in the dark for 30 min at 90°C. To terminate the reaction, the vessel was cooled to – 20°C followed by lyophilisation. For sequence analysis, MALDI-TOF MSMS of tagged oligosaccharides was applied (sample preparation cf. *MALDI-TOF MS of the cellulase digest*). Mass spectra were recorded on an API QSTAR Pulsar I (Applied Biosystems/MDS Sciex, Toronto, Canada) hybrid mass spectrometer equipped with a MALDI ion source. MSMS spectra were recorded by selecting the ion of interest in the Q1 quadrupole, generating fragments by collision with argon in the collision cell and analysing the fragments in the TOF analyser. All spectra were measured using external calibration.

RESULTS AND DISCUSSION

Total hydrolysis of the polysaccharide biosynthesised by *Acetobacter xylinum* followed by HPAEC/PAD analysis (Figure 1) gave the following monosaccharide composition: 53.0% Glc; 21.2% Man; 13.0% Fru; 12.8% GlcN/GlcNAc.

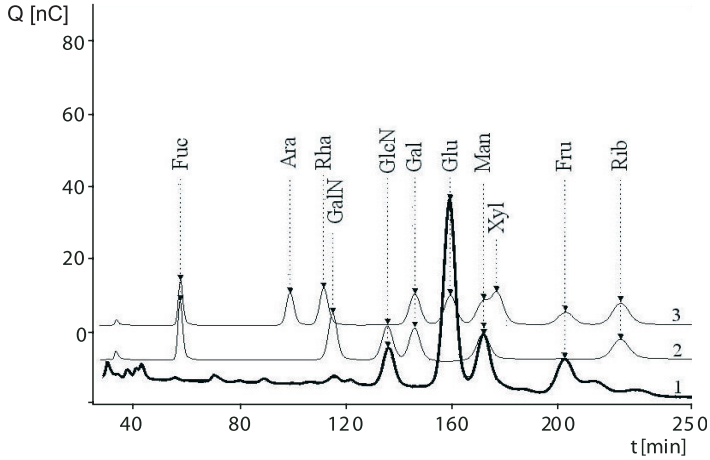


Figure 1. HPAEC/PAD of the polysaccharide total hydrolysate (1), and monosaccharide standards (2, 3)

Partial hydrolysis of the modified bacterial cellulose with cellulase Econase CE afforded a mixture of oligosaccharides of DP ≤ 9, which was fractionated by GPC on Biogel P4

(Figure 2). MALDI-TOF MS analysis of the fractions showed that the main components were oligosaccharides of hexoses (Glc, Man, Fru) of DP ≤ 3 as well as chito-oligosaccharides composed of GlcN and GlcNAc. Most interestingly, the mass spectra indicated the presence of several minor hetero-oligosaccharides of DP ≤ 9 containing GlcN, GlcNAc and one hexose residue.

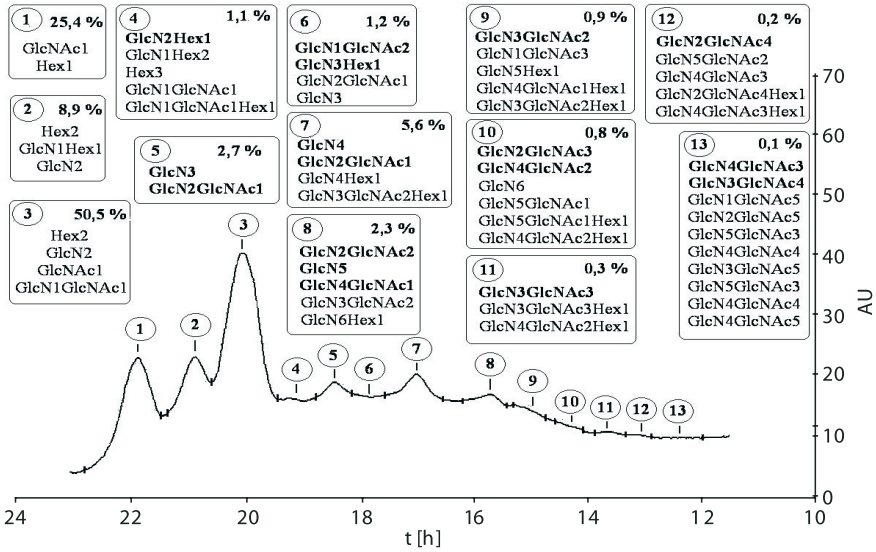


Figure 2. GPC / MALDI-TOF MS results of the cellulase digest of modified bacterial cellulose

Main components are in bold letters.

For sequence analysis of hetero-oligosaccharides, GPC fractions were tagged with 2-aminoacridone (AMAC) at the reducing end. MALDI-TOF MSMS was applied for fragmentation of the ions of the interesting homologue, which were previously selected in the quadrupole of the mass spectrometer. In the case of AMAC derivatives, the mass increment of 194 Da allows for unambiguous identification of Y-type fragment ions and straightforward readout of the oligosaccharide sequence from the reducing end.

Fragment Ion	Peak Assignment	Ratio of Peak Intensities
Y1	$GlcN_1$-amac : Hex_1-amac	0.55 : 1
Y2	$GlcN_2$-amac : $GlcN_1Hex_1$-amac	0.42 : 1
Y3	$GlcN_3$-amac : $GlcN_2Hex_1$-amac	0.25 : 1
Y4	$GlcN_4$-amac : $GlcN_3Hex_1$-amac	0 : 1

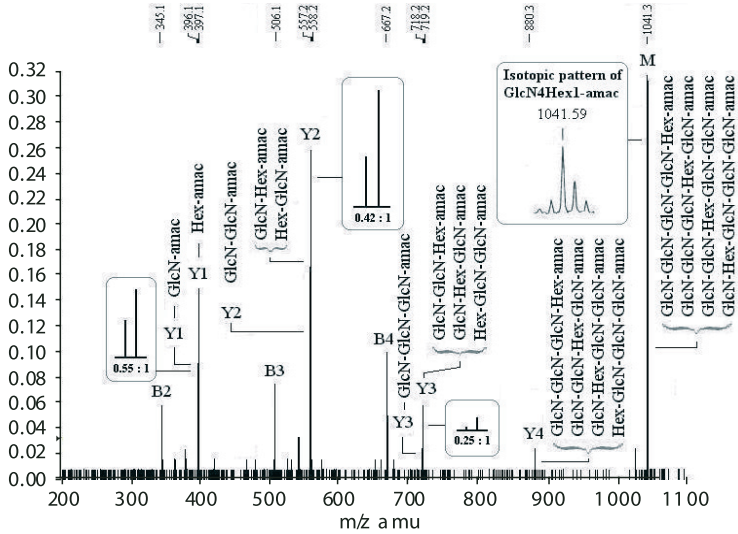
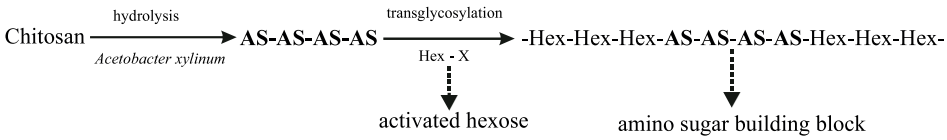


Figure 3. MALDI-TOF MSMS of the AMAC derivative of $GlcN_4Hex_1$. Relative intensities of Y-type fragment ions indicated that the hexose residue was more likely positioned at the reducing end and never at the non-reducing end

CONCLUSIONS

1. Bacterial cellulose from *Acetobacter xylinum*, which was modified with chitosan, is composed of 53.0% Glc, 21.2% Man, 13.0% Fru, and 12.8% GlcN / GlcNAc.
2. Modified bacterial cellulose is a copolymer of hexoses and amino sugars (not a composite of cellulose and chitosan), as hetero-oligosaccharides of GlcN, GlcNAc and one hexose residue were detected by MALDI-TOF MS in the cellulase digest. The distribution of the hexose residue within the sequences of chito-oligosaccharides (cf. Figure 3.) reflects the cleaving specificity of the cellulase. The cleaving rate for different types of glycosidic bonds decreases in the following order (reducing end on the right): Glc-Glc > GlcNAc-Glc > GlcN-Glc > Glc-GlcNAc > Glc-GlcN > GlcNAc-GlcNAc > GlcN-GlcN.
3. Modified bacterial cellulose is a linear polysaccharide as fragmentation mass spectra of oligosaccharides obtained by enzymatic degradation showed only fragment ions of linear oligosaccharides (B- and Y-type ions) but none of branched oligosaccharides.
4. Cellulase partial hydrolysis of the polysaccharide pellicle gave as main products oligosaccharides of hexoses (DP ≤ 3) and chito-oligosaccharides of DP ≤ 9. This fact indicates, that chito-oligosaccharides are building blocks for the biosynthesis of modified bacterial cellulose by *Acetobacter xylinum* [2]. Most probably chitosan is depolymerised to cell wall permeable chito-oligosaccharides, which act as a carbon source. Activated hexose residues (Hex-X) are linked to the initial building blocks by transglycosylation.



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CHAPTER IV

SOURCES AND PRODUCTION

ADVANCES IN CHITIN SCIENCE — Vol. VIII
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INFLUENCE OF THE REACTION CONDITIONS ON THE ISOLATION OF CHITIN FROM *PARALOMIS GRANULOSA* (SNOW CRAB)

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Abstract: The influence of the order of application of the different steps related to the elimination of proteins and minerals on the quality of the isolated chitin is demostrated. The content of residual mineral elements of the chitin isolated according to our method was the lowest (1.02%). This fact indicates the effectiveness of the method for the removal of calcium carbonate and other minerals from shells. Futhermore, this chitin shows the higher crystallinity index (88.92%), the highest deacetylation degree (99.6%) and its crystallinity index is ranged between the higher ones (92.64%). If we observe the quantity of protein extracted, our method shows a higher protein concentration in the medium (22.6 mg/ml). Taking into account the reduced time of treatment and the fact that it is a single-stage process, our method is an interesting proposal for industrial chitin isolation.

INTRODUCTION

Chitin industrially obtained is in α-chitin form and is isolated from exoskeletons of crustaceans, more particularly from shrimps and crabs. This biopolymer has many potential and usual applications only limited by its insolubility in ordinary solvents [1]. One of the major problems related to the preparation of chitin is keeping its structure as close as possible to the native form [2]. For this reason, it is necessary to reduce the partial deacetylation and chain degradation caused by demineralisation and deproteinisation reactions.

The most common derivative prepared from chitin is chitosan, coming from its partial *N*-deacetylation [3]. Besides the different parameters related to the deacetylation process, the nature of the source, the physical structure of original chitin and its isolation process are also factors which determine the physicochemical characteristics of the chitosan sample. Consequently, the selection of an appropiate source of chitin and the control of the parameters for demineralisation and deproteinisation reactions appears to be important in the obtention of chitosan. The proper adjustment of the process conditions will make possible to produce chitin with similar structure to its native form and to prepare chitosans with well-controlled characteristics.

The aim of this work was to compare the quality of the chitin obtained from Snow crab shells (*Paralomis granulosa*) by applying the conditions reported in literature by different authors [4–11] to the one obtained in our laboratory. The conditions we propose are included in the range of the normally used [3], but our results show an improved quality of the chitin obtained. The quality of the products was determined by the crystallinity index monitorised by X-Ray diffractograms, the ash content, the protein content and the acetylation degree measured by FT-IR.

MATERIALS AND METHODS

The raw materials (shells) were supplied by Idebio S.L. (Salamanca, Spain). This material belongs to a type of crustacean *Paralomis granulosa* (Chilean Kingcrab). The freshly caught crabs were boiled in water for 12 hours to remove soluble organics and some of the binding proteins [12]. After this, the shells were dried at 50°C for 24 hours, grounded with an electric mill and sieved to obtain samples with a particle size minor than 1 mm.

Hydrochloric acid and sodium hydroxide were purchased from Panreac (Barcelona, Spain) both of analytical reagent grade, and BSA from Sigma (Madrid, Spain).

Obtention of chitins

Demineralisation. For this stage of the isolation process, several methods quoted in literature have been applied. Table 1 describes the methods (the one applied in our laboratory also included) with the conditions and references in each case.

Table 1. Methods proposed for the first stage: Demineralisation

Method	Treatment (hrs)	Acidic concentration (HCl)	Temperature (°C)	Ratio Solid-Acid	Number of treatments
Our method [13]	2.5	2 N	25°C	1:10	One
Tolaimate et al. (2003) [10]	2.5	0.55 M	25°C	1:10	Four: 15+15+60+60′
Muzzarelli et al.(1980) [4]	5	1.57 M	25°C	1:10	One
Broussignac (1968) [6]	24	1.4 M	25°C	1:10	One
Mima et al. (1982) [5]	24	1 M	25°C	1:10	Two: 12 hr +12 hr
Shimahara et al. (1992) [8]	48	2 M	25°C	1:10	One

Some of these authors put forward the idea of repeating the treatments several times in order to remove residual minerals more efficiently without damaging the sctructure of chitin.

Our laboratory applied different conditions albeit included in the range mencioned by Roberts [3]. In our case, demineralisation was carried out for 2.5

hours at room temperature using a single treatment and the amount of acid was stoichiometrically greater than all the minerals present in the shells (ash content 33.97%) to ensure complete reaction (1:10 HCl 2N) [13].

In all cases, demineralisation was followed by measuring the evolution of pH within time in order to control the acid consumption.

Deproteinisation. As above, several methods mentioned in literature have been compared on the basis of some characteristics of the product obtained (Table 2).

Table 2. Methods proposed for the second stage: Deproteinisation

Method	Treatment duration (hours)	Alkali concentration (NaOH)	T (°C)	Ratio Solid-Alkali	Number of treatments
Our method [13]	3	15%(w/v)	65	1:10	One
Muzzarelli et al. (1980) [4]	2	2%(w/w)	65	1:30	One
Mima et al. (1983) [14]	6	1M	80	1:10	Two: 3 hr+3hr
Broussignac (1968) [6]	2	5%(w/v)	90	1:10	Three: 40′+40′+40′
Hackman et al. (1974) [9]	72	1M	100	1:10	Three: 24hr+24hr+24hr
Tolaimate et al. (2003) [10]	3	0,3M	85	1:10	Three: 1 hr+1hr+1hr
Karusppawamy (1977) [11]	24	5%(w/v)	100	1:10	One
Shimahara et al. (1982) [8]	36	1M	100	1:10	One

In our case, the deproteinisation reaction was performed using NaOH 15% (w/v) at 65°C for 3 hours in a single treatment [13].

Some of the authors propose repeating the treatments several times in order to reduce the degradation of the final product (number of baths given in table 2). The reduction of the proteins retained was indicated by the absence of colour of the medium at the end of the last treatment. This fact was lately confirmed by element analysis of chitins and Bradford assay in the medium.

After each step of the process, washings with water were carried out up to neutrality and samples were dried at room temperature (25°C) for 24 hours.

Characterisation of chitins

Demineralisation. The ash content of the samples obtained after the application of every method, was determined by gravimetric measures using an oven at 700°C for 7 hours (15). Crystallinity indexes were calculated with the equation proposed by Segal and Creely (16). X-ray diffraction measurements were made on powered samples using a Philips X-ray powder diffractometer model XPERT with nickel filtered CuK radiations.

Deproteinisation. The effectiveness of the methods proposed at this stage was evaluated by measuring the proteins remaining in the medium at the end of the chemical treatment with alkali. In the case of having applied several baths, we

took samples of medium at the end of every one. For this purpose, we used the Bradford assay with bovine serum albumin as protein standard. Apart from this, we measured the residual proteins retained in final chitins. Taking into account the nitrogen percentage obtained by element analysis we estimated the percentage of protein in the samples. The results obtained with each method were compared.

As in the previous stage, crystallinity index was calculated from X-ray diffractograms so that we could monitorise if the native assembly of chitin was disturbed during the different treatments.

Acetylation degrees of purified chitins were obtained by FTIR, measured by the potassium bromide disc method with a Perkin-Elmer, Model 221 double-beam spectrophotometer and using the ratio of absorbances at 1320 cm^{-1} and 1420 cm^{-1} with the baselines proposed by Brugnerotto et al. [17].

RESULTS

Within the isolation processes, the influence of the order of application of the different steps related to the elimination of proteins and minerals on the quality of the isolated chitin is demonstrated. The obtained results when the demineralization was preceded by deproteinisation allows us to observe that concerning the chitin, the residual content in mineral elements stays high (ash content= 4.14%) and moreover chitin becomes collapsed. These results do not support the claims made by some authors about the necessity of the inverse order to reduce ash content in final chitins.

Demineralisation. The results obtained by applying the different methods for demineralisation are presented in Table 3.

Table 3. Results obtained after the first stage: Demineralisation

Method	Treatment duration (hours)	Ash content*(%)	Crystallinity index** (%)	Number of treatments
Our method (13)	4	1.03	88.92	1
Tolaimate et al. (2003) (10)	2.5	2.85	85.29	4
Muzzarelli et al. (1980) (4)	5	3.93	84.03	1
Broussignac (1968) (6)	24	3.39	86.51	1
Mima et al. (1982) (5)	24	3.47	84.81	2
Shimahara et al. (1992) (8)	48	3.81	84.98	1

* RSD= 10%; n=10
** RSD= 6,5% ; n=10

The efficiency of demineralisation has been followed by measuring the residual ash content of the isolated chitin.

The content of residual mineral elements of the chitin isolated according to our process was the lowest (1.02%). This fact indicates the effectiveness of the method for the removal of calcium carbonate and other minerals from shells.

Futhermore, this chitin shows the higher crystallinity index (88.92%). To obtain these results it was not necessary to apply several treatments as reported by some authors and we applied the treatment with alkali for less time (4 hours), making the process more economic in order to implement it in industry.

Prolonged demineralisation times, even to 48 hours, result in a lower drop in the ash content but they cause polymer degradation as it is shown by the crystallinity index. This point was previously reported by Brzeski [18].

The chitin contents calculated by a mathematical method using the data of nitrogen percentage obtained by element analysis of the samples, were 98% in all cases.

Deproteinisation. The results obtained by applying the different methods in this stage are presented in Table 4.

Table 4. Results obtained after the second stage: Deproteinisation

Method	Treatment duration (hours)	Protein in the medium (mg/ml)	Acetylation degree* (%)	Crystallinity index* (%)
Our method [13]	3	22.60	99.60	92.64
Muzzarelli et al. (1980) [4]	2	13.43	96.50	91.03
Mima et al. (1983) [14]	6(3+3)	14.43	92.10	91.46
Broussignac (1968) [6]	2(40+40+40)	24.17	90.12	91.85
Hackman et al. (1974) [9]	72(24+24+24)	1.81	71.17	90.60
Tolaimate et al. (2003) [10]	3(1+1+1)	14.72	98.33	91.16
Karusppawamy (1977) [11]	24	9.10	89.52	92.57
Shimahara et al. (1982) [8]	36	4.14	91.51	92.14

* RSD=1,5%, n=10
** RSD=6,5%, n=10

At the end of the deproteinisation there was no protein retained to any of the final chitins, its elimination being complete at the end of the processes.

Shahidi and Synowiecki in 1991, reported that a minimum period of 1 hour was needed to extract over 90% of the proteins and that a 2 hours extraction method was required for removal of all the proteins present in the shells [19]. This would explain the results we have obtained, as time treatments applied are over 2 hours.

In all cases, crystallinity of the chitin preparations seemed to increase as the protein content decreased.

Although this time the results are not so different as in demineralisation, the chitin obtained by our method shows the highest deacetylation degree (99.6%) and its crystallinity index is ranged between the higher ones (92.64%).

At the same time, if we observe the quantity of protein extracted (remaining in the medium), our method produces a higher protein concentration (22.6 mg/ml).

Taking into account the reduced time of treatment and that we have not renewed alkali during the process, our method is the best option to isolate chitin.

CONCLUSIONS

It is demonstrated that the proposed method provides the chitin with a less mineral content, a highest degree of acetylation and a highest crystallinity index. It is clear then, that our process allows to prepare chitin highly acetylated preserving its original crystalline structure.

As our method is the one which provides a higher quality chitin with only one treatment, it is strongly suggested that the multi-stage process is not more efficient than the one carried in one stage.

Taking into account the reduced time of the treatment and the relatively mild conditions of both stages, the proposed method is an interesting alternative for industrial chitin isolation.

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CHITOSAN – PRODUCTION, MODIFICATION, APPLICATION

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Abstract: The production of activated chitosan in a simple way and on favourable economical and ecological conditions has been described. The chitosan has a good reactivity and is easily to handle, thus being particularly eligible for the production on technical scale.For modifying chitosan in a two step process a way was shown with chitin taken as a basis without interim isolation. This method is ecologically and economically favourable and produces derivates with high yields and degrees of substitution well to be controlled. The versatile applicability of chitosan could be well pointed out with the anti graffiti protective layer as an example.

Key words: chitosan, new method of production, modification, alkylation, anti graffiti protective layer

INTRODUCTION

A new ecological and economical method of production of chitosan on technical scale will be described. The synthesis way applying this new method will be shown. Finally a new very special application of chitosan will be introduced.

Macromolecular natural substances, such as cellulose, starch and chitin, represent a class of materials steadily gaining in importance. These three compounds, all similar in structure, only differ in the position of the glycosidic oxygen bridges, respectively in the acetate and amino groups of chitin and chitosan.

The constantly growing economic importance of chitosan, the deacetylated chitin derivate, has been widely known in different areas such as the water industry, paper, cellulose and leather industry, pharmacy and cosmetics.

PRODUCTION OF CHITOSAN

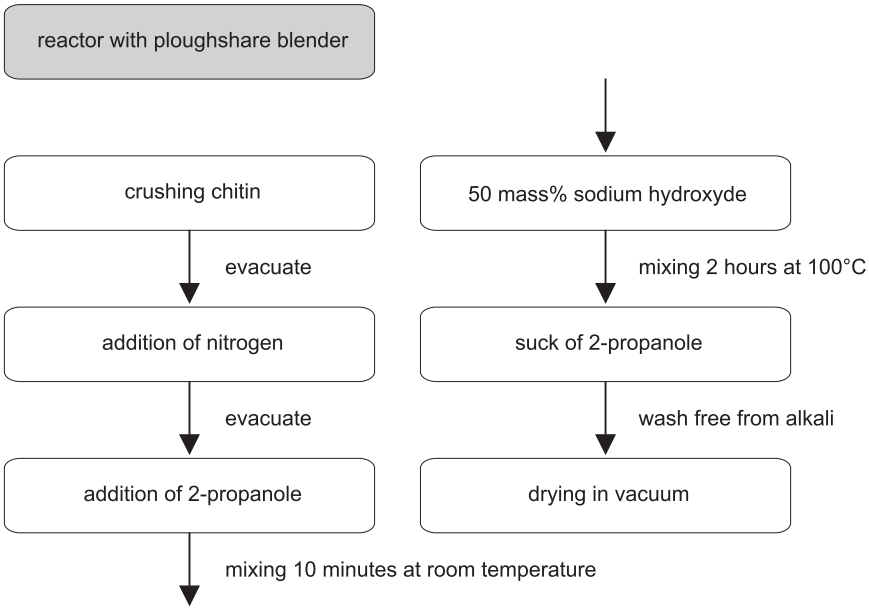
The preparation of chitosan from chitin has been well known for a long time [1]. With the first methods molten alkali hydroxides were mainly applied in order to separate the acetyl groups from chitin. By damaging the reactor material which was mainly made of stainless steel these reactions proved to be very aggressive. Deacetylation with alkalines in solution became more and more accepted.

With the conventional methods of producing chitosan from chitin by deacetylation with alkalines large quantities of alkali are used at a high concentration, a multiple of the quantity of chitin. [2, 3]. The deacetylation requires long reac-

tion times of up to several hours on drastic conditions or rather several days on milder reaction conditions and high reaction temperatures of up to 150°C. This procedure is less economically and ecologically [4, 5, 6]. A further negative aspect is the large amount of remaining alkali solution.

With the new method a distinctly reduced alkaline concentration in a water/alcohol mixture is applied. The quantity of alkali has been strongly reduced. The reduction of reaction time and temperature increases the economical and ecological importance of the message discussed. The reactor material, mostly consisting of stainless steel, is not damaged under the conditions used so that the production of chitosan on technical scale should be possible.

The reaction is carried out in a reactor with ploughshare blender. Previously crushed chitin is filled into the reactor and the system is than evacuated. Nitrogen is used as protective gas. After the addition of alcohol (e.g. isopropanole) the slurry is mixed for ten minutes at room temperature. Subsequently an aqueous solution of sodium hydroxide (50% M/M) is added. The reaction is finished after two hours at 100°C. Then the alcohol is removed by distillation, the product is washed and dried in vacuum.



The formed product has a yellowish to ochre colour and trickles easily. The degree of deacetylation can be controlled between 75 and 90% and was determined by means of ^{13}C-NMR-spectroscopy.

All products are soluble in 2% acetic acid.

MODIFICATION OF CHITOSAN

As polymer analogous reaction at hydroxy and amino groups of chitosan alkylation and acylation can be carried out. *O*- and *N*-alkyl and acyl derivates are formed. Also grafting reactions onto the chitosan are known.

For these reactions the transformation of chitin to chitosan is extended. In this procedure the mixture is cooled down to 30°C and the alkylation agents are added. The mixture reacts for five hours at 80°C, then the alcohol is removed, the product washed free of alkaline and then dried in the vacuum.

As alkylation agents are applied among others: methyl chloride, ethyl chloride, sodium chloroacetate, ethylene oxide, propylene oxide, 1-epoxybutane and 1,2-epoxyoctane.

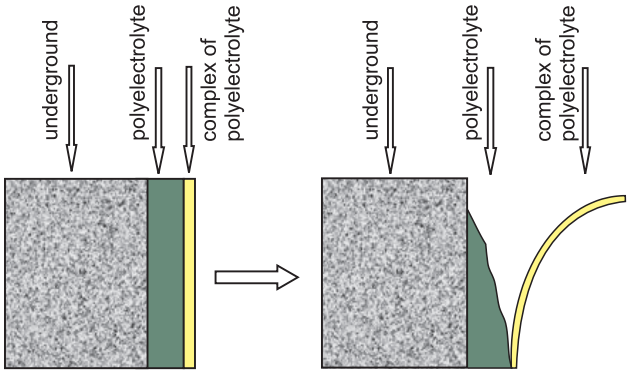
In this process in which chitosan is available in an activated very reactive form, the synthesis leads to products with high yield.

Depending on the degree of substitution they are highly swelling or clearly soluble in water.

APPLICATION OF CHITOSAN AS ANTI GRAFFITI PROTECTION LAYER

As a very special application chitosan is used as a part of a temporary anti-graffiti protective coating.

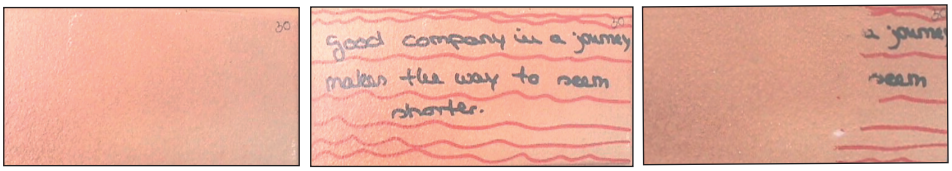
On building sites an anionic polyelectrolyte solution, i.e. carboxylmethyl cellulose, is sprayed as a sacrifice layer. After that a film forming chitosan solution is applied as a protective layer. At the interface between both oppositely charged polyelectrolytes a semi permeable membrane is formed which is water vapour and air permeable. In contrast to the sacrifice layerthe polyelectrolyte complex layer is not soluble in cold water thus preventing the protective layer from coming off due to the influences of weather.



Structure and removal of protection layer

Here the protective layer was sprayed on clinker and after that painted with marker and paint. The right part of the clinker is untreated. After cleaning the clinker with a high-pressure cleaner at 60°C and a pressure of 40 bar the clinker is clean again in the place where the protective layer had been.

The same effect is observed in the case with sandstone, a highly sensitive material as endangered to graffiti because of its high absorbency. The protective coat could also be tested successfully with a heat insulatation compound system that was painted with dispersion paint.

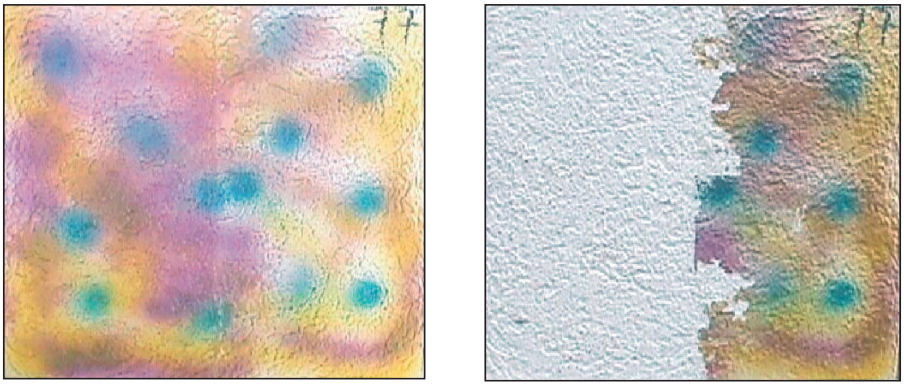


1. layer (middle and left) CMC
2. layer (left) chitosan

painted with marker

after cleaning with high-presure cleaner (60°C, 40 bar)

The properties of temporary protective paints are very favourable. Due to chitosan they are not only deodorant, but also anti-bacterial, cold water proof, bio-degradable, and ecologically beneficent as they are solvent resistent when applied.



left side is treated with protection layer sprayed with graffiti colours

after cleaning with Kärcher (60°C, 40 bar)

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CHITIN DEACETYLATION AT LOW TEMPERATURES

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Amongst known procedures for chitin deacetylation, the alkaline hydrolysis process based on the action of high temperatures and concentrations of sodium hydroxide has become the most widespread technique.

Depending on the basic material type, the processing temperature ranges between 100 and 120°C, the sodium hydroxide concentration – between 50 and 55%. Such a technique requires the employment of expensive corrosion resistant equipment, high energy inputs, which ultimately lead to a higher cost of the product.

As is known, the low accessibility of chitin acetamide groups is due to its high degree of crystallinity (up to 85%), vitreous state of the amorphous phase (T_{vitr}=+55 and +105°C) (6), and well-developed system of intermolecular bonds (1). Therefore, in order to actualize the deacetylation reaction, severe conditions are applied to enable the breaking of the hydrogen bond in the $NH\text{-}COCH_3$ group.

However, aggressive media at high temperatures contribute to the destruction of chitosan, the breakage of the polymer molecular chain at glycosidic linkages (2), which results in the reduced molecular weight, sorption capacity, and viscosity of its solutions.

The VNIRO personnel have developed a process for producing chitosan (3) by way of "cold" deacetylation at ambient temperatures of 20–22°C while simultaneously reducing the sodium hydroxide concentration down to 35–40%. This process compared to conventional procedures ensures a lower degree of the polymer destruction, its increased molecular weight and viscosity (Fig. 1–2). At the same time, the duration of the deacetylation process creates certain difficulties in its practical application.

In order to shorten the reaction time and produce a polymer with a high degree of deacetylation and a high molecular weight at the same time, some efforts have been made to modify the "cold" process of chitin deacetylation.

The research was conducted on chitin produced from the shell of the Kamchatka crab containing 5% of moisture, 6.2% of nitrogenous substances, and 0.16% of mineral substances.

Works of S. Mima et al. (4) have shown that periodic washes of the intermediate product with water help obtain highly deacetylated chitosan without destructing the molecular chain. The temperatures of physical transitions were found to be equal to 250 K, 328 K, and 378 K (5).

On the grounds of the above, the chitin deacetylation process was carried out in two steps. First, "cold" deacetylation of chitin was implemented at the ambient temperature (20–22°C) using 40% solution of sodium hydroxide during 5 to 7 days, after which the suspension was separated, and chitin was washed with distilled water. Dry chitin became turgescent, which facilitated the access of sodium hydroxide to acetyl groups and its partial deacetylation.

Chitin was then treated for 2 to 3 hours with a pre-strengthened solution of sodium hydroxide at 50 to 60°C.

An important advantage of the modified deacetylation process proposed is a considerable reduction in the time required to carry out the reaction (three times) as compared to the "cold" process, and the production of chitosan with a high degree of deacetylation (Fig. 1).

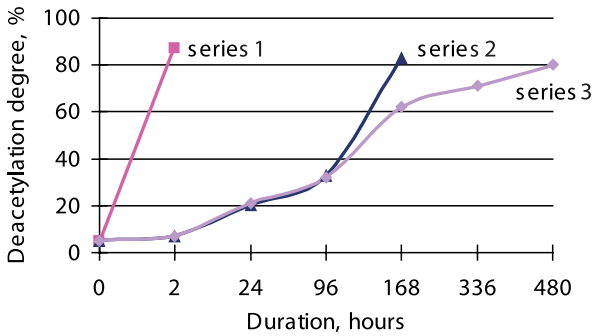

Figure 1. Chitosan deacetylation defree vs. production process
1 – conventional; 2 – modified; 3 – "cold"

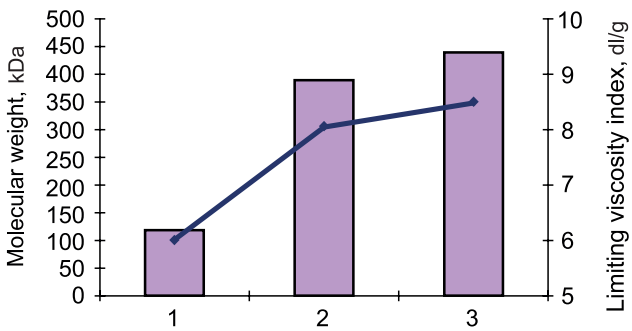

Figure 2. Characteristics of chitosan produced using various processes
1 – conventional; 2 – modified; 3 – "cold"

Figure 2. shows the effect of deacetylation conditions upon the qualitative characteristics of chitosan.

The qualitative characteristics of chitosan samples produced using the modified and "cold" processes are identical. The polymer produced using the conventional process is characterized by lower values of molecular weight and limiting viscosity index.

The X-ray diffraction analysis of chitosan samples obtained using various techniques showed the similarity of their structure (Fig. 3).

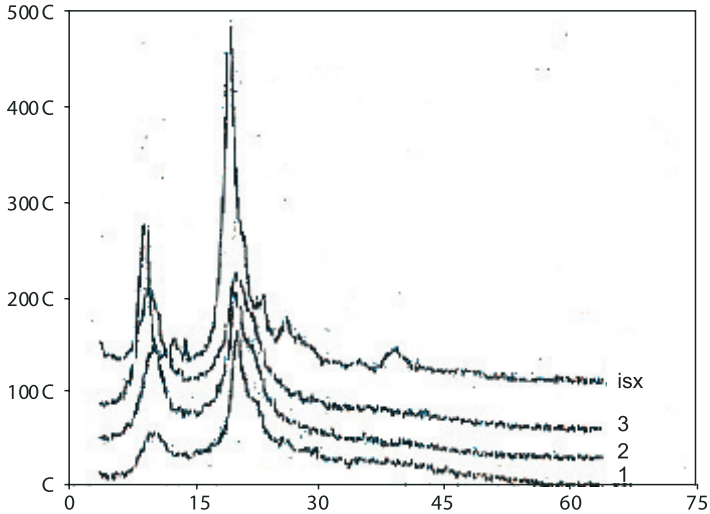

Figure 3. Diffractograms of samples chitosan end chitin
1 – conventional; 2 – modified; 3 – "cold"; isx – chitin

The diffractograms of all the three samples are characterized by the presence of a wide reflex at 2θ=20°C and a wide plateau in the region of 2θ=12–17°C, which gives evidence to a poorly ordered structure with a low crystallinity degree of 15 to 20%. The pronounced maximum on the diffractogram of the initial sample (chitin) is intrinsic to a higher crystallinity degree of approximately 60%.

The research conducted has demonstrated the feasibility of modifying the deacetylation process at low termperatures, at the same time the reaction duration is reduced threefold as compared to the "cold" process. The values of molecular weight, viscosity, degree of deacetylation (DD) of chitosan produced using this process are comparable with the characteristics of the "cold" deacetylation polymer.

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ALKALINE SOLUTIONS OF CHITIN DEACETYLATION AT HOMOGENOUS CONDITIONS

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Most of known technologies for chitin and chitosan production, using both enzymatic preparations and the strains of proteolytic microorganisms, are directed to the maximum conservation of molecular mass of these biopolymers. However, further deacetylation of chitin in tough conditions can lead to its significant destruction, and therefore to the loss of the final product – chitosan.

As it is well known, at chitosan treatment with concentrated alkaline solutions (40–50%), cleavage of polymer acetyl groups occurs with formation of chitosan, and with free NH_2 – group content not less than 75%. The process is usually carried out at high temperature (100–140°), where chitosan is formed rather quickly, as far as at heterogeneous conditions the reaction of deacetylation takes place predominantly in amorphous sites of the polymer. However, further process of the reaction is slowed down significantly because of the poor availability of hydrolized acetamide groups in crystal area of the polymer. In this connection distribution of residue acetamide groups in Chitosan obtained can possess block character and vary, depending on the reaction conditions, as well as on aggregate state and properties of chitin itself (sources of chitin, particle size, the rate of crystallization, humidity, the method of extraction etc.).

To eliminate the influence of these factors onto the reproducibility of the chitosan properties, the attempts were undertaken to turn from heterogeneous conditions for the reaction of deacetylation to homogeneous ones. One of such attempts is a study of possibility for chitosan production in 40% NaOH solution at prolonged keeping of alkaline chitin suspensions (solutions) at room temperature [1]. Such method of deacetylation allows to obtain chitosan with deacetylation rate (DAR) up to 90%.

Another attempt to turn to homogeneous conditions is a study of possibility for chitin deacetylation in alkaline solutions, obtained after freezing-unfreezing of chitin alkaline solutions.

Chitin, like cellulose, is characterized by swelling in alkaline solutions, and the swelling effect increases as the temperature is lowering. It was shown earlier [2], that at multiple freezing-unfreezing of chitin suspensions in 8% NaOH solution, the alkaline chitin solution is formed, and the process is not accompanied by noticeable polymer deacetylation. In more recent works [3], there was shown

a possibility to carry out the process of chitin dissolution in alkali in one step. The process of chitin swelling and dissolution leads to polymer activation, and its structure amorphization. One can suppose that the reaction of deacetylation performs practically identically in all sites of the polymer, that allows to obtain chitosan, characterized by uniform distribution of acetamide groups as well as free amino groups along the polymer chain.

The rate of chitin swelling and dissolution in alkali at freezing-unfreezing depends mostly on its aggregate state, on the method of its production and particle dispersion. So, humid chitin immediately after its production, can be dissolved at greater degree and form more homogeneous solutions, than dry chitin. Homogeneity of the chitin alkaline solution obtained depends directly on the degree of its disintegration.

Experimental conditions

To investigate the process of chitin dissolution in alkaline solutions and its further deacetylation at homogeneous conditions, chitin from Antarctic shrimp (*Euphausia superba Dana*) and from Barents sea shrimp (*Pandalus borealis*) was used. Chitin was extracted from frozen shells by enzymatic deproteinization with proteinases of microbial and animal origin, protosubtilin G20X and medicinal pancreatin respectfully. Deproteinized shells were purified from carbonates with hydrochloric acid solution and then dried or frozen humid.

Dry or unfrozen chitin was mechanically ground and suspended in aqueous solution of NaOH with alkaline concentration from 13 to 24%. Chitin concentration in the suspension varied from 1 to 10%. Alkaline suspension of chitin was frozen in cryostat, and then unfrozen at room temperature. In this case chitin swells significantly, forming alkaline solution – viscous flowable opalescent liquid of yellow-brown colour. After this step alkaline solution of chitin losses its flowability, and the gel is formed, that is ground mechanically to particle size 3–5 mm, then washed from alkali with distilled water. Obtained so chitosan is dried at 50–55°C.

The degree of deacetylation of chitosan samples was determined by the method of conductometric titration; solution viscosity – in rotary viscometer, and molecular mass - by the method of capillary viscometry in the viscometer Ubellode.

RESULTS AND DISCUSSION

In the present study we investigated the behaviour of chitin in 10–30% alkaline solutions in broad temperature range, including low temperature providing suspension freezing, room temperature using for suspension unfreezing and keeping, and elevated temperature using for thermal treatment of solutions obtained. After single freezing-unfreezing of chitin suspension in alkaline solutions of given above concentrations, the process of polymer dissolution can be observed with formation of viscous flowable solution. Keeping of such solution at room temperature with the lapse of time leads to its gelation. Stability of chitin alkaline solution during storage till the moment it looses flowability depends

on the same causes, as the solution homogeneity, that is – depends on the initial chitin humidity, its aggregate state and dispersibility.

Peculiarities of chitin deacetylation were investigated in the solution, obtained after single freezing-unfreezing of shrimp chitin suspension at different concentrations of alkali. It was shown (Fig. 1), that with increasing of alkali concentration almost linear growth of chitin deacetylation rate (DAR) took place up to the level, conditioned by the terms of the system unfreezing. Increasing of Mole correlation alkali/chitin also leads to linear growth of chitin DAR value (Fig. 2). As in the previous case, linear growth of chitin DAR value has a certain limit for each of investigated alkali concentration.

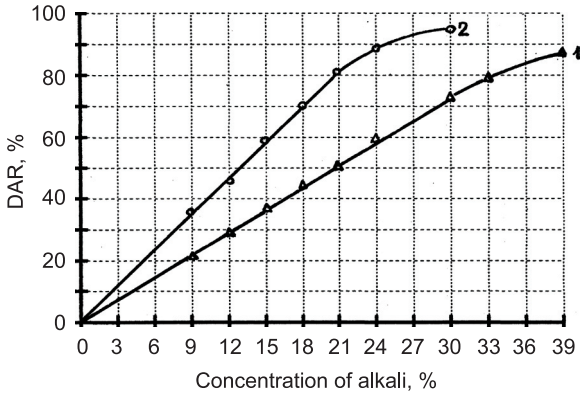

Figure 1. Dependence of chitin DAR value on the concentration of alkali used
1. Deacetylation without preliminary gelation of alkaline chitin
2. Deacetylation with preliminary gelation of chitin alkaline solution at room temperature

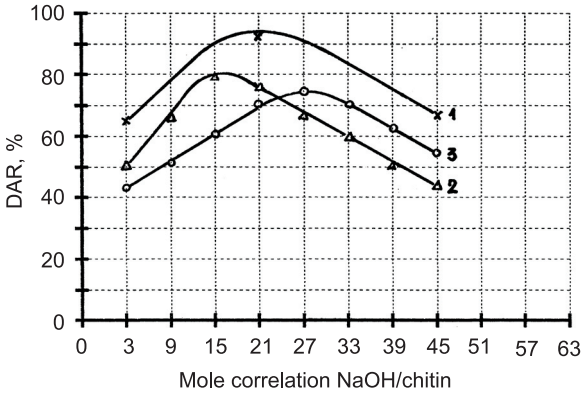

Figure 2. Dependence of chitin deacetylation rate on the Mole correlation NaOH/chitin at unfreezing in equal conditions
1. C_{NaOH} = 24%; 2. C_{NaOH} = 17.5%; 3. C_{NaOH} = 16.8%.

Study of the process of chitin deacetylation in alkaline solution with time demonstrated, that this process has step-character, differing from the analogous process at traditional heterogeneous conditions (Fig. 3). Step-character of the process can vary depending on the source and conditions of chitin production, on

its aggregate state, on particle dispersibility. The reaction of deacetylation takes place practically during all the time of chitin alkaline solution keeping. On reaching of certain DAR value, for example 42% at the point A, the solution gelation happens, and the reaction of deacetylation is slowed, but after a while, beginning from the point B, DAR value began to grow again. The process of DAR value growth, starting from the point B is accompanied with the syneresis of gel, stable in the A-B region. The solution gelation on reaching chitin DAR value of 42% in this certain case is related to decrease of chitin interaction with environment, and to strengthening of inter-chain contacts, that leads to gel formation. The period of stability for alkaline solution before gelation can vary from several hours to several days.

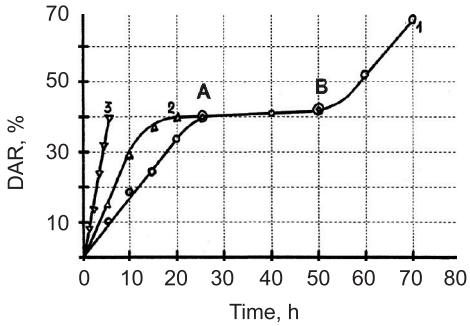

Figure 3. Dependence of chitosan deacetylation rate on the exposure time of chitin solutions at different temperatures 1. 21–22°C; 2. 37°C; 3. 70–75°C

Temperature increase is usually used to accelerate the reaction of deacetylation. At heating of chitin alkaline solution to 80–90°, they gelate during first 5–20 minutes, and the deacetylation reaction performs within the gel, and DAR value reaches 40–45% during 4–5 hours.

While keeping chitin alkaline solution at room temperature, DAR value, providing complete chitosan dissolution, can be reached during 120–150 hours. But if we begin to heat the gel, formed from partly deacetylated chitin, starting from the point A, it is possible to reduce the time and to reach DAR value of 80% during 4–5 hours; and thereby to obtain soluble chitosan.

Carrying out of chitin deacetylation in alkaline solutions opens possibility to regulate the process with temperature and time factors. Polymers obtained with given DAR values, can maintain a structure and high molecular mass, with uniform distribution of amino-groups. Comparing with traditional methods, production of chitosan by the described here technology, is carried out in alkaline solutions of lower concentrations, that decreases the rate of polymer destruction. Chitosan thus obtained, is not electrified at grinding absolutely, it forms highly viscous solutions, showing in some cases properties of Newton liquids (Fig. 4); in some cases 1% solutions of these chitosans look like gels.

Chitin derivatives with low DAR values, obtained from alkaline chitin solutions, and insoluble in acid solutions (only swelling in acid solutions at different rate), demonstrate characteristics of efficient sorbents for metal ions, toxins and other compounds.

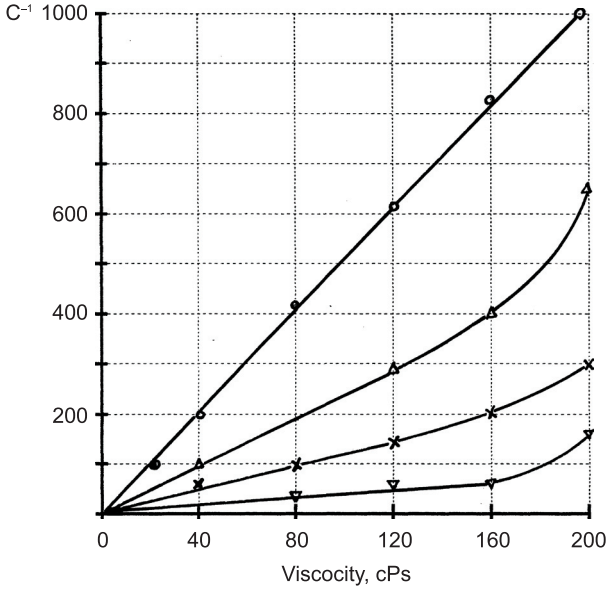

Figure 4. Flow curves for 1% chitosan solutions, obtained from chitin alkaline solutions
1 – chitosan from "humid" chitin
2 – chitosan from dry chitin with preliminary swelling
3 – chitosan from dry chitin without preliminary swelling
4 – chitosan, obtained by traditional method

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CHAPTER V

APPLICATION OF CHITIN AND CHITOSAN

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PROPERTIES OF NEW POLYMER ALLOY BY HIGH AFFINITY OF CHITIN AND BACTERIAL CELLULOSE

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Key words: Chitin, Bacterial cellulose, Polymer alloy

INTRODUCTION

Chitin, a naturally abundant mucopoly-saccharide, is known to be biodegradable and its hydrolysates are susceptible to metabolic pathway in animals. Chitin is expected to apply for biomedical materials due to biodegradability and low toxicity on the administration to animal body, such as wound healing in animals [1–3]. Two forms of chitin, called α- and β-chitin have been known. α-Chitin is the tightly compacted, most crystalline polymorphic form and β-chitin is the form where the packing of polymer chain is lose [4]. Both α-chitin from Crab or Shrimp shells and β-chitin from squid pen were found to dissolve in calcium chloride dihydrate saturated methanol [5] (Chitin solvent) and regenerated to chitin hydrogel prepared by precipitation with addition of large excess of water to the chitin solution. In addition, β-chitin slurry was also prepared by repeated mechanical agitation of β-chitin powder in limited amount of water. Both chitin hydrogels contain more than 95% of water. The hydrogel is very useful for several areas, such as preparation of sheet, substrate for graft polymerization and so on.

Bacterial cellulose (BC) is an extracelluar cellulose biosynthesized by Acetobacter xylinum in the Sharman Hestrin (SH) medium containing glucose as a carbon source [6]. Original BC is a gelatinous material which is made up of a network of fibrils. Therefore, BC is a highly hydrated hydrogel containing more than 99.5% of water. Since BC is highly purified cellulose, mechanical property of dried BC is very high.

Since both the chitin hydrogel and BC are highly hydrolated material, hybridyzation of them is very interesting. In addition, treatment with calcium chloride dihydrate saturated methanol solution of chitin and BC will be a more preferable combination of these two polysaccharides. Thus, two types of composites were prepared mentioned above.

Although chitin sheet showed somewhat brittle character arising from strong hydrogen bonding, composite of BC with the chitin hydrogel will improve the property. The material is regarded as a new type of polymer alloy composed by

natural polymer system and is suitable for artificial soft tissue which require biodegradable, biocompatibility and appropriate strength.

PREPARATION OF CHITIN AND BACTERIAL CELLULOSE HYBRID

Chitin coated bacterial cellulose (CCBC)

One of the method to prepare hybrid of chitin and BC is chitin coated bacterial cellulose (CCBC). BC was biosynthesized by Acetobacter xylinum ATCC 23769 in SH medium containing 2% glucose as a carbon source. The cultivation was performed at 28°C for 7 days and the pellicle was treated with sodium dodecyl sulfonate (SDS), 4% sodium hydroxide aqueous solution and water. All the treatments were done in reflux condition for 4 hours. BC membrane was pretreated by dipping in calcium chloride dihydrate saturated methanol solution for 24 hours. Treated BC membrane was dipped in 1% chitin solution of calcium chloride dihydrate saturated methanol for 24 hours. Excessive calcium was removed by immersing in methanol and the hybridyzed BC membrane was air-dried.

Bacterial cellulose and chitin composite (BCCOM)

Purified BC membrane was mechanical agitated in calcium chloride dihydrate saturated methanol solution. Suspension of BC was mixed with calcium chloride dihydrate saturated methanol solution and mechanical agitated. The mixture was dialysed against water to remove calcium and obtained gel was air-dried.

SPECTROPHOTOMETRIC ANALYSIS OF CCBC AND BCCOM

The SEM images of surface structure of original BC, CCBC and BCCOM indicated that typical microfibril network was clearly observed in the surface of BC. The surface structure of the hybrids depended on the preparation method. Although microfilbril was partly observed in CCBC, mechanical agitation led uniform surface of BCC. FT-IR, CP-MAS NMR and XRD analyses were performed to elucidate interaction of chitin and BC. CP-MAS NMR spectra of chitin, BCCOM and BC, one of the example is shown in Figure 1.

MECHANICAL PROPERTIES OF HYBRIDS

Table 1 shows the mechanical properties of BC, CCBC and BCCOM. Although the interaction of chitin and BC was not clearly observed by the spectroscopic measurements, mechanical properties depended strongly on the preparation method. Since mechanical agitation of BC destroyed microfibril network completely, maximal stress of BCCOM was very low compared to BC and CCBC. CCBC showed highest strength of all probably due to the best combination of microfibril and chitin. The fact was apparent by the SEM observation of breaking surface after the mechanical measurement (Figure 2). In the case of CCBC, microfibril network was fully occupied by chitin, however spaces were remained in BC and BCCOM. These results strongly suggest that CCBC had highest mechanical strength.

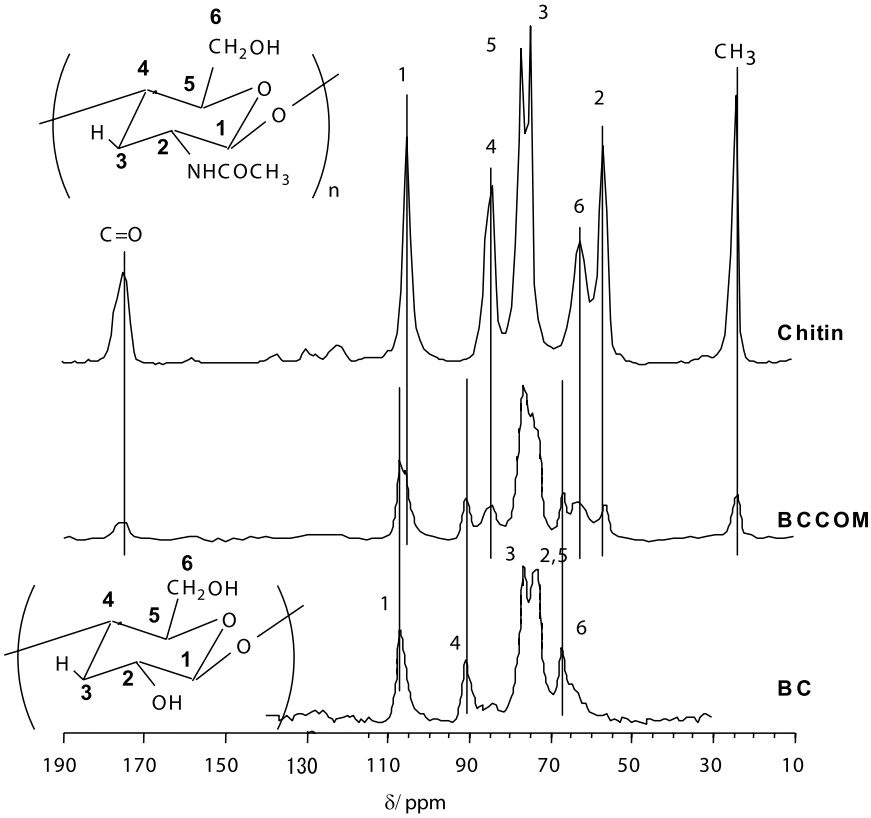

Figure 1. CP-MAS NMR spectra of chitin, BCCOM and BC

Table 1. Mechanical properties of BC, CCBC and BCCOM

	Stress (MPa)	Strain (%)	Young's modulus (GPa)
BC	132.01	5.02	2.60
CCBC	154.57	3.78	6.10
BCCOM	34.03	1.73	2.77

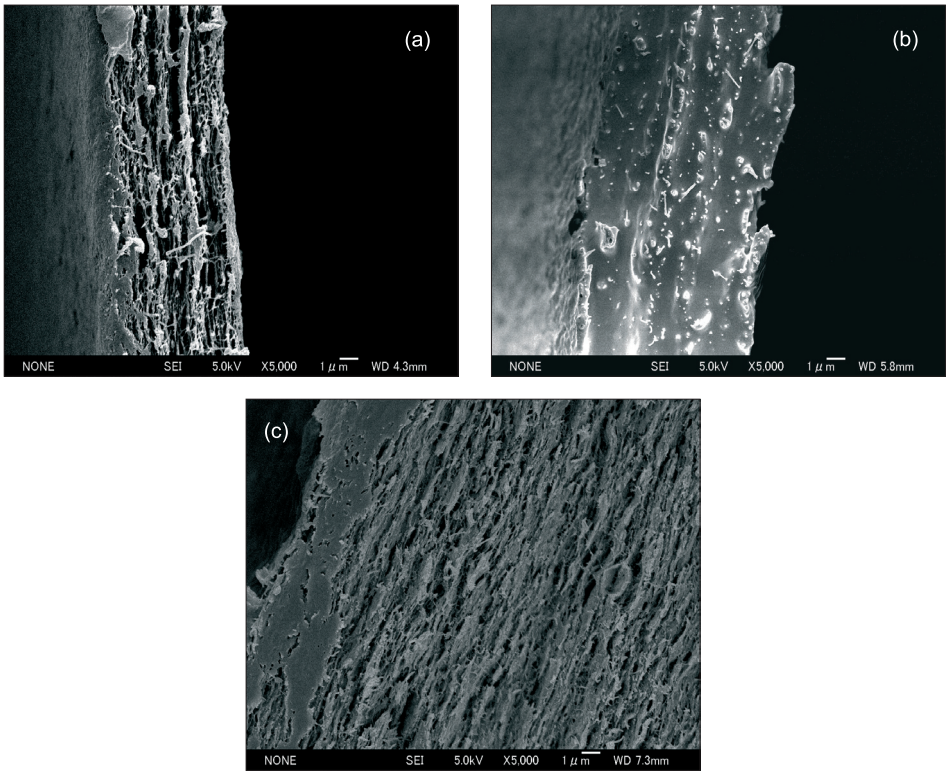

Figure 2. SEM images of breaking surface structure of original BC(a), CCBC(b) and BCCOM(c)

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NOVEL BACTERIAL CELLULOSE/CHITOSAN WOUND DRESSING MATERIALS

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Key words: modified bacterial cellulose, chitosan/bacterial cellulose hydrogel dressings

INTRODUCTION

An effective wound healing requires the wound be maintained in wet condition. Such conditions are provided by modern dressings in form of hydrogels. The best dressing is the patient's own skin which is permeable for vapour and protects the deeper layer tissue against mechanical injuries and infection. Since many years, except of the transplantation of patient's own skin, applied are biological dressings of pig skin or human cadaver skin. Disadvantage of such dressings are their antigen properties limiting the span of the application. The method is, moreover, very expensive and therefore, rarely applied.

In the Institute of Chemical Fibres (IWCh), Poland an ecological technology has been developed for the manufacture of natural hydrogels based on chitosan-modified bacterial cellulose [1]. Modified bacterial cellulose combines properties of both cellulose and chitosan. The modification of the bacterial cellulose occurs already during the microbiological synthesis by introducing selected bioactive polysaccharides like various chitosan forms and its derivatives to the culture medium. It has been found that glucosamine and *N*-acetylglucosamine units are incorporated into the cellulose chain [2]. Such wound dressings can be applied in treating burns, bedsores, skin ulcers, hard-to-heal wounds and wounds requiring frequent dressing change.

MATERIALS AND METHODS

Modified bacterial cellulose (MBC) has been obtained during microbial synthesis under static conditions with the use of acetic bacteria *Acetobacter xylinum* (ŁOCK 0805) from Pure Culture Collection delivered by Institute of Microbiology and Fermentation, Technical University of Łódź. The biosynthesis proceeds in 7 days in the Hestrin-Schramm standard medium modified with different chitosan forms, at temperature 30°C [3].

Amount of aminosaccharides released from dressings under lysozyme degradation was estimated colorimetrically by DNS method [4]. Antibacterial activity tests were performed by quantitative method against *Escherichia coli* (ATCC 11229) and *Staphyloccocus aureus* (ATCC 6538) according to Polish and Japanese standards [5, 6]. Cytotoxicity tests were carried out in Medical Academy Wrocław, Poland according to appropriate standard [7]. Tests on animals for clinical investigations were carried out in Central Animal Laboratory in Katowice, Poland [8].

RESULTS AND DISCUSSION

Some properties of bacterial cellulose/chitosan hydrogels

Two types of hydrogels A and B modified with chitosan salts were obtained directly in static culture of MBC. The advantage of the manufacture technology of MBC hydrogels is that dressings in desired size and shape can be obtained, characterized by a water content of 99.0–99.5% and polymers content 0.5–1.0% (cellulose 90–93% dry weight and chitosan 7–10% dry weight)

Three-dimensional, interwoven structure of modified bacterial cellulose is composed of cellulose with chains linked by strong hydrogen bonds to form microfibrils. This novel material is characterized by: a-cellulose content over 90%, high average degree of polymerization (DP_w>1500), high water retention value (WRV>140%), high elasticity and durability.

A specific feature of chitosan, essential for its medical application, is its biological activity resulting from the susceptibility to degradation influenced by lysozyme, an enzyme present in tissue fluids (Table 1). Mono- and oligoaminosaccharides, being the products of enzymatic chitosan degradation, stimulate tissue granulation which, in turn, accelerates the wound healing without large scars.

Table 1. Susceptibility of MBC to enzymatic degradation by lysozyme

Time of enzymatic degradation [days]	Amount of aminosaccharides released [mg/cm^3]	
	Symbol of hydrogel	
	A	B
0	0.000	0.000
1	0.106	0.081
3	0.113	0.113
6	0.113	0.133

Bacterial cellulose/chitosan dressings are an innovative concept characterized by unique biological features like biocompatibility, non-toxicity and ability to complete post-use degradation.

One of the factors affecting wound healing process is an infection caused by natural human microflora or microorganisms present in the environment. Ap-

plication of wound dressing materials possessing good antibacterial and barrier properties against microorganisms may be a solution to this problem.

Bioactivity tests (Table 2) revealed that hydrogel dressings are characterized by bacteriostatic activity against both *Escherichia coli* and *Staphylococcus aureus*, and bactericidal activity against *Escherichia coli*.

Table 2. Bioactivity tests of hydrogel MBC dressings

Symbol of hydrogel	*Escherichia coli* (ATCC 11229)			*Staphylococcus aureus* (ATCC 6538)		
	Total bacteria number [CFU]	Bacteriostatic activity	Bactericidal activity	Total bacteria number [CFU]	Bacteriostatic activity	Bactericidal activity
0 (unmodified BC)	5.2×10^8	–	–	3.2×10^8	–	–
A	$< 2.0 \times 10^1$	8.7	3.4	2.9×10^6	2.0	–1.8
B	$< 2.0 \times 10^1$	7.4	3.7	1.3×10^7	1.4	–2.4

Studies on biomedical properties of hydrogels

Novel wound dressing materials should maintain appropriate conditions within the wound that would aid regeneration of broken tissue and protect newly formed skin from external infections. Such dressings should also be safe i.e. should not be toxic for live cells nor be allergenic or irritable for the patient's skin.

Cytotoxic *in vitro* tests were carried out by direct contact method on mouse fibroblasts 3T3/Balb. The results proved that wound dressings from modified bacterial cellulose have no toxic effect on mouse fibroblasts (Table 3).

Table 3. Cytotoxicity tests for hydrogel dressings after 72 hours

Tested sample	Morphological changes	Average cell number $\times 10^6$				Toxicity degree
		live	dead	total	standard deviation SD	
Hydrogel A	none	2.53	0.052	2.58	0.0571	0
Hydrogel B	none	2.63	0.054	2.68	0.0668	0
Mother culture	none	2.57	0.052	2.62	0.0294	0

Wound dressings based on modified bacterial cellulose, tested on guinea pigs, showed good stimulation of factors accelerating granulation tissue forming (Fig. 1), good adhesion to the wound bed and good isolation of the wound from the environment, thus providing protection against secondary infection [8].

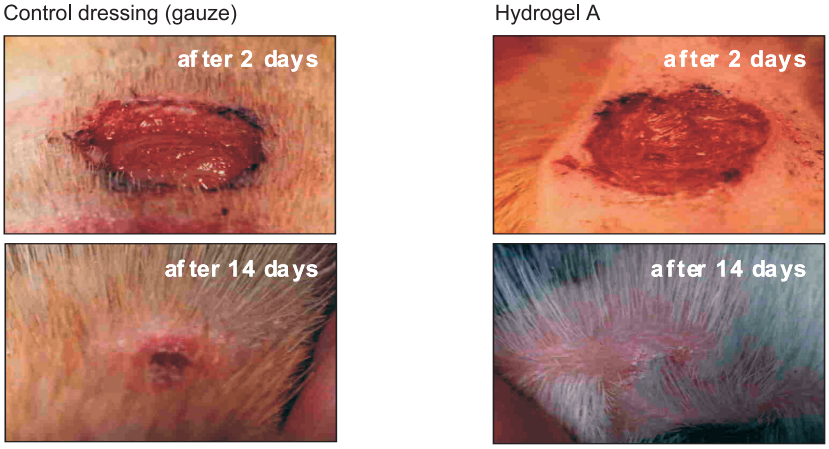

Figure 1. *In vivo* tests of hydrogel wound dressings from modified bacterial cellulose

CONCLUSIONS

A method to manufacture hydrogel dressing materials from chitosan-modified bacterial cellulose has been developed. Wound dressings with specified dimensions and shapes, characterized by valuable biological properties have been prepared directly in biosynthesis process. Hydrogel wound dressings proved to be non-cytotoxic. After contact of cell cultures with dressings, high fibroblast proliferation was observed. Bioactive hydrogel dressing materials have been elaborated providing optimal moisture conditions for rapid wound healing and stimulating wound healing process without irritation or allergization.

ACKNOWLEDGMENTS

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CHITOSANS FOR SIZING NATURAL AND SYNTHETIC YARNS

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Abstract: Strategies and preliminary results of a research project on the use of chitosan (CTS) as a fibre protecting agent for the weaving process (sizing) are presented. Chitosan may replace some existing sizes in the weaving industry which frequently are of low biodegradability and high demand on COD or BOD especially in weaving of synthetic yarns.

Key words: Chitosan, Sizing, Textiles

INTRODUCTION

Within the production of textiles the sizing process for the weaving industry is of utmost importance. Due to the high speed weaving a yarn is exposed to high forces of abrasion. Friction between yarn gearing units and friction between warp and weft yarns have to be taken into account. Especially with natural yarns their hairiness and roughness are problematic. So the function of a size is to provide a protecting film on the yarn. This requires [1, 2]:

– good film forming properties of the size used,
– high tenacity and high elongation capability of the size film,
– high reduction of hairiness meaning good glueing/adhesion properties.

At present two classes of sizes are employed: Sizes based on natural stuff like derivatives of cellulose or starch and synthetic sizes of the polyvinylalcohol-, polyester- and polyacrylate type. Often combinations are used depending on the kind and structure of the yarn to be woven.

After weaving in most cases the size has to be removed in order not to disturb following textile finishing processes. Since currently the load of size on a fabric ranges from about 3–30 weight-% a huge load for the size removal washings and effluents in textile finishing industry arises. Besides COD and BOD aspects some of the synthetic sizes are not biodegradable [3].

Regarding these problems a joint research project has been launched in order to investigate the utilization of chitosans (CTS) or derivatives – well known film formers and biodegradable materials [4–8] – for yarn sizing. Another aim of the project is to check whether lower loads on the yarns and thus lower prob-

lems with effluents can be achieved without loosing weaving performance. Some problems and some results of this research are presented now.

The structure and aims of the projects are sketched in Fig. 1.

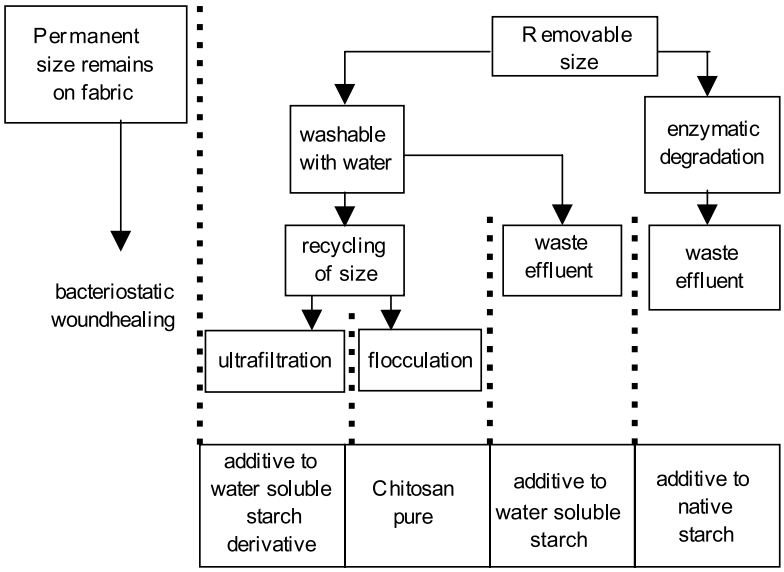

Figure 1. Project structure for use of CTS as sizing agent in yarn weaving

This structure implies two strategies: One where some residual chitosan may remain on the yarn to impart some antimicrobial property of CTS to the final product and the other to have quick size removal. Size removal may be envisaged as done destructively as enzymatic or acid degradation or only by washing retaining the chance for size recyclation by ultrafiltration techniques.

MATERIALS AND METHODS

In Fig. 2 a scheme is given of one of the testing equipment for sizing performance. It has been deviced by ITV Denkendorf as a quick analysis of the properties of a size [9]. It comprizes size add-on and conditioning, and roughness/hairiness measurents following frictional load as is occurring in a weaving process. Other size testings like adhesion power or yarn breakage counting not shown).

CTS with degree of deacetylation of 75 resp. 85% with mean molecular range from 90 to 300 kDa have been used for sizing experiments and for substrates for derivatization.

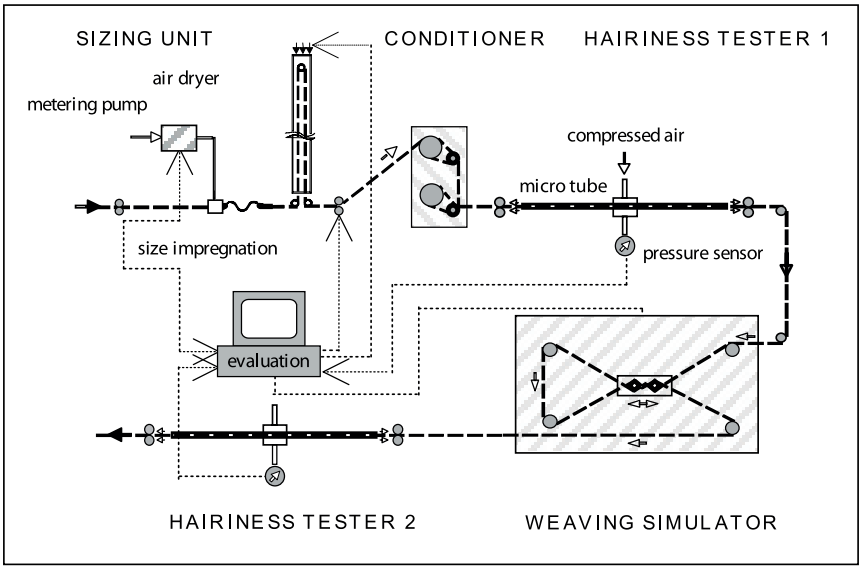

Figure 2. Scheme of testing unit for yarn roughness in sizing evaluations

PRELIMINARY RESULTS

Very soon during project work it turned out that redissolution of CTS-films – once prepared at 85°C drying temperature on yarn or fabric – within a short time scale (seconds to minutes) is demanded. The demands and facilities of the industrial project partners normally uses this technique. So many studies have been directed to use CTS-derivatives of various solubility. Some of the structures tested are sketched in Table 1 (each with high and low degree of substitution).

Table 1. Derivatives tested for quickly redissolvable CTS derivatives

	substitution with	Comment
$-SO_3Na$	SO_3 /Triethylamine	non film forming
$-CH_2-COONa$	Na-Chloroacetate	slow dissolution
$-CH=CH-COONa$	Maleinic acid anhydride	brittle films
$-CH_2-CH_2-OH$	Chloroethanol	slow dissolution
– Lactose	Reductive alkylation	slow dissolution
$-CH_2-CHOH-CH_2OH$	Propylenoxide	difficult preparation

At the present state the requirements of film dissolution times << 3 min for industrial hot desizing could not be met with these derivatives (starting MW for derivatization 230 kDa).

Related to these demands some measurements on water uptake of CTS-films are presented in dependence on storage time. It shows that after a sudden increase in water content a gradual loss is observed. Due to this ageing process

redissolution time of a CTS-film either in water of slightly acidic (warm) medium increases in an industrially unacceptable extent.

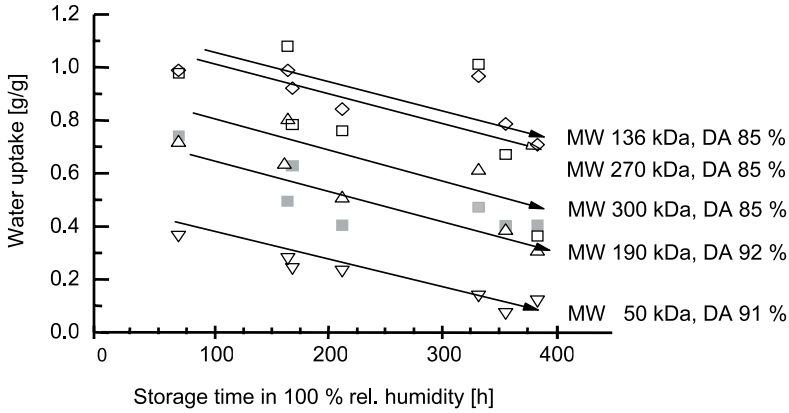

Figure 3. Time dependence of the weight of CTS-films stored in humid air (film casting from pH 5 solutions in acetic acid)

This loss of water even in water vapour saturated surrounding may be interpreted as being due to structural changes within the polymer chains of the films. A sketch of structural changes as proposed is drawn in Fig. 4. Similar questions have been raised earlier by Ogawa et al. [10]. This ageing behaviour is less pronounced with films of CTS prepared from hydrochloric or tetrafluoroboric acid solutions.

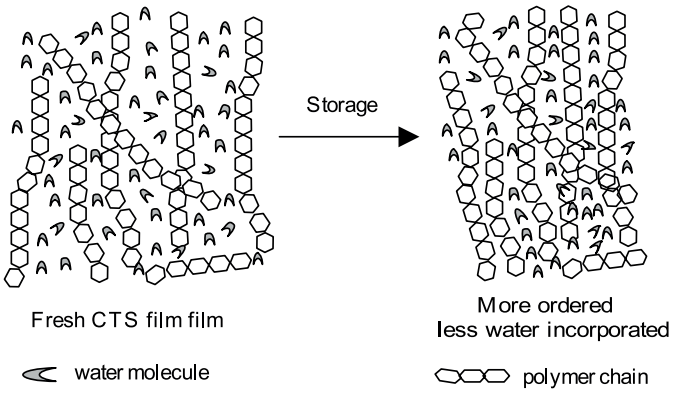

Figure 4. Model for structural changes on storage of CTS films (100% rel. humidity, 25°C) (c.f. [10])

Blending with molecular lubricants, i.e. structure breaking additives, are investigated now to prevent changes during storage.

For the part of size removal for low temperature/long time processes very efficient enzymatic recipes have been found during project work. Fig. 5 shows enzymatic degradation of dissolved CTS by several enzymes measured as viscosity drop vs time. Using these results one of the project partners has designed a textile auxiliary which works efficient in CTS-degradation even from a CTS-loaded fabric (both natural and synthetic type). The degree of CTS-size removal may be

monitored by sample immersing in hypochlorite solution, followed by extensive rinsing and soaking in solutions of potassium iodide/starch and determination of the colour development. This testing procedure may be useful for any other field where an analysis of CTS on surfaces may be needed.

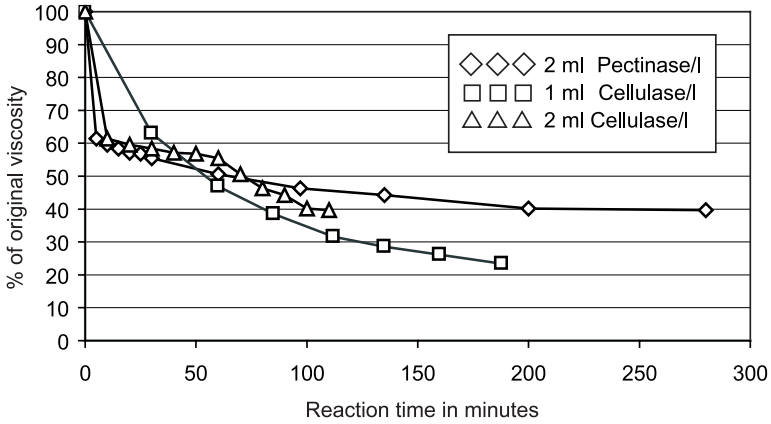

Figure 5. Viscosity/time of solutions for CTS (DA 85%, MW 230 kDa), enzyme: 2.5 g/l, pH 6, 50°C

Tests under industrial conditions using CTS alone as size for polyester yarn showed promising results. Even at the low add-on of about 2 wt-% only about 7 breaking failures per 10^5 counts have been found which is higher than conventional but conventional sizing has higher sizing wt-% load.

Further work for performance improvement of pure CTS-sizes will be directed to decrease molecular weight of the CTS used in order to increase solubility and dissolution rate of CTS or its derivatives. Strategies which have perspectives in industrial application are listed in Table 2 together with remarks on feasibility.

Table 2. Intended ways for molecular mass reduction of CTS samples

Reagent	Pro	Con
Acid	–	needs long time
Ozone	without solvent	Product has too many aldehydic groups, cross links at > T = 80°C, → oxydized CTS
Peroxygen compounds	–	same as with ozone
Na-Nitrit (pH ~ 4)	quick very fast	only few aldehydes problems with stirring homogenously, product has (few) aldedyd groups
Enzymatically (e.g. Cellulase/Hemicellulase)	in progress	in progress

Molecular mass reduction will provide too a way for obtaining less viscous solutions which at present are difficult to handle in technical sizing processes

but providing higher mass concentration in the sizing bath. This might be useful when using pure CTS as film former on synthetic yarns.

For cotton sizing mixtures of CTS with conventional starch based sizes have been tested since starch is a low-price material and therefore the high-price CTS has to used economically. The addition of CTS distinctly improves smoothness of the yarns to a degree suited for industrial application.

OUTLOOK

At present one states promising properties for the use of CTS as yarn sizing additive or as pure size. Many properties of the material used have to be improved in future. Further work tackling the questions of molecular weight, derivatives and dissolution rate have to be overcome. Following this the recycling story can be solved.

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NON-WOVENS MADE FROM DIBUTYRYLCHITIN AS NOVEL DRESSING MATERIALS ACCELERATING WOUND HEALING

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Key words: dibutyrylchitin, non-woven fabrics, dressings, clinical research, wound healing

INTRODUCTION

Chitin, the natural most abundant polysaccharide containing nitrogen, is degraded by biological enzyme, has biocompatibility on cell level, induces human cells to promote the restoration of the wound, enhances the healing tension of the wound and has high permeability for the substances of medium molecular weight in serum [1]. Chitin might be an ideal raw material for bioactive dressing materials, but due to its low solubility can be hardly conversed into useful forms like films, fibre or non-woven matrix.

Dibutyrylchitin, an ester chitin derivative, is easily soluble in common organic solvents and has both film- and fibre-forming properties [2]. Preliminary investigations of biological properties of dibutyrylchitin materials showed its good biocompatibility, lack of cytotoxicity, inflammatory and immunomodulating effects and ability to accelerate wound healing [3, 4]. It was established that dibutyrylchitin dressing materials fulfil all basic requirements set up for medical devices by EN ISO 10 993.

The first clinical investigations of medical properties of dibutyrylchitin have been carried out at Polish Mother's Health Institute in Lodz, Poland. Dibutyrylchitin samples under investigations were used in form of non-woven materials made at Technical University of Lodz. Preliminary results of the clinical investigations are shown for the first time in the present article.

EXPERIMENTAL AND RESULTS

Preparation of dibutyrylchitin and dibutyrylchitin fibres

Synthesis of dibutyrylchitin (DBC) was carried out at ca 20°C during 4 hours using 5 g of $HClO_4$ (70–72%) and 160 g of butyric anhydride (98%) per 10 g of krill

chitin (MIR, Gdynia, Poland, molecular weight 286,7x10^3 daltons) [5]. DBC with intrinsic viscosity value of 2.29 dL/g (determined in dimethylacetamide solutions at 25°C) was obtained with the yield of 93%. Elemental analysis of DBC gave: C – 55.80% (calculated value 55.98%), H – 7.51% (calculated value 7.29%) and N – 4.10% (calculated value 4.08%) suggesting that degree of chitin acylation was equal to 2.

Wet spinning of DBC fibres was made at Institute of Chemical Fibres, Lodz, on apparatus commonly used for preparation of rayon fibres [6]. A typical dope containing 17% of DBC in DMF without filtration was added to the reservoir of the spinning system and extruded through a spinneret (300 holes, 80 μm diameter of the hole) to a coagulation bath. The filaments were coagulated in water, drawn in hot water, collected on rollers with a rate of 40 m/min and dried.

Preparation of non-woven dressing materials

DBC fibres used in non-woven manufacturing process were characterised by length equal to 60 mm, linear density of 4,2 dtex and tenacity of 12.1 cN/tex. Non-woven samples were prepared on the laboratory carding and calendering machines at Technical University of Lodz. The surface mass of obtained non-woven fabrics was equal to 28 g/m^2 and thickness was equal to 0.44 mm.

Non-woven fabrics were cut into pieces of 5 x 5 cm and γ-sterilised with dose of 2.5 Mrad.

Clinical investigations of DBC non-woven dressing materials

Surgical staff of Dept. of Paediatric Surgery was provided with a number of DBC petals for medical application (Fig.1.) According to data available in literature:

- no cytotoxic effect of DBC was found in vitro,
- no adverse reaction was observed in animals (mice, rabbits) after intradermal administration, as well as intraperitoneal implantation of DBC,
- microscopic evaluation of foreign body reactions concerning DBC were less evident than these around Maxon and Dexon thread; cytokins levels in peritoneal fluid of laboratory animals after intraperitoneal implantation of DBC and Maxon or Dexon were comparable.

Biological investigation of DBC dressing materials carried out at Medical Academy in Wroclaw, Poland, showed that they fulfil all basic requirements set up for medical devices by EN ISO 10 993.

On the other hand chitin and its derivatives have been since long used in humans:

- in cosmetics,
- in some wounds' dressings production,
- in pharmacology (as ingredient of drugs' capsules),
- in ophtalmology,
- in some dietary systems, etc.

It is now of little doubt that chitin and its derivatives are harmless and may be beneficial for humans.

Based on these facts was the acceptance of Ethic Committee of Polish Mother's Health Institute for the project forecasting the application of DBC in surgical paediatric patients.

It has been planned to apply DBC in the group of 30 children with following indications:

– burns,
– wounds of various etiology,
– bed sores – in the patients immobilized for long periods regardless of reason,
– lesion of parietes as a result of multifactorial processes (eg. sepsis).

Preliminary results (n=21)

– **Burns**

There were 6 patients in this group.

a) Five of them suffered thermal burns, their age ranging from 7–18 m (mean 11,5 m) and burned area was 5–20% of total body surface; depth 2a. All the burns healed up within 1–2 weeks after DBC application (Fig. 2a, 2b, 2c, 3a, 3b, 3c).
b) One patient (boy 17 year old) suffered electric burn locally to the left forearm. After removal of necrotic tissue, DBC was applied as a pretreatment before surgery (split – thickness skin transplant) (Fig. 4).

– **Wounds (other then burns)**

a) *Postoperative.* This group consisted of 3 patients. All of them have got small areas of granulation tissue healing "per secundam". Two of them were after plastic operation of microtia and one after excision of huge naevus (Fig. 5).
b) *Posttraumatic.* There were 4 patients in this group. Two of them sustained injury to the digits with partial necrosis of soft tissues. After removal of necrotic tissue DBC was applied and there was no need of further surgical interference. The other two children had extensive wounds of limbs and skin loss due to necrosis. The petals of DBC were applied on the row surface – as a pretreatment before surgery. In one patient the wound healed up spontaneously and rapidly and he avoided surgery. In the other one the split – thickness skin transplant was performed (Fig. 6, 7a,7b,8).
c) *Posttraumatic/postoperative.* Three patients from this group sustained severe injuries to the lower limbs: open fractures of tibia with the loss of soft tissue. All were repeatedly operated on by orthopedic surgeon. As a consequence the residual chronic ulcers with tibial bone exposed were seen in all cases. The application of DBC resulted in the healing of wound in two cases. The third child is still under observation (Fig.9a, 9b, 9c).

– **Various entities leading to skin/epidermis loss**

a) The local treatment with the use of DBC was introduced in 1,5 year old child with foci of necrosis of the skin in the course of general sepsis. As a result of removal of necrotic tissue and application of DBC the healing was achieved (Fig. 10a, 10b).

b) Similar indication was found in 7 year old black boy with epidermolysis. His treatment is still under way.
c) In 3 months old infant who has been treated conservatively for omphalocele, the residual granulation tissue was covered with DBC and it healed up soon. The child awaits surgery but the operation is by no mean urgent.

– **Bed sores**
There were two patients in this group – both disabled, neurologically impaired after meningomyelocele operated on in infancy. Extensive chronic bed sores were infected, covered with pus. The pieces of DBC did not stick to the bed sore and were flowing freely on its surface, making no contact with the tissue. The conservative treatment was discontinued.

DISCUSSION

As far as thermal burn patients are concerned the depth of the wound was evaluated as 2a in all cases. It means there was no necrotic tissue and no indication for operative treatment. In all cases healing was quick and uneventful and in no case infection developed. As regards the only child with electric burn – the first step of procedure was the removal of necrotic tissue. The application of DBC, as preparation for surgery, and operation itself followed. The non-septic wounds are by definition indication for surgical treatment. As a matter of fact all presented patients were primarily treated this way. In 3 of them, after plastic operation, there were small unhealed patches with granulation. There were no signs of infection and no necrotic tissue. All these places healed up soon after the application of DBC. Another 4 children suffered mechanical injury, and wounds were complicated by the presence of necrotic tissue. The first stop in all cases was the removal of necrosis followed by the use of DBC. Two of them were considered the candidates for further surgery but this was the case only in 1 patient. Surprisingly the other one healed up soon enough to avoid surgery. The biggest surprise was the uneventful progress made by 2 children operated on repeatedly for open, complicated leg fracture. The wounds were clean, without necrosis but with exposed bone in the centre. In spite of this they healed up quickly. The third such a patient hopefully will be going the same way. DBC was applied as well in 3 another children with various entities. They all have two things in common: the lesion of the skin/epidermis and the absence of necrosis and infection.

The results were at least promising but too few cases have been observed until now and the etiology was diverse.

It turned out that in another suggested indication for DBC application: bed sore, no benefit was seen. Extensive bed sore is connected always with infections, suppuration, non – viable tissue and made up case for surgery rather then for conservative treatment.

Presented observations are preliminary. Further evaluation is necessary.

CONCLUSIONS

– Preliminary results of DBC application are highly promising: DBC seems to promote wounds' healing.
– Selection of the patients for this treatment has to be meticulous: the tissues to be covered with DBC should be viable, possible without infection. Otherwise the results are doubtful – as was the case with bed sores.
– To obtain evidence based proofs of beneficial effects of DBC further randomised trials with referential group are necessary.

FIGURE INDEX

1. The flake of DBC.
2a. Burn of chest.
2b. DBC application.
2c. The wounds are healed up.
3a. Burn of right lower limb.
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4. Deep electric burn. DBC applied after debridement.
5. Microtia – DBC on small residual granulation after operation.
6. Crushing of distal part of 4th digit of left foot. DBC applied after removal of necrotic tissue.
7a. Wound devoid of necrotic tissue.
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8. DBC was applied. Wound is ready for surgery. 9a. Residual granulation. Bone exposed.
9b. DBC applied.
9c. Wound healed up.
10a. Wounds devoid of necrosis, covered with DBC.
10b. Healing nearly completed

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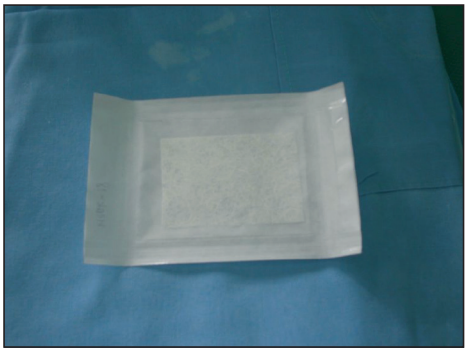

Figure 1. The flake of DBC

Figure 2a. Burn of chest

Figure 2b. DBC application

Figure 2c. The wounds are healed up

Figure 3a.Burn of right lower limb

Figure 3b. DBC application

Figure 3c. Healing nearly completed

Figure 4. Deep electric burn. DBC applied after debridement

Figure 5. Microtia – DBC on small residual granulation after operation

Figure 6. Crushing of distal part of 4th digit of left foot

Figure 7a. Wound devoid of necrotic tissue

Figure 7b. DBC application

Figure 7c. Healing nearly completed

Figure 8. DBC was applied. Wound is ready for surgery

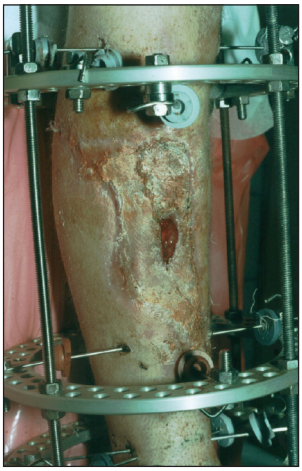

Figure 9a. Residual granulation. Bone exposed

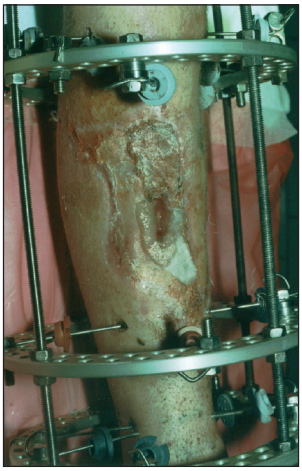

Figure 9b. DBC applied

Figure 9c. Wound healed up

Figure 10a. Wounds devoid of necrosis, covered with DBC

Figure 10b. Healing nearly completed

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CHITOSAN PHYSICAL HYDROGELS: A NEW THERAPEUTIC WAY TO BURN INJURIES TREATMENT

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Key words: Chitosan, physical hydrogels, burn treatment

INTRODUCTION

Chitosan is largely studied in pharmaceutical and medical fields for its biodegradability, biocompatibility, bioactivity and its interesting physicochemical properties [1]. Its chemical structure mimics the glycosaminoglycans constituting an important part of the extra-cellular matrixes of mammalian living tissues and then must be regarded as a decoy for biological media.

The aim of our study was to obtain physical hydrogels of chitosan. Hydrogels know a great interest for the diversity of their properties. Their capacity at absorbing water, make them a biocompatible decoy at a larger scale and opens new trends for the research on biomaterials.

Burns are among the most common injuries encountered by mankind [2]. When applied to extended burns and chronic wounds, classical treatments, are insufficient in preventing the scar formation and promoting healing. Thanks to chitosan hydrogels, we propose a new route to treat burn injuries. This paper deals with the mechanism of formation of chitosan gels, their nanostructure and results on the healing on extended third-level burns processed on pigs.

MATERIALS AND METHODS

We used a squid pen chitosan with a low Degree of Acetylation (DA) and a high weight-average molecular weight.

To obtain hydrogels, chitosan was dispersed in water and hydrochloric acid was used to achieve the stoechiometric protonation of the NH_2 sites. After complete dissolution of chitosan in water, 1,2-propandiol was added. Then, the mixture was stirred and let to evaporate at 50°C up to gelation [3].

Table 1. Characteristics values and experimental methods used for the determination of the physiochemical features of chitosan

DA	2.6% ± 0.1%	^{1}H NMR
Mw	540 000 g/mol ± 27000 g/mol	SEC
% H_2O	8% ± 1%	TGA

We used a gas chromatographic method to evaluate the proportion of the alcohol all along the gelation. The chromatograph was equipped with a Poraplot column of diameter 5mm, the injector temperature was 240°C, and with column temperature 240°C. We used an ionization flame detector and helium as gas vector and 1 μL injections.

Rheological measurements were performed at room temperature with a RMS 800 rheometer operating with a plate-plate geometry (diameter 25 mm).

The study of the variation of chlorine ions content, in the gelation media, was performed with an anionic-exchange chromatography constituted of a Dionex AG9HC column of 4mm diameter and 5 cm length. The eluent was a 12/5 molar mixture of Na_2CO_3/$NaHCO_3$ in ultra-pure water. The apparatus was equipped with a conductimetric detector. After gelation, for each sample, chitosan was precipitated with NaOH and then diluted in distilled and de-ionized water.

The hydrogel nanostructure was studied by small angle X-ray scattering. The experiments were performed on BM2-D2AM beamline at ESRF (Grenoble, France).

Formation of physical gels

We prepared physical hydrogels without any chemical reaction or chemical cross-linkers. Whatever the DA, it is always possible to form a physical hydrogel from a solution of chitosan [3]. For that it is only necessary to observe three conditions.

- The polymer concentration must be maintained above the critical concentration of chain entanglement (C*),
- A change in the balance between hydrophobic and hydrophilic interactions must be induced in order to favor hydrophobic interactions,
- Gelation must be performed homogeneously throughout the sample.

As there is no known azeotropic composition between water and 1,2 propandiol, and considering data found in the literature [3–5], we considered the evaporation of alcohol in the gelation conditions as negligible.

In this work, we evaluated the amount of propandiol in the medium during the gel formation.

Whatever the initial polymer concentration, we confirmed that during gelation, only water evaporated. Then, after complete elimination of water, corresponding to the full gelation, the composition remained constant and only chitosan and 1,2-propandiol were present in the medium. The role of the alcohol was to reduce the dielectric constant of the medium, by the progressive concentration of the less polar solvent, possibly participating in the formation of hydrophobic junctions between polymer chain segments, and also acting on the acid dissociation, hence reducing the charge density carried by the polyelectrolyte, as shown

below. Rheological measurements confirmed that the gel was fully formed after evaporation of the water contained in the initial solution medium.

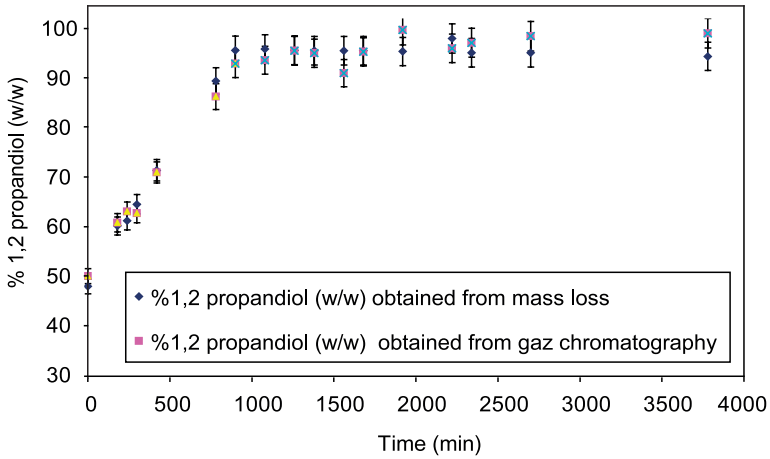

Figure 1. Evolution of the mass fraction of 1,2 propandiol in the hydro alcoholic medium during gelation, with an initial chitosan concentration of 3% (w/w) (samples in the form of disks with a diameter close to 30 mm and a thickness close to 10 mm)

Evaluation of Cl^- ions concentration

In order to evaluate the changes in the mixture composition, we studied the variation of the concentration of Cl^- ions with time. We could clearly evidence a decrease of Cl^- before gelation up to $3{,}6*10^{-7}$ mol. In fact, during water evaporation, HCl was liberated, in relation with a medium of decreasing dielectric constant, where HCl had a lower solubility and became a weak acid. As a consequence during evaporation, both water and HCl were eliminated. This phenomenon was responsible for a decrease of the apparent charge density of chitosan chains and contributed to displace the balance between hydrophilic and hydrophobic interactions up to the point of gelation.

Rheological characterization

We studied the evolution of the real (G′) and imaginary (G″) part of the complex shear modulus as a function of the frequency at different gelation steps. It is generally considered that when G′<G″, the behavior of the material is of liquid type, but when G′>G″, in a large frequency range, the material behaves as a gel. This transition is shown on Fig. 2. We observed that the crossing point between G′ and G″ shifted from high to low frequencies, when the gelation time increased.

The shift of the crossing point can be interpreted in terms of an increase of the time of disentanglement of polymer chains as the gelation time increased, i.e. as the number of physical crosslinks increased. After 19 hours (1140 min.) of evaporation time, G′(ω) and G″(ω) curves were parallel, and do not display the crossing point, at least in the assessable angular frequency range. We could thus deduce that on a rheological point of view, the gel formation was achieved between 15 and 19 hours. If we now consider the kinectics of water evaporation (Fig. 1)

we observed that the gel formation corresponded to the end of evaporation, corresponding to the equilibrium plateau. This confirmed that alcohol molecules played an important role in the formation of hydrophobic interactions. Indeed, in spite of the evolution of the charge density of chitosan, their amphiphilic structure could allow interactions between acetylated groups to form hydrophobic connections.

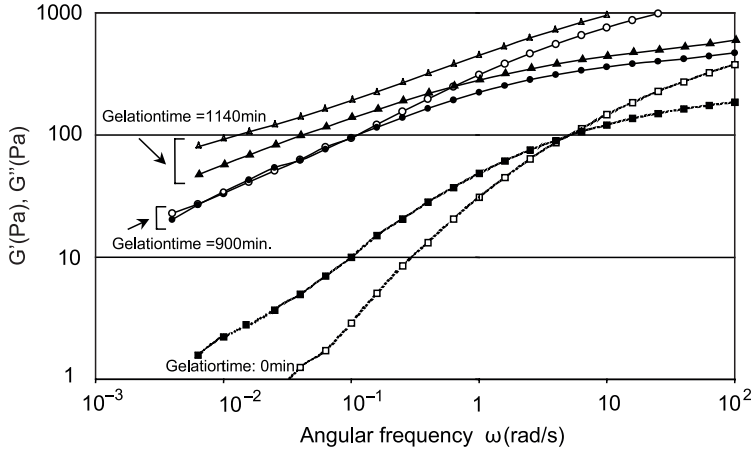

Figure 2. Rheological study of a chitosan physical hydrogel. $[\text{chitosan}]_{init}$=3% (w/w), plate-plate geometry with disks of diameter=25mm, measurements performed at 25°C, at a shear rate of $50s^{-1}$ G'': (■;●;▲); G' (□;○;△)

Structure of the gels

We studied the nanostructure of our hydrogels by SAXS, and the anisotropic evolution of this nanostructure by *in situ* compression under the X-ray beam. The so-called butterfly patterns could be interpreted in terms of the presence and anisotropic development of (*i*) nano-cavities of water and (*ii*) chain aggregates interacting by hydrophobic junctions. The scattering laws in parallel and perpendicular direction were analyzed from Guinier laws. It seemed that the most appropriate modeling is a collection of cylinders with diameter and thickness of about 100Å (Figure 3).

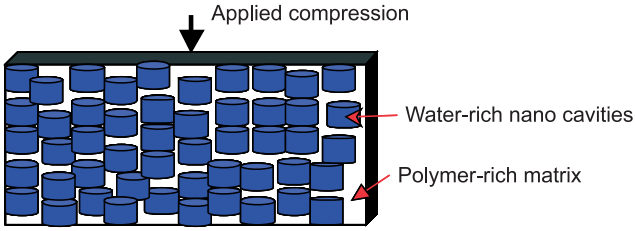

Figure 3. Modeling of the nano-structure of chitosan hydrogels, determined by *in situ* compression under synchrotron beam

This nanostructure was sensitive to compression and the nano-cavities flattened under stress. This geometrical evolution was reversible in a large applied

deformation range with the nano-objects fully recovering their initial state. This property could be particularly interesting for our concept of materials, decoys of biological media, since such anisotropic structural evolution could also be evidenced with the same type of experiments the same experiments performed on soft human tissues such as skin.

Application to third-level burn treatment: *in-vivo* tissue engineering

In-vivo experiments were performed on pig skin. We processed dorsal third-level burns. Then, the protocol for each injury both concerned the epidermis, dermis and hypodermis. After a complete elimination of the necrotic tissue, the defects were filled with our hydrogels.

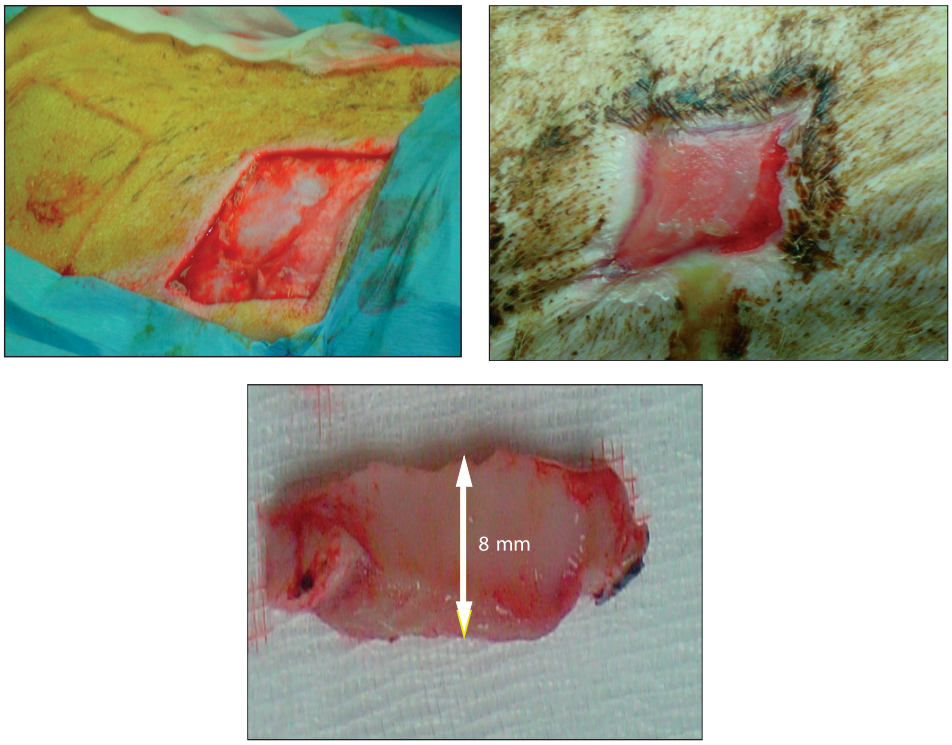

Figure 4. third-level burns processed on pig skins at day: (a) 0; (b) 18, with the use of chitosan hydrogel; (c) 18 view of the thickness of skin reconstructed with chitosan hydrogel

We obtained promizing results on wounds treated with a chitosan hydrogel. After 9 days we first observed the reconstruction of blood vessels, then, the birth of an hypodermis. As we can see on figures 4 b and c, at day 18, a new skin with a large thickness was formed, and the epidermalisation was in progress. We were typically in the presence of a mechanism we call *in-vivo* tissue-engineering.

CONCLUSIONS

In this work, we developed a new way for the production of true chitosan physical-hydrogels. We confirmed that these gels were essentially cross-linked thanks to hydrophobic interactions, in contrast with chemical crosslinking gelation routes. The mechanism of gelation was based on the evaporation of water and HCl thus displacing the equilibrium between hydrophilic and hydrophobic interactions up to a critical value where the network was formed.

We pointed out that our biomaterials were nano-organised. The nanostructures provided the materials with a bio-inspired behaviour allowing us to term them decoys of biological media. Indeed, chitosan hydrogels showed very good results on third-level burns healing. This healing was faster than with a classical treatment and the neo-formed skin was very flexible as for a native skin.

ACKNOWLEDGMENTS

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THE NON-WOVEN COATED BY VARIOUS CHITOSAN FORMS FOR SPECIAL APPLICATIONS

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Key words: non-woven, chitosan forms

INTRODUCTION

A most important problems in the manufacture of sanitary products is the utilization of sanitary pads, nappies and towels after use. The man-made non-wovens used for the manufacture of last generation disposal products mostly do not indicate the susceptibility for biodegradation. Sanitary products could make up 50% of the waste from a household goods. They should never be flushed down toilets, which causes blocked household drains and maintenance problems at sewage treatment plants. The cost to each local authority of disposing of disposables is hundreds thousands of USD per year, so sanitary products schemes now play a key role in local authority waste strategies.

The idea of an introduction of non-woven that indicates necessary endurance during use and will easily disintegrated after use is still the major priority of the sanitary industry sector.

Chitosan as well as its derivatives are often used for the preparation of the biodegradable films. Use of chitosan in form of salt causes the solubility of formed films in solution having pH < 7. However, an application of microcrystalline form of this biopolymer shows the resistance for the dissolution in neutral pH as well as prolongation of the biodegradation due to the relatively high crystallinity of formed films.

The aim of study was to determine the effect of the application of various chitosans differed in average molecular weight, their aqueous solution in in- or organic acids to create useable properties of formed films and non-wovens for:

– control of their dissolution and disintegration in aqueous solution,
– swelling behavior,
– biodegradation affinity.

MATERIALS AND METHODS

Materials

Chitosan

Chitosan was made by Vanson Ltd. (USA). The properties of biopolymers used are presented in Table 1.

Table 1. Properties of chitosan used

Symbol of samples	Form	$\overline{M}_V$	DD	WRV	Solubility in 1% acetic acid	Moisture content	Heavy metal content[1]	Ash content[2]
		[kDa]	[%]	[%]	[wt%]	[wt%]	[wt%]	[wt%]
V3	Powder	166.3	76.2	121.0	100	7.8	0.005	0.3
V6	Powder	593.6	76.5	109.0	97.3	6.2	0.006	0.4
V9	Flakes	755.3	71.3	143.0	99.9	9.6	0.010	0.4
V12	Flakes	267.4	82.4	99.0	99.6	8.9	0.010	0.9
V14	Flakes	848.8	82.4	136.0	99.9	13.3	0.003	0.5

$\overline{M}_V$ – average molecular weight; DD – degree of deacetylation; WRV – water retention value,
[1] – heavy metal content determined by PN-82/C-84002.15 Standard,
[2] – ash content determined by ISO 3451-1:1997 Standard.

Oriented fiber pulps

For the preparation of non-wovens two types of sources made of synthetic and natural origin (polypropylene: PP non-woven or viscose: VS non-woven) were used:

- oriented polypropylene fiber pulp used for the manufacture of non-woven with surface weight of 25 or 50 g/m^2;
- oriented viscose fiber pulp used for the manufacture of non-woven having surface weight of 25 or 50 g/m^2;

Analytical methods

Degree of deacetylation (DD) of chitosan was determined by potentiometric titration [1], average molecular weight ($\overline{M}_V$) by viscometry [2, 3], water retention value (WRV) by gravimetrical method [2]. $\overline{M}_V$ and DD of microcrystalline chitosan (MCCh) were determined by modified method described before [2, 3].

The test in artificial urine or artificial blood (test media) was carried out based on the DIN 53923 Standard, whereas the swelling of the chitosan films in artificial urine or artificial blood was determined according to WRV method [4].

The insoluble part of films was determined gravimetrically according to WRV procedure [2] taking into account known dry weight of initial and swelled film during 1h ca. 0.3 g of sample immerse in 100 ml destilled water or artificial urine.

Surface weight of non-wovens was determined using PN-EN 29073-1:1994 Standard, thickness of non-wovens was determined using PN-EN ISO 9073-

2:2002 Standard, breaking strength and elongation of non-wovens were determined using PN-EN 29073-3:1994 Standard.

Methods

Preparation of chitosan salts films

2,4 g of chitosan was dispersed in destilled water (150 ml) for 24h. Then suitable amount of acetic acid, lactic acid or hydrochloric acid was added to obtain final concentration as fallow: 0,8%; 0,8% or 0,2%. Chitosan solution was filtered using the cheese-cloth and filled to 200 ml by destilled water. Than suitable amount of 1% (w/v) sodium hydroxide was dropped till pH ranged 5.4–5.5. The buffered solution was filled with destilled water to 240 ml.

Buffered chitosan salts films were formed by air-drying on PTFE plate (ca. 6 x 4 x 0,3 cm) for ca. 24h.

Preparation of modified non-wovens in aquoues chitosan salt solution

Oriented fiber pulp (made of polypropylene or viscose) were submerged in aqueous chitosan salt solution for 5 or 10 min. The excess of binding agent was impressed using FULARD apparatus with gab 0,25 mm or 0,65 mm. Than formed non-woven was dried in owen for 24 h at 40°C.

RESULTS AND DISCUSSION

The swelling behavior of chitosan salts films

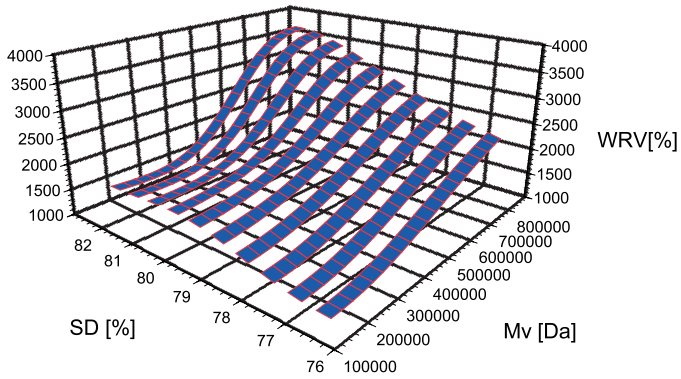
Chitosan films formed of the buffered solution of organic acids (pH 5.5) shows WRV in range of 1000 to over 4000% (Fig. 1.).

The full solubility of chitosan hydrochloride films (buffered at pH 5.5) has been noted for each chitosan used. Than, no WRV results was collected for these samples.

The partial dissolution occurred for films prepared from both organic acid solutions.

The films made of chitosan salts immersed in artificial urine show significantly lower WRV (Fig. 2.) in comparison to WRV determined in distilled water (Fig. 1.). Partial dissolution and disintegration was found for each films prepared from both organic and inorganic acid solutions.

(a)



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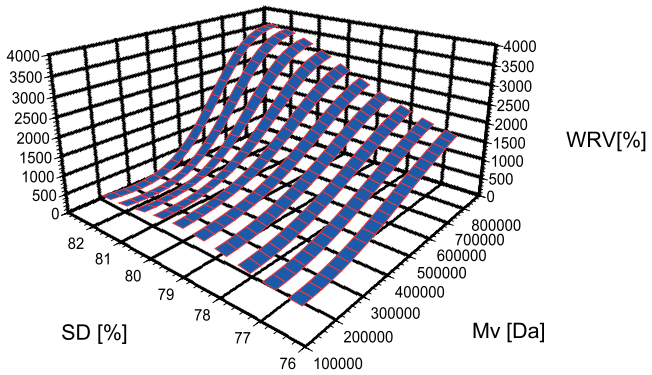
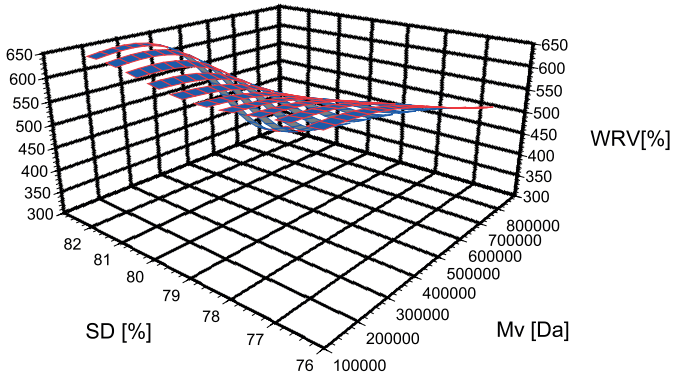


Figure 1. The effect of DD and $\overline{M}_v$ on swelling properties of chitosan salts films in water:
(a) – made of 0.8% (v/v) solution of acetic acid;
(b) – made of 0.8% (v/v) solution of lactic acid;

(a)



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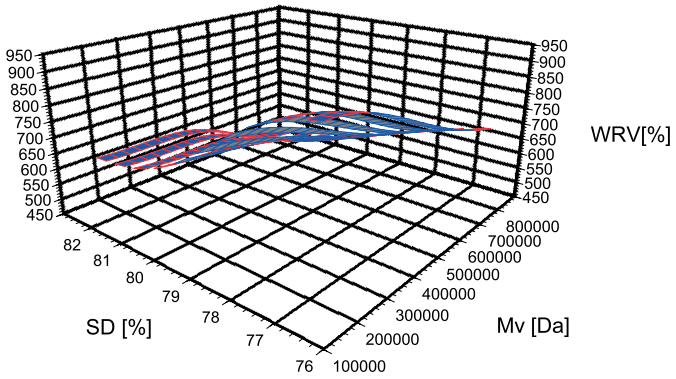

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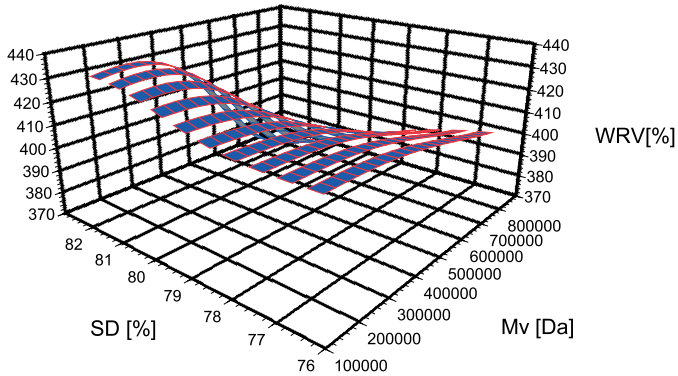


Figure 2. The effect of DD and $\overline{M}_V$ on swelling properties of chitosan salt films in artificial urine:
(a) – made of 0.8% (v/v) solution of acetic acid;
(b) – made of 0.8% (v/v) solution of lactic acid;
(c) – made of 0.2% (v/v) solution of hydrochloric acid

The increase in DD and the decrease in $\overline{M}_V$ caused the improvement in WRV of chitosan salts films after immersing in artificial urine except samples of films made of chitosan lactate. In above-mentioned case the decrease in DD and $\overline{M}_V$ yielded in an increase in WRV of formed films.

Figure 3. shows the effect of distilled water or test medium (artificial urine or artificial blood) composed according to DIN 53923 Standard on swelling of the films formed of chitosan salts.

The increase in the swelling of chitosan salts (chitosan acetate or chitosan lactate) films has found if distilled water as tested solution was used. The chitosan hydrochloride films show full dissolubility in distilled water during the test. Application of artificial urine caused the swelling of tested samples by ca. 1350% (chitosan acetate), whereas the use of artificial blood performed the decrease in WRV of all tested samples to max. 340%.

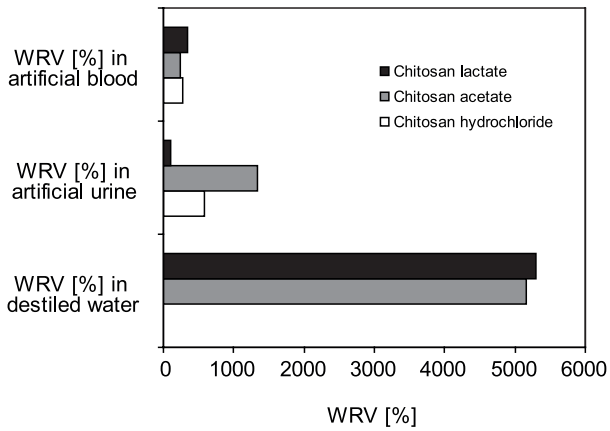


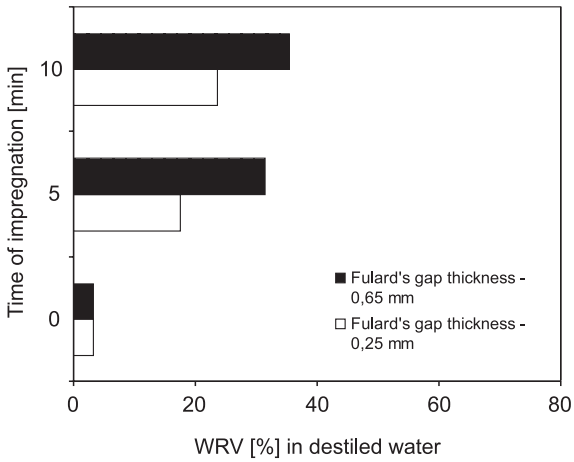
Figure 3. Effect of type of tested fluids on the swelling of chitosan salts films

Effect of modification by chitosan lactate on properties of non-wovens

Effect of impregnation in chitosan lactate on swelling of PP non-wovens in distilled water or artificial urine

Figure 4. shows the effect of impregnation time in chitosan lactate on the swelling of modified polypropylene non-wovens differ in surface weight in distilled water.

(a)



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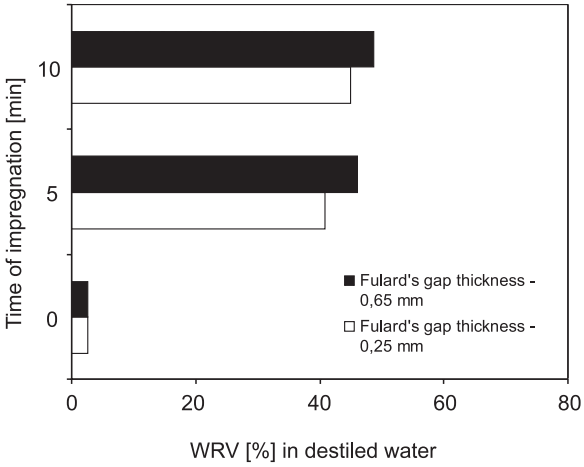
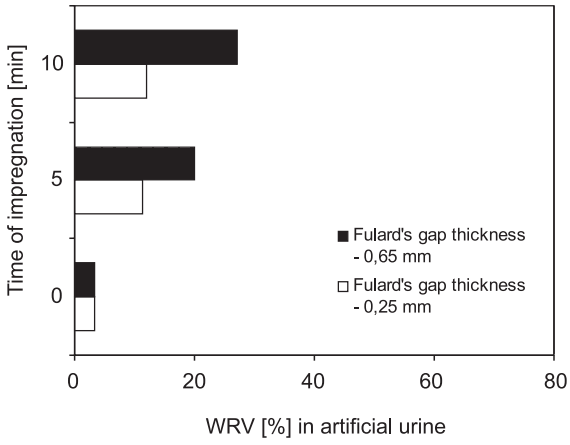


Figure 4. Swelling in distilled water of polypropylene non-woven (PP non-woven) modified by chitosan lactate:
(a) surface weight of initial oriented PP fiber pulp – 25 g/m^2;
(b) surface weight of initial oriented PP fiber pulp – 50 g/m^2;

The prolongation of impregnation time resulted in increase in swelling of modified non-wovens in distilled water by 30–50% as compared with non-impregnated non-wovens. Increase in surface weight of PP-non-wovens caused the increase in their WRV.

Application as a test medium – artificial urine – yielded in decrease in the swelling of chitosan lactate PP non-wovens by ca. 50% (Figure 5.).

(a)



(b)

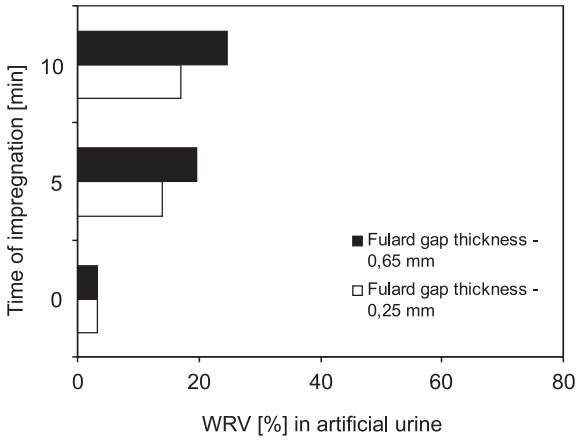


Figure 5. Swelling in artificial urine of polypropylene non-woven modified by chitosan lactate:
(a) surface weight of initial oriented PP fiber pulp – 25 g/m^2;
(b) surface weight of initial oriented PP fiber pulp – 50 g/m^2;

Mechanical properties of modified non-wovens

Figure 6. shows the comparison of mechanical properties of VS or PP non-wovens modified by chitosan lactate

Increase in surface weight of modified non-wovens caused the increase in breaking strength, both PP or VS non-wovens. PP non-woven modified by chitosan lactate shows ca. 3.5 times lower breaking strength as VS non-woven impregnated in the same conditions. Moreover, modified PP non-woven indicated ca. 5-times elongation at break as compared with modified VS non-wovens. The Young modulus of modified viscose non-wovens shows the much higher value as obtained for PP non-wovens prepared in similar conditions.

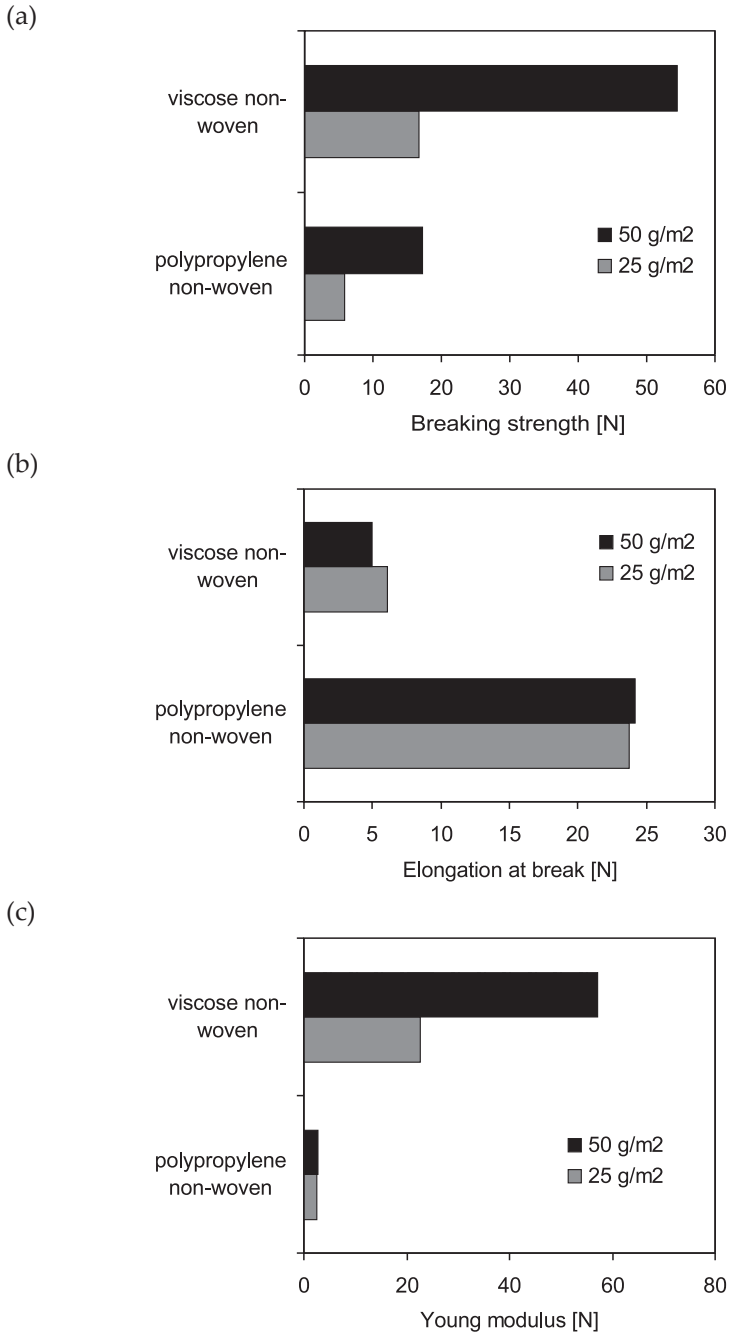


Figure 6. Mechanical properties of PP or VS non-woven modified by chitosan lactate: (a) – breaking strength; (b) – elongation at break; (c) – Young modulus;

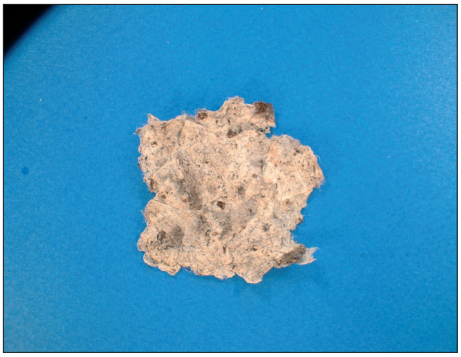
Biodegradability of modified by chitosan lactate non-wovens (viscose or polypropylene)

The biodegradability of modified non-wovens (viscose or polypropylene) by chitosan lactate was carried out by standard soil test.

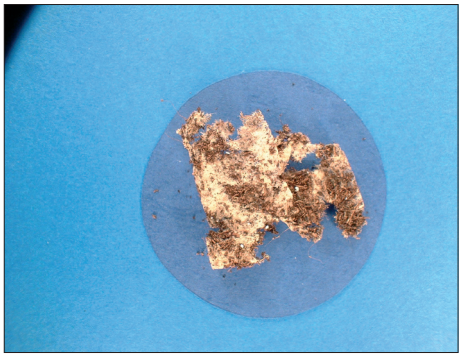
The pictures shown below present the form of initial and form of VS non-woven after 1, 2 or 3 months of soil test.

Soil test performed for the VS non-woven modified by chitosan lactate (surface weight of 50 g/m^2)

Initial VS non-woven modified by chitosan lactate



VS non-woven after 1 month of soil test



Rest of VS non-woven after 2 month of soil test

Rest of VS non-woven after 3 month of soil test

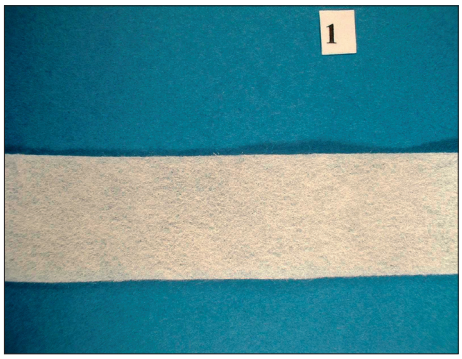
The biodegradability of VS non-woven modified by chitosan lactate during soil test indicated the full disintegration of non-woven structure after 1 month and before 2 months of study. The prorogated test shows only disintegrated particles of viscose fibres after 3 months of study.

The pictures shown below present the form of initial and form of PP non-woven after 1, 2 or 3 months of soil test.

One month test shows the disintegration of non-woven structure and the presence of microorganisms colonization of the PP fibres. Prolongated test performed the further disintegration of modified PP non-wovens with visual increase in colonization of microorganisms.

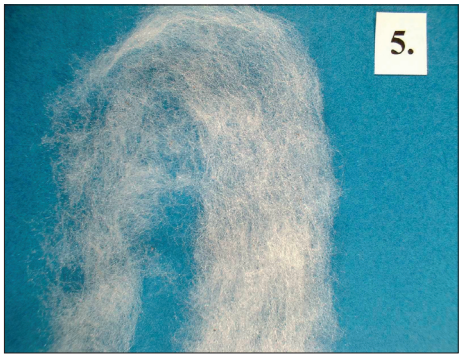
The biodegradation in the presence of microorganisms characteristic for soil results in more dynamic disintegration and degradation of VS non-wovens modified by chitosan lactate.

Soil test performed for the PP non-woven modified by chitosan lactate (surface weight of 50 g/m^2)



Initial PP non-woven modified by chitosan lactate

PP non-woven after 1 month of soil test



Rest of PP non-woven after 2 month of soil test

Rest of PP non-woven after 3 month of soil test

CONCLUSIONS

The presented study being the base for the application of novel, modified non-wovens for disposable products shows below conclusions:

- optimal useable properties of chitosan films were obtained for films made of chitosan lactate;
- increase in mechanical strength of modified non-woven was found if the viscose origin is applied;
- viscose non-wovens modified by chitosan lactate showed better affinity to biodegradation as compared with polypropylene non-woven. Moreover, the microorganisms colonization of modified polypropylene non-woven after 1st month of soil test was noted.

Chitosan salts modified non-wovens seem to be optimal source for the application in sanitary disposable products due to possibility of non-wovens properties control by various forms of chitosan and improvement in biodegradability.

ACKNOWLEDGMENTS

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APPLICATION OF CHITOSAN IN TEXTILE AND PAPER INDUSTRY

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Abstract: Heppe GmbH is a producer of chitosan in a big variety. The company explored in co-operation with institutes and business partners the application of chitosan in the textile industry. The R&D department focused their activities on two aspects. First the combination of chitosan and silver to achieve bacteristatic properties and to bind odour and second: the application as sizing agent in the production process. In these projects different varieties of chitosan and derivatives were investigated. There are two applications: short period usage or permanently binding on the fibre. The tests in weaving mills in Germany showed positive effects for the production process besides the advantages of economical and ecological datas, for instance: minimisation of sizing agent consumption, uniform size pick-up on warps, higher stability, less dust in the weaving mill, reduction of thread breaks by 50%, increased dye affinity, less pollution in waste water and bacteriostatic properties. Another sphere of activity in Heppe GmbH is the paper industry. Together with our partners we investigated special chitosan qualities to improve the properties of paper. For instance chitosan can increase the wet strength, air permeability, colour absorption and print quality.

Key words: textile industry, paper industry, chitosan

INTRODUCTION

Chitosan is usable in many industrial fields. For an industrial application of chitosan it is very important to have defined chitosan in stable qualities and specifications. It is well known that chitosan has many properties to improve the quality of products in many fields, for example in agriculture, cosmetic, textile or paper industry. For the use in textile and paper industry chitosan is distinguished by its bacteriostatic and coating effects. Heppe GmbH work together with many industrial partners to establish chitosan in industrial production processes. One of the biggest problems for introducing this product is the necessary adaption of existing equipment and recipes.

RESULTS AND DISCUSSION

Textile Industry

Chitosan application in the textile industry has a wide range. Chitosan can be used in sizing or coating or respectively to produce chitosan fibers. The waste water treatment in textile industry is also an application field for chitosan. The present work represents our results in sizing and coating tests.

Sizing: Two institutes for textile research, one company, which produces chemicals for the textile industry and two weaving mills were involved in this textile project. Sizing is normally a temporary process before weaving to smooth the fibres. After weaving the textiles are desized and chemicals like PVA, acrylates, etc. are concentrated in the waste water. This means high costs for the companies to treat their water.

With the use of chitosan it is not necessary to remove the size totally. The special properties of chitosan can be used in the finish product (i.e.bacteriostatic effects, improving the colour absorption and others). Every application field requires a special chitosan to achieve the optimal properties for its use. Because of this it is necessary for certain cases to combine different chitosans to receive best sizing properties and for example bacteriostatic effects.

The use of chitosan opposed a lot of advantages but also some problems.

1. Chitosan is a very good sizing agent and it is usable without any additives but it is to expensive and the viscosity is to high. Combinations with starch is a practicle way for the application of chitosan and if necessary the adding of wax to decrease the costs and to simplify the applying process. The recipes must be individual adjusted to the yarn and the weaving mill.
2. We observed some differences in the effects of chitosan between natural (cotton) and synthetic fibres (PES). The cotton fibre showed an improvement of 4% of the weaving efficiency. The synthetic ones had no improvement. Both applications showed no negative influences on the following processes like washing, bleaching and dying. So the chitosan have to be tailormade for different yarns.

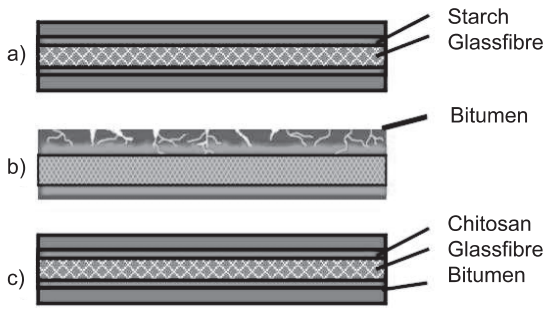

Figure 1. Glassfibre for roofing felt

Economical effects: For the synthetic yarn costs can be saved by the reduced costs of the sizing agent (up to 25%, approx 500–700.000 €/year) and the positive effect of the biodegredability of the size. For the cotton fibre the user has has to

pay more for the size (9%) but saves on an additional yield of the weaving efficiency of 4%. The company can save 250.000 €/year.

In following processes were positive effects observed. For instance the colour absorption has improved and the intensity and colourfastness was higher because of the use of chitosan.

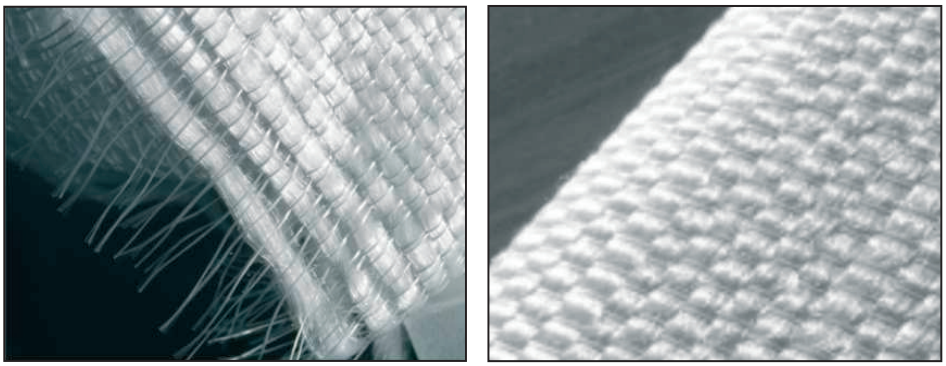

Figure 2. Glassfibre coated with chitosan

Figure 3. Silvercoated socks inside

Coating: Another application of chitosan is the coating of glassfibrefleece for roofing felt. Normally starch and some additives are used as coating agents. The glassfibre is coated with starch and covered with bitumen (see Figure 1a). By the years the bitumen will be brittle. The water can penetrate into the bitumen and the starch is swelling, so the roof has to be repaired (see Figure 1b). Chitosan can inhibit the swelling and therefore the durability of the roof is longer (see Figure 1c).

Chitosan is also useable in the production of silver coated yarn, what is produced by a galvanic process. The problem is the roughness of the yarn after treatment. In the weaving process arise a high number of thread breakage and this reduces the efficiency. By coating the fibre with chitosan after the galvanic process the smoothness was improved and we observed no negative influences on the finished product. The quantity of the chitosan is only 1 kg for 1000 kg of yarn.

Paper Industry

Chitosan has its application in the pulp, for coating, in the finishing, as biodegradable glue and in the waste water treatment. We have done some experiments in the pulp. It is possible to improve the colour intensity. Only 0,4 up to 1,5 kg Chitosan per one ton of paper were needed.

The advantages are:

- reduce the porosity, this is adjsutable by the amount and the molecular weight of chitosan
- reduce of the basic weight
- better print quality and more intensive colours
- the surface is more smooth

By coating finished paper with chitosan the surface quality will be improved. That means the smoothness and the ink absorbancy will be better.

CONCLUSIONS

Both industries are automated and works with high speed. For example a paper machine has a speed up to 1500 m/min. Small changes in the process can have big consequences. One day on a paper machine can cost up to 250.000 €. The influence on production cycles are actual after 1 week, because the material cycles have big amounts. To introduce the chitosan successfully to the industry it is necessary to have stable defined qualities.

Another problem: It is an additional substance and it needs a dosage system in the process. This means additional fees for the user and changes in the production equipment.

Possible solution: Both industries use starch. The focus of our company is the production of chitosan-starch combination products. The advantages for the customer are:

- no additional product, the user works with normal procedures
- the products have the same easy handling like normal starch, but the properties are improved. The customer doesnt need any additional equipment.

ACKNOWLEDGMENTS

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NEW METHOD OF CHITOSAN SPINNING: APPLICATION TO REINFORCING PLATE

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INTRODUCTION

Visceral surgery, especially hernia reduction, often requires the use of fiber-based reinforcing plates (herniaplasty). Nowadays, surgeons insert polyester or polypropylene fiber reinforced materials [1]. However, the implantation of this kind of synthetic polymer woven or non-woven fabrics neither leads to a well-ordered wound-healing, nor to a regenerated tissue close to the native state. Moreover a permanent inflammatory process is observed.

Our work consists in developing a new concept of reinforcing plates [2] principally made from chitosan fibers preventing from post-operative adhesion and promoting the tissue regeneration, in addition to their stiffening function. Indeed, chitosan is not only known to be bioabsorbable [3], biocompatible and bioactive, but also as anti-adhesive and tissue regenerative promoter. The aim of our study was to optimize the conditions and the parameters of each step (solubilization/degassing, coagulation, stretching and drying) of a new method of spinning in order to obtain chitosan fibers showing well adapted mechanical features and physical, physicochemical, structural and biological properties for the considered medical application. In this paper, we will first detail step by step the innovative spinning process of chitosan fibers: from the laboratory prototype to the industrial pilot. We will particularly study the influence of the elimination of the formed salts and the stretching on the macro- and micro-morphology of the fibers. Finally, first *in-vivo* experiments will be shown.

MATERIALS AND METHODS

Studied chitosan

A weakly acetylated chitosan was used. It was produced from endoskeletons of calmars: purchased from France Chitine (MAHTANI CHITOSAN, chitosan

114. batch n° 15/11/02).

Table 1. Characteristics of the chitosan used in this work: degree of acetylation (DA), water content, weight-average molecular weight MW, weight-average degree of polymerization DP_w and polydispersity index IP

DA (%)	Water content (%)	MW (g/mol)	DPw	IP	[η] (mL/g)
2.7 (± 0.5)	7 (± 2)	540 000 (± 20 000)	3 330 (± 30)	1.5 (± 0.2)	1 657 (± 35)

Experimental spinning machine : laboratory prototypes 1 and 2

Laboratory prototype 1: chitosan was dissolved in an acetic acid aqueous solution in order to obtain a polymer concentration of 2% w/w. The chitosan dope was degassed several hours at ambient air. A laboratory scale extrusion was used, constituted of a metering pump (from 0.7 to 35 mL/h), a syringe (20 mL) and a needle (22 mm length, 0.8 mm internal diameter) as a spinneret. The chitosan dope was extruded through the spinneret so that the monofilament falled straight down. The linear extrusion rate was maintained at 0.6 $m.min^{-1}$ for most experimental runs. The coagulation method was an innovative concept. It consisted in coagulating the chitosan monofilament by contact with gaseous ammonia [4]. The alkaline coagulation bath classically used in a wet-spinning process of chitosan fibers was therefore un-useful [5]. The extrusion spinneret was directly put down in the coagulation chamber. At the laboratory scale, the filament was stretched by hand at the end of the coagulation chamber. Then, the fiber was immersed in two washing baths (1L) containing demineralised distilled water. Finally, the hanging chitosan fiber was dried at ambient air under stretching.

Laboratory prototype 2: A second innovation dealt with the absence of any aqueous bath after the spinneret. The gaseous coagulation step was directly followed by the drying under hot air (~90°C). Thus, we could consider a "pseudo-dry-spinning" process of chitosan fibres.

Industrial spinning pilot

From the laboratory prototype 2, we processed an industrial pilot where all parameters were precisely controlled. When the chitosan yarn coagulated in the ammonia gas, the ammonium acetate produced with the fiber could be easily hydrolysed into gaseous acetic acid and ammonia and then eliminated. For that, we used an oven where wet and hot air was circulating around the fiber.

Chitosan fibers analysis

The ammonium acetate content in the fibers was evaluated from ^{1}H-NMR (Brucker, 250 MHz), the acetate methyl protons of the salt was located at 2 ppm. Chitosan fibers were observed using a transmission optical microscope and a Scanning Electron Microscope (SEM). Small Angle X-ray Scattering (SAXS) and Wide Angle X-ray Scattering (WAXS) experiments were performed at the European Synchrotron Radiation Facility (ESRF) in Grenoble on the BM2/D2AM beam-line to study the influence of the stretching on the macro- and micro-morphologies of the produced chitosan fibers.

In vivo experiments

To check the harmlessness of chitosan fibers, *in vivo* experiments were carried out. The first experiments on New Zealander rabbits consisted in putting staples of five fibers in different locations for one, four and twelve weeks.

RESULTS AND DISCUSSION

Influence of washing

In a first step, we studied the washing time necessary to eliminate the major part of the salts formed with chitosan fibers during the coagulation with ammonia gas. From the spinning process of the laboratory prototype 1, we produced several runs of fibers with different washing times. We concluded that it was necessary to wash them for at least two minutes in repeatedly renewed distilled water. These results were compared with those of a sample of fibers made from the laboratory prototype 2. The fibers were dried under hot air and let at ambient air. Thanks to ^{1}H NMR spectra analysis, we noticed that the unwashed fibers, after one month at ambient air, showed a proportion of ammonium acetate of about 5% close to that of the washed sample (about 2.5%) and the same content after three months. Then, our objective was to improve and accelerate the hydrolysis of the salts without any washing of the fibers.

In a second step, a kinetics study was processed with different fiber samples produced with the pilot. The objective of these experiments was to evaluate the efficiency of the hydrolysis by wet air. Every ten to fifteen hours, the determination of the content of ammonium acetate (from ^{1}H NMR) was made in parallel with the evaluation of the weight-average molecular weight (MW) of chitosan (measured by gel permeation chromatography).

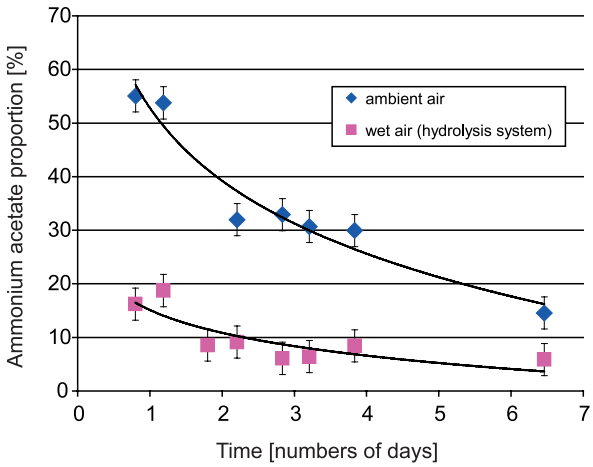

Figure 1. Influence of wet air or ambient air with time on ammonium acetate content (% in mol./mol.) in the fibers produced with the pilot

It was clear that a wet air was necessary to eliminate ammonium acetate after hydrolysis. The proportion of salt reached the expected value (<8%) after only

two days under wet air, whereas the proportion of salt in the fibers at ambient air was still over 30%. Moreover, it seemed important, for an industrial application, to reduce the time of the salt removal to ensure that the weight average-molecular weight of chitosan was not lowered. After one week, the difference in MW between fibers dried in ambient air and wet air was not significative (below 5%) and the MW of the fibers dried in wet air was about 500 000 g/mol.

Influence of the stretching

Morphology of chitosan fibers

The first morphological characterizations were performed by transmission optical and scanning electron microscopies to measure the diameter and observe the surface state of the chitosan fibers.

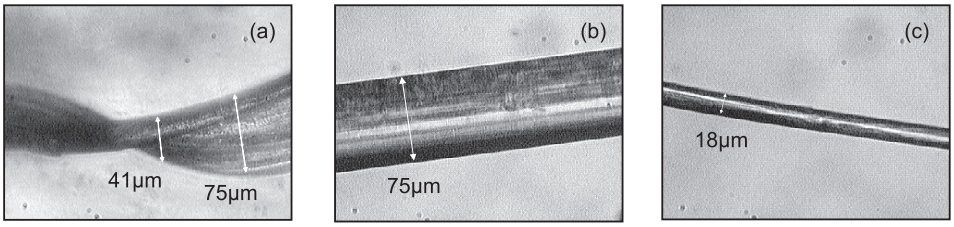

Figure 2. Transmission optical microscopy photographs of (a) un-stretched fiber when drying; (b) stretched fiber during drying; (c) stretched fibers when coagulating and drying (produced from the laboratory prototype 1)

We notice that it was very important to stretch the fibers during drying. Indeed un-stretched chitosan fibers were irregular and distorted owing the shrinking due to the elimination of water and formation of hydrogen bonding between the macromolecular chains during drying. This behavior was confirmed by scanning electron microscopy photographs showing the smooth surfaces of the chitosan fibers produced with the laboratory prototype 2 (Fig. 3).

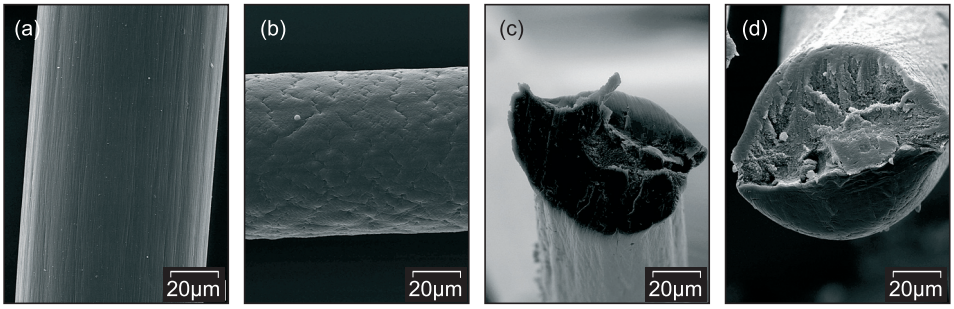

Figure 3. SEM photographs of the surface and the cut of the stretched [(a) (c)] and un-stretched [(b) (d)] chitosan fibers when drying

Moreover, we also noticed an increase of the mechanical properties of the fibers with stretching. Indeed, the stretched fibers were less brittle in agreement with our objective to improve the stretching step so as to achieve a maximum orientation of the macromecular chains.

WAXS patterns

Figure 4. WAXS patterns of un-stretched (a) and stretched (b) chitosan fibers when drying, containing SAXS information at the centre of the patterns. The chitosan yarns were put vertically and perpendicular to the incident beam

In order to quantify the orientation of the chains in the chitosan fibers, we studied the fiber diffraction patterns of stretched and unstretched fibers. The anisotropy was increasing with stretching. We could calculate the Hermans function f [6] of the equatorial intensity of the diffraction spots, which specifically corresponds to the orientation of the (020) reticular planes (the c axis being the chain axis). For pattern (a), f_{020} is equal to -0.09, as a result of a nearly random orientation of the (020) planes of the un-stretched fibers. For pattern (b), f_{020} is equal to –0.4, showing that the 020 planes of the stretched fibers are almost oriented in zone with the spinning axis.

In vivo experiments

Whatever the localisation, no inflammatory response due to the implantation of chitosan yarns was observed after rabbits euthanasia, even after twelve weeks. Moreover a neo-tissue encapsulating the implant was still observed. This was particularly encouraging for biological applications of these fibers.

CONCLUSIONS

The study of the processing parameters for chitosan fiber ingineering, like washing and stretching, shows the interest of our innovative "pseudo-dry spinning" process. The washing step is not necessary to eliminate ammonium acetate salt. Moreover, the stretching step plays an important role on the surface state, the diameter and the orientation of the macromolecular chains. A detailed analysis of the mechanical properties of the chitosan fiber in various stretching conditions is on going. We will also continue to observe the crystalline and microscopic morphology of the chitosan fibers. Finally, in vivo experiments contribute to comfort the use of this biomaterial for herniaplasty.

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APPLICATION OF CHITOSAN AND BIOREMEDIATION ON SHRIMP-POND EFFLUENT TREATMENT

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Abstract: One of the most serious problems which came along with Thailand fast growing shrimp industry is the deteriorating environments. Increasing use of high protein content feed dramatically decreases the quality of the water in the shrimp pond. Farmers commonly solves this problem by their most convenience method but most seriously environments damaged way, exchanging water with the natural water body. However, a more environment-friendly alternative is to apply bioremediation system for the effluent treatment. This efficient alternative does not only reduce the environmental impact but can also produce profitable by-products. Chitosan, a biopolymer founded in shrimp crust, can be added back into the shrimp-pond effluents, to enhance the treatment efficiency of the treatment. Since this biopolymer is shrimp-originated, its addition will create no harm to the eco-system in the pond and is also safe for human. Chitosan was applied as coagulant into effluents from shrimp (*Peneaus monodon* and *Macrobrachium rosenbergii)* pond and shrimp bioremediation pond. The bioremediation system consisted of red tilapia (*Oreochromis* sp.), seaweed (*Entermorpha* sp.), and sedimentation pond. The results showed that the treatment system could efficiently remove nitrogeneous source (ammonia, nitrite, and nitrate). The accumulation rate of ammonia, nitrite, and nitrate were only 0.02, 0.05 and 0.21 [mg-N/l/d], respectively. These values were 2–3 times lower than those in the control set. Moreover, the shrimp growth rate of chitosan applied set was higher than those in control set up to 1.2 times (0.17 and 0.21 g/shrimp/day, respectively).

Key words: bioremediation, chitosan, coagulant, nitrogeneous source

INTRODUCTION

Thailand has become the leading country in marine shrimp production from farming since 1991 [1]. The intensive marine shrimp farming technology has been developed for 10 years. During this period, shrimp-farming area has been expanded very rapidly in almost every coastal province of the country. The pollution loading and toxic materials deteriorate the coastal natural resources and environments. The environmental impacts from untreated effluent have raised concerns about the sustainability of shrimp farming. This has prompted the search for cost-effective methods of improving effluent water quality prior to dis-

charge into receiving waters. A potentially alternative is biological treatment to remove suspended particulate and nutrients. The organic and inorganic matter in the effluent would fertilize many aquatic organisms, such phyto-plankton and zooplankton, which could serve as food to shellfish (molluscs, shrimps, etc.) and many other species of fish [2, 3]. With this approach, the farmers can earn more income from these cultured species as byproducts while reducing the impacts on the coastal environment caused by the intensive marine shrimp farms.

Chitosan, a bio-polymer obtained from chitin, had grown attraction as an alternative synthetic flocculants. The protonization of amino groups in solution makes chitosan positively charged and since most natural colloidal particles, including bacteria and macromolecules, are negatively charged, attractive electrostatic interactions may lead to flocculation. Chitosan is a biodegradable and non-toxic so it has been used in various areas of water treatment, such as removal of organic substances from drinking water, treatment of wastewater from distillers, removal of oil from wastewater, treatment of food processing wastes, as well as other applications [4].

This study was conducted to study the efficiency of integrated aquaculture system combined with application of chitosan on water quality change and aquacultue species growth.

MATERIALS AND METHODS

Shrimp Pond

The two commercially valuable aquaculture species in Thailand, i.e. pacific white shrimp (*Penaeus monodon*) and giant freshwater prawn (*Macrobrachium rosenbergii*), were grown in the two same dimension earthen-ponds, with 1,600 m^2 surface area and 1.2 m depth. Aeration was supplied via 1 HP electric paddle wheels aerator. The salinity at starting period was set at 10 ppt in both ponds. *P. monodon* were stocked at the rate of 80,000 postlarva/pond (50 postlarva/m^2). A commercial diet was fed at the rate of 3% of shrimp body-weight per day. On the other hand, *M. rosenbergii* were stocked at the rate of 30,000 heads/pond (18.75 heads/m^2) and feeding rate was set at 2% on body-weight per day.

Fish Pond

Red tilapia (*Oreochromis sp*.) is one of the most popular cultured species in many tropical countries because of its resistance to poor water quality and diseases, tolerance to a wide range of environmental conditions, and good growth rates. Algae and detrital matter in the extensive aquaculture are already sufficient nutrient sources for red tilapia. In this study, red tilapias, with 90 g in average weight were stocked in the 400 m^2 earthen-pond, at the rate of 5 fishes/m^2. Aeration was supplied via a 0.5 HP electric paddle wheels aerator. No artificial feed was added.

Sedimentation Pond

The suspended solid was reported to cause negative effects on photosynthesis of aquatic plants by attach on its leaves [5]. Hence, the 400 m^2 sedimentation pond

was applied to reduce suspended solid in fishpond-effluents prior discharge to seaweed-pond.

Seaweed Pond

In this study, the green algae genus *Enteromorpha* was chosen to be an aquatic plant in the treatment system. This seaweed had potential for commercial exploitation because of its abundant and varied chemical composition, and quality and concentration of basic nutriments for other living organisms [6]. Seaweed was stocked at the rate of 2.5 kg/m^3 in the 400 m^2 earthen-pond.

Recirculating System

Water exchange rate was set at 10% of shrimp-pond volume per day. Chitosan was added into *M. rosenbergii* pond at the rate of 10 mg/L every 10 days. Water samples were collected for analysis every 4 days. A schematic diagram of this study was shown in Fig. 1.

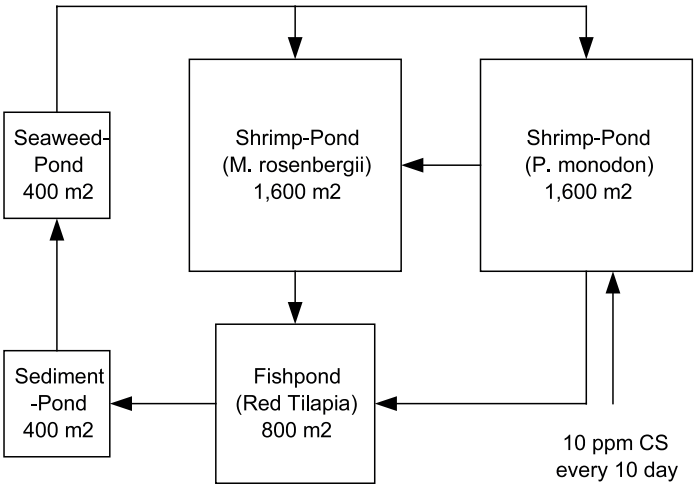


Figure 1. A schematic diagram of integrated system based on shrimp-fish-seaweed with an application of chitosan

Analytical procedures

The filtrate of water sample were collected and the concentration of NH_3, NO_2^-, NO_3^- and PO_4^{3-} were determined by the USA standard methods [7].

RESULTS AND DISCUSSION

Water quality profiles in shrimp-ponds in bioremediation system were compared between the ponds with and without chitosan and control set as shown in Fig.2. No significant differences were found between the water quality in shrimp-pond with and without application of chitosan for nitrogenous waste. The integrated system could efficiently control NH_3-N levels in both shrimp-ponds and treatment ponds. The NH_3-N levels in treatment ponds are always maintained

at low level (below 1.0 mg/L). However, NO_2-N levels are high in both shrimp-ponds and treatment ponds, i.e. the range of 0.5–4.0 mg/L variation. The reduction of NO_2-N, in shrimp-ponds at the end of experiment, might come from growth of available phyto-plankton. To control nitrite at low level may require better fine-tuning of the operating condition of the treatment system, such as recirculating rate and size of the treatment (fish, algae) ponds.

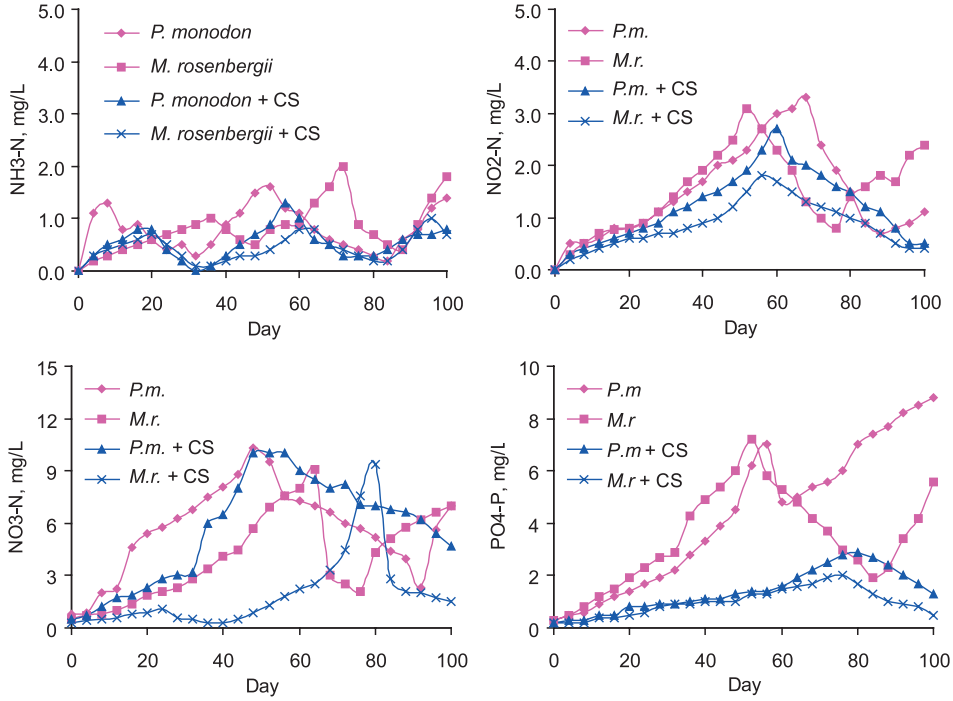
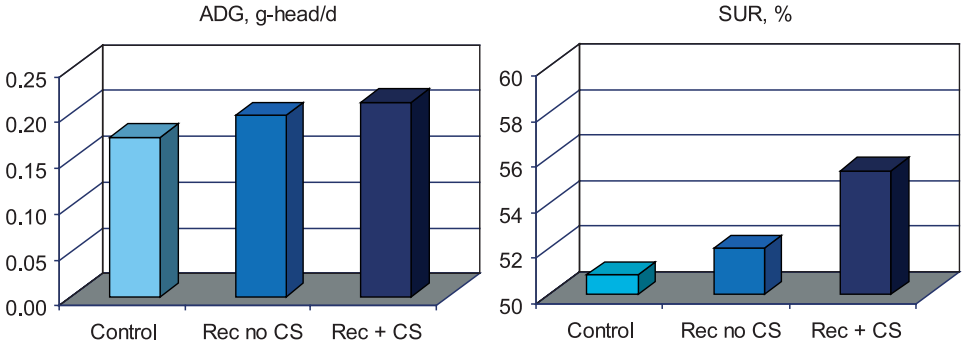


Figure 2. Water quality trends in shrimp-ponds with and without application of chitosan

One of the problems with the biological treatment system is the reduction of the phosphate. Applying chitosan showed good results in reducing PO_4-P levels in the pond, lowering the maximum level of phosphate more than 50% of the pond without chitosan. Furthermore, chitosan also improved growth, survival



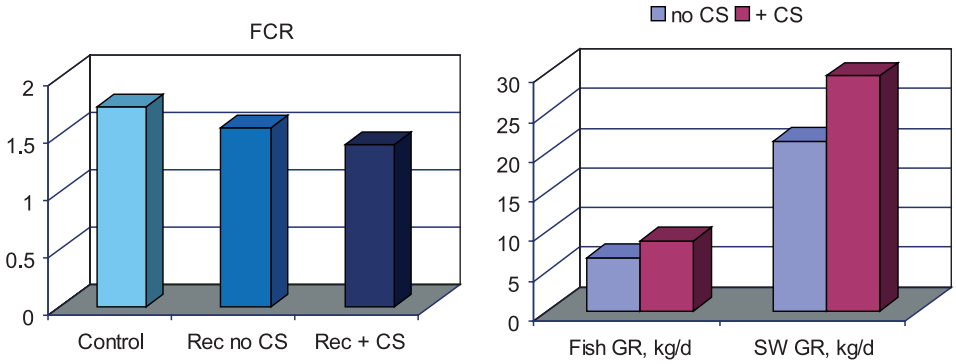


Figure 3. Average daily growth (ADG), survival rate (SUR), feed conversion ratio (FCR) and growth rate of fish and seaweed in the integrated system with and without an application of chitosan

and food conversion rate of the shrimp in the system (Fig. 3). The production of fish and seaweed in the integrated aquaculture system were also increased by chitosan as well.

CONCLUSIONS

Bioremediation by using aquatic animals and plants was tested and proved to be efficient for treatment of shrimp-pond effluents, especially the nitrogen waste. Adding chitosan into the system enhanced the treatment of phosphate which is normally difficult to remove by biological means. Besides higher growth and survival rate of the shrimp, chitosan also increased the production rate of the fish and seaweed.

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DIBUTYRYLCHITIN, THE MANUFACTURING OF MICRO- AND NANO-STRUCTURES

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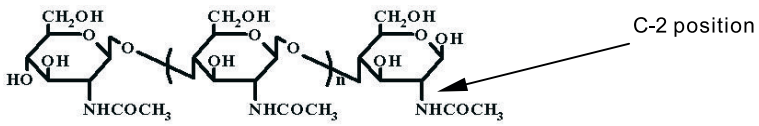
Key words: dibutyrylchitin, microspheres, porous fibres, electrospinning

INTRODUCTION

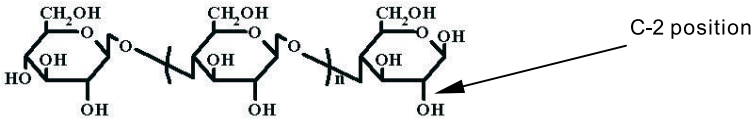
Natural polysaccharides are now extensively used for the development of wound dressing materials, solid dosage forms for delivery of the drug and scaffolds for tissue engineering. Some polysaccharides which are degraded by human colonic microflora have been studied as colon-specific drug carrier systems. Polysaccharides like chitin are found to induce granulation tissue with angiogenesis and less scar formation. It is closely connected with the primary objective in wound care, which is the promotion of rapid wound healing with the best functional and cosmetic results. Recently, chitin and chitin derivatives attracted attention due to their unusual biological properties, interesting from the point of view of medical applications. Dibutyrylchitin, abbreviated DBC, is a valuable ester derivative of chitin. The method of synthesis of dibutyrylchitin with degree of substitution about 2 was created by L. Szosland from Technical University of Lodz [1, 2]

Chemical structure of dibutyrylchitin

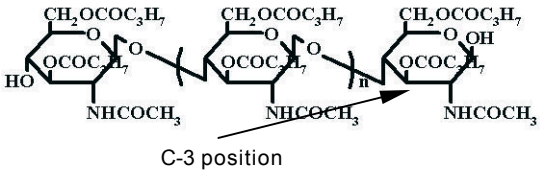
Polymers sometimes show very slight difference in chemical structure eg. chitin -A), may be regarded as cellulose -B) with hydroxyl at position C-2 replaced by an acetamido group. Chitin resembles cellulose in its low solubility and low chemical reactivity. These properties restrict any practical applications.
A) Chitin



B) Cellulose



C) Dibutyrylchitin (DBC)



An introduction of bulky butyric groups into chitin chain at position C-3 and C-5 was found a very successful method of improving chitin solubility [1,2]. New ester derivative of chitin, known as dibutyrylchitin -C), was obtained.

Some properties of dibutyrylchitin:
- easy solubility in popular organic solvents,
- fibres, strong transparent films and microspheres formation capabilities
- possibility to restore to chitin product without destroying of macrostructure of dibutyrylchitin products,
- biocompatibility, biodegradability, bioresorbability,
- exhibit no irritation reaction,
- lack of cytotoxity, inflammatory and immunomodulating effects,
- ability to accelerate wound healing [3–6]

EXPERIMENTAL AND RESULTS

Synthesis of dibutyrylchitin as a raw material for the manufacturing of micro- and nano-structures

Dibutyrylchitin was prepared from krill chitin, complying with requirements worked out by L. Szosland [1, 2] and described in Polish Patent [2]. Krill chitin, with viscosity average molecular weight value $\overline{M}_v$=286,7x10^3 daltons, was purchased from Sea Fisheries Institute, Gdynia, Poland. Before the processing chitin was purified from proteins and calcium carbonate. An esterification process of chitin undergone in heterogeneous conditions using perchloric acid and butyric anhydride. Procedure and conditions are in accordance with Polish Patent. DBC as the final product of esterification reaction, was characterized with intrinsic viscosity value determined for the polymer solutions in *N*,*N*-dimethylacetamide (DMAc) at the temperature equal to 25°C. Three lots of dibutyrylchitin polymer were synthesized. The intrinsic values of viscosity determined for their solutions in DMAc were as follows:

[η]=1,5 dL/g for DBC-1, [η]= 2,05 dL/g for DBC-2 and [η] =2,75 dL/g for DBC-3

Manufacturing of different forms of dibutyrylchitin products

Dibutyrylchitin films containing metronidazole

Metronidazole (MTZ) was chosen because it is one of the best drugs protecting patients from anaerobically infected burned wounds. Transdermal use of MTZ might diminish the hard side effects, caused by metronidazole during it's oral administration.

Samples with diameter size of ca 45 mm and an average thickness of 40 μm. were cast from 5% (w/v) methanolic solution of DBC-1, containing 0,2% (w/v) of metronidazole (Fluka Chemie AG). Similar films of DBC-1 without the drug were also cast.

Metronidazole release to water was performed in constant temperature 37°C during sufficient stirring. Concentration of the drug was measured from the absorbance at 319 nm, using a UV/VIS spectrometer. Water solution over the films without drug was used as reference one. Actual amount of MTZ that was released from DBC-1 films was calculated on the basis of the calibration curve.

An experimental curve of the drug release in vitro from DBC-1 thin films loaded with metronidazole during films formulation is described with the best approximation by the following logarithmic equation: $M_t/M_\infty = k \ln(t)+b$, where M_t/M_∞ – fractional release drug amount, t- time release, k and b- constants for measured system. The half-release time was found: $t_{1/2}$=~15min. [7]

Dibutyrylchitin microspheres containing metronidazole

Microspheres were prepared from DBC-1, using a solvent-evaporation method. In agreement with this method solvent was slowly evaporated under reduced pressure from the previously formed emulsion in methanolic solution of DBC-1/silicone oil system. The diameter size range of obtained, air dried microspheres was 10-200 μm.

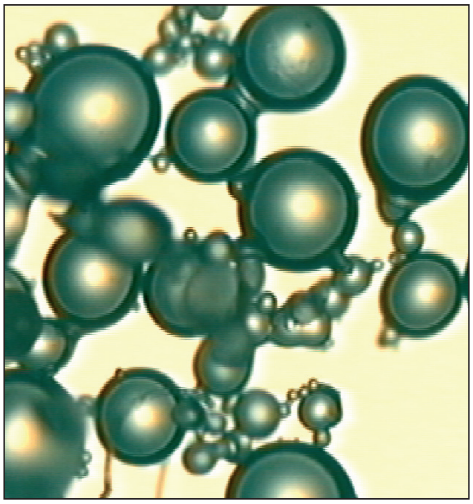


Figure 1. Metronidazole loaded microspheres made from DBC-1 are visible in the right hand side [7]

For metronidazole loading two different methods were applied. Method I – MTZ solution was mixed with DBC-1 solution before microspheres preparation. Metronidazole in the amount of 0,2 g, dissolved in 2 ml of methanol was added to 50 ml of 10% (w/v) DBC methanolic solution. Method II – preformed DBC-1 microspheres (2,0 g) were equilibrated in 10 ml of water solution containing 0,1 g MTZ. After 48h water was subsequently evaporated and dried microspheres with the drug were obtained.

Metronidazole release investigations were carried out in the same way as described earlier for the dibutyrylchitin thin films. It was observed that approximation of the results by loga-

rithmic equation is independent on the form of carrier (microspheres or thin film) or the method of drug loading (either during carrier formulation or to the previously formulated microspheres). The half-release time of the drug quantity ($t_{1/2}$) was determined and equal to ca. 60 min. In every case MTZ was monomoleculary dispersed in the investigated samples.

Dibutyrylchitin porous fibres

DBC-3 were used for fibres manufacturing from solutions in ethanol. Parameters of the spinning process were established on the basis of rheological investigation [8], preliminary tests and also earlier experience concerning the spinning process of porous fibres [9, 10]. Wet spinning of DBC fibres was made at the Technical University of Lodz applying of the laboratory stand.

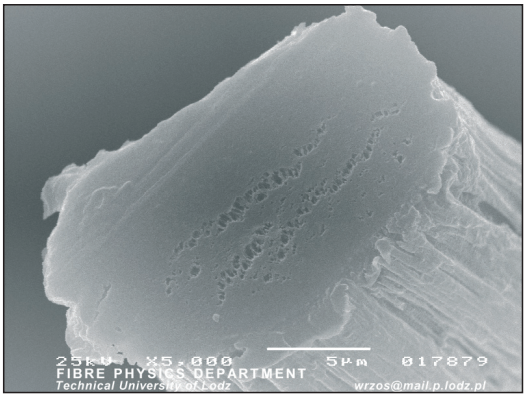


Figure 2. Transverse section of DBC porous fibres, SEM micrograph

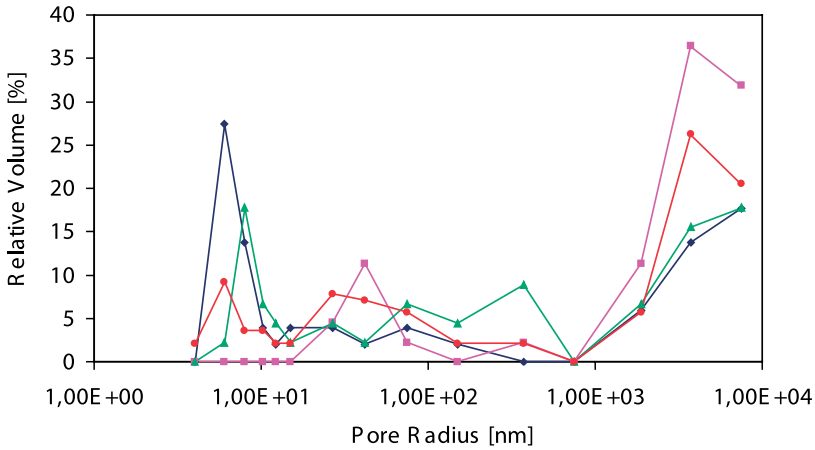


Figure 3. Pore size distribution curves vs. pore radius

Dibutyrylchitin electrospun fibres in the form of flat textile

Possibilities of electrospun nanofibres application in new materials are as follows: wound dressings, drug delivery systems, scaffolds for synthetic skin, artificial organs etc.

Sample of the DBC-1 and DBC-2 was used for fabrication of flat textile materials consisting of random laid nanofibres. Electrospinning was carried out from ethanolic solutions of DBC-1, DBC-2 at tip to collector distance from 10 up to 23 cm under voltage from 6 up to 25 kV.

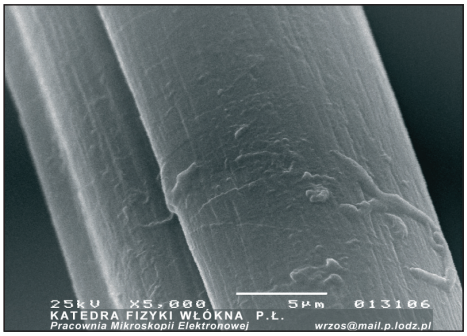


Figure 4. DBC nanofibres in the form DBC classical fibres of flat textile Both in the same scale

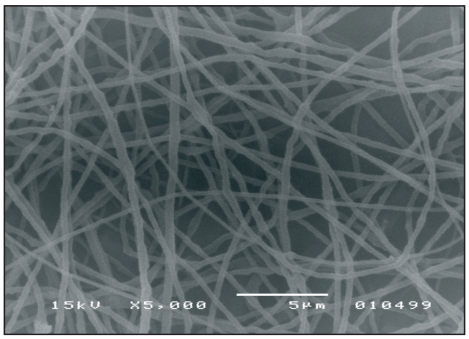


Figure 5. Nanofibres vs. classical fibres, SEM micrographs [11, 12]

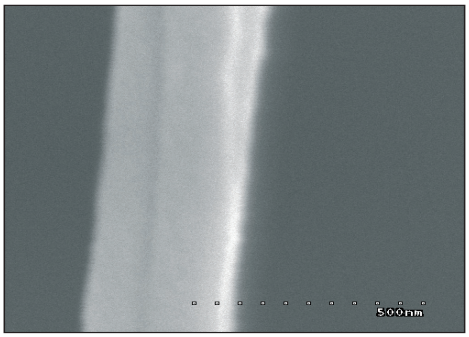


Figure 6. Single DBC nanofibre. The whole scale is 500 nm

CONCLUSIONS

Dibutyrylchitin (DBC) polymer is easily overworked into diversified forms of products. Thanks to its attractive and flexibility properties DBC has a potential to take up deserved position in the medical device industry. It seems to be truth that dibutyrylchitin can be the material of tomorrow.

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MICRONIZATION OF THE CHITIN/CHITOSAN MONOMER DERIVATIVES BY DENSE GAS ANTI-SOLVENT PRECIPITATION TECHNIQUES

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Abstract: There is growing interest of fine particles with a narrow particle size distribution in the pharmaceutical area and in medicinal research in order to obtain high surface area as the particle size is one of the critical factors for the determination of appropriate routes of administration. In this work we have used dense-gas anti-solvent crystallization techniques for the straightforward production of micron-sized powders of chitin/chitosan monomer derivatives D-glucosamine hydrochloride, D-glucosamine sulphate salts, and *N*-acetyl-D-glucosamine. The use of these crystallization methods permit to avoid the problematic communition and homogenization down-stream processes steps required after conventional crystallization methods. Semi-continuous ASES and batch GAS procedures are the two extremes of dense gas anti-solvent precipitation techniques using compressed CO_2, and the results obtained on the crystallization of these monomers are shown herein.

Key words: Compressed fluids, compressed CO_2, crystallization, micro-particles, D-glucosamine sulphate, D-glucosamine hydrochloride, *N*-acetyl-D-glucosamine.

INTRODUCTION

The pharmaceutical and food industry are recently tempted to use chitin and its derivatives due to the optimal biodegradability, biocompatibility and recent FDA approval [1]. Current treatments for allergies typically involve the use of steroids to depress the immune system [2]; however, there are undesirable side effects with steroid therapy [3]. Steroids, as well as synthetic drugs, are expensive to manufacture, involving a complex process which requires complex quality control and GMP (Good Manufacturing Practice) standards to meet requirements of Health and Safety Authorities. In view of these difficulties, it remains a problem in finding effective treatments for allergy. Recently, it has been demonstrated, that intranasal deliver of chitin derivatives, such as chitosan or its monomers, is particularly effective in the treatment of allergies (seasonal, respiratory aller-

gies, allergy to aeroallergens, asthma, food allergies, atypical dermatitis) providing a more natural an alternative to steroid treatments [4]. However, this kind of allergy treatment is only effective if the materials, delivered via intranasal, have microscopic particle size. This fact shows up the importance of developing new processes for the micronization of chitin derivatives as powders. Within the last two decades, new cost-efficient processes for the production of micron-sized crystalline particles have emerged using compressed fluids (CF), either in the liquid state or above its supercritical point [5]. Most of these technologies have in common the use of CO_2 as CF since it is non-toxic, non-flammable, cheap and easy recyclable, as well as their final purpose: to produce micro-, submicron- or even nano-crystals with a narrow particle size distribution. Moreover, such crystallization methods permit to avoid the problematic comminution and homogenization down-stream steps, which are often required after conventional crystallization processes. The existing methods using CF can be classified in different groups according to the solvating behavior of the CF in the process. Gas Anti-Solvent (GAS) [6], and Aerosol Solvent Extraction System (ASES) [7] are two different high pressure crystallizations techniques were the CO_2 behaves as anti-solvent. In both systems the compressed carbon dioxide, miscible with the organic solvent and immiscible as partially so, with the solute to precipitate, behaves as anti-solvent over and initial solution of the compound to crystallize, inducing its precipitation. In this work we have used GAS and ASES techniques, for the straightforward production of micronized powders of the chitosan derivatives, and it is foreseen to extent the crystallization to other mocromolecular chitin/chitosan derivatives.

MICRONIZATION PROCEDURES

The equipments used in the micronisation of these compounds by ASES (A) and GAS (B) procedures are depicted in Figure 1. In the ASES a constant flow of CO_2 is continuously supplied inside autoclave R1, with a high-pressure piston pump P1. The working pressure, P_w, inside R1, is kept constant using a back pressure regulator valve, V4. A circulating water jacket is used to keep constant the working temperature, T_w, inside the autoclave.

When constant values of T_w and P_w are achieved, an organic solution of the compound to be crystallized is fed to the autoclave trough a nozzle (100 μm diameter), by a high pressure pump P2 at a constant flow. In this process the supercritical CO_2 diffuses into the sprayed droplets causing a rapid expansion of the solvent, decreasing its solvating capacity and therefore forcing the solute to precipitate or crystallize as particles. The solid micro-particles, produced during the spraying of the solution, are collected at P_w on a stainless steel filter housing F1 and the mother liquid is depressurized after the back-pressure regulator valve, V4. In the GAS process a known volume of a solution of the compound is loaded in the vessel R1, and a circulating water jacket keeps constant T_w inside R1. The initial solution was then pressurized up to P_w by the addition of CO_2 thereby the organic solvent is expanded several folds until filling all the autoclave volume. During the expansion the solvent power of the initial organic solvent is reduced, causing a sharp solution saturation increase and the precipitation of small and

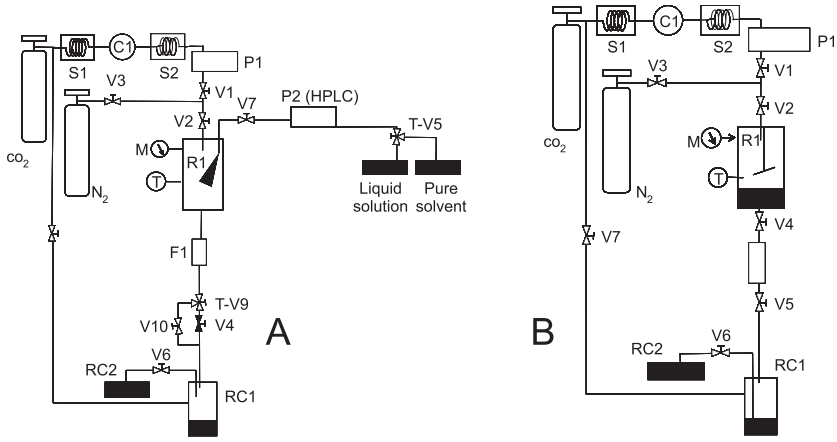

Figure 1. Equipment schemes used in ASES (A) and GAS (B) crystallizations. P, pump; R1, high pressure vessel; S, heat exchanger; V, valve; C1, flow-meter; M, manometer; T, thermopar; F1 collector filter; RC, solvent recycling collector

uniform particles. After complete homogenization of the mixture and thermal equilibration, the valve V4 is opened in order to filter the micro-particles produced on F1. The CO_2 evolved in the processes as well as the mother liquid can be easily separated and recycled if required.

POWDER CHARACTERIZATION

Successful generation of the monomer derivatives as micro-powders has been achieved by ASES and GAS anti-solvent techniques using methanol or ethanol as solvent, and liquid or $scCO_2$ as anti-solvent following the described procedures.

Table 1. ASES and GAS crystallization experiments of the compounds

	P (MPa)	T (°C)	Yield (%)	Median of diameter of particles (μm)[a] X50 %
D-Glucosamine hydrochloride (Methanol/CO_2)				
Unprocessed	–	–	–	188.9
ASES	12	35	95	5.95
GAS	10	25	70	18.4
D-Glucosamine sulphate (Methanol/CO_2)				
Unprocessed	–	–	–	88.55
ASES	12	35	98	5.36
GAS	10	25	95	8.61
N-Acetyl-D-glucosamine (Ethanol/CO_2)				
Unprocessed	–	–	–	120.05
ASES	18	35	90	11.92
GAS	10	25	70	6.91

[a] Volumetric particle size distributions, measured with the light scattering technique (Beckman Coulter/LS Particle Size Analyzer), are given as 50% quantiles

The particle size distribution of the processed materials were analysed by light scattering technique (LS) and those are compared to their unprocessed precursors in Table 1.

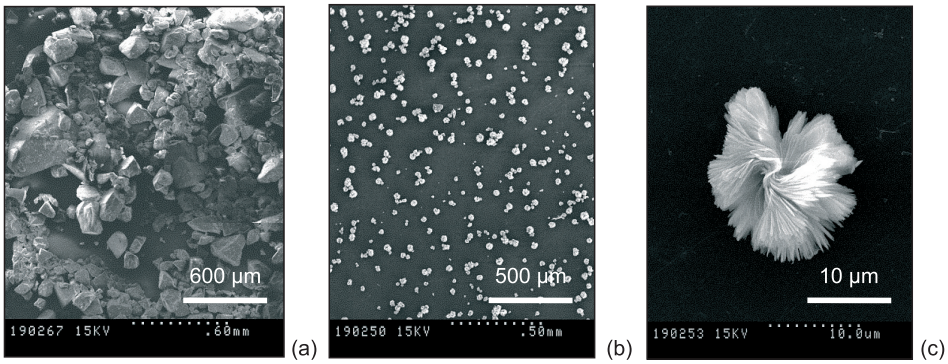

Figure 2. The SEM image of unprocessed D-glucosamine sulphate (a) is compared to SEM images of processed particles of this compound by GAS method at different scales (b) and (c)

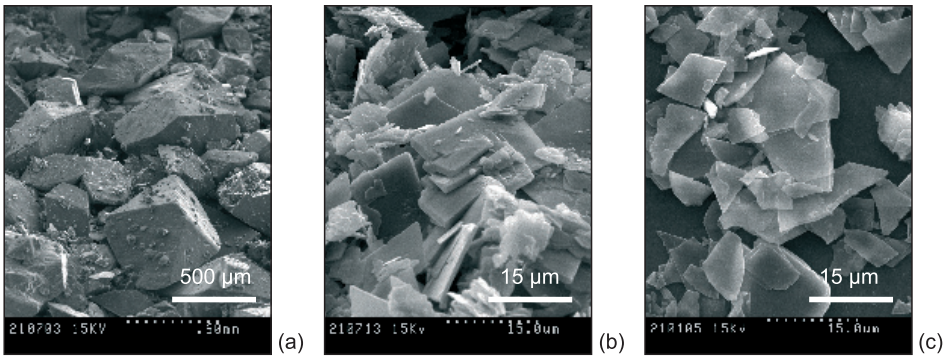

Figure 3. The SEM image of unprocessed D-glucosamine hydrochloride (a) is compared to SEM images of processed particles of this compound by GAS (b) and ASES (c) methodologies

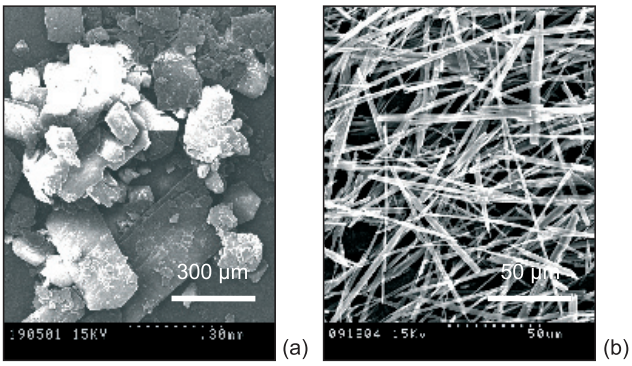

Figure 4. The SEM image of unprocessed *N*-acetyl-D-glucosamine (a) is compared to SEM image of processed particles of this compound by the ASES methodology (b)

The crystallinity of the processed samples was ascertained by powder X-ray diffraction, and the morphology of the processed particles was observed by scanning electron microscopy (SEM). SEM images of the particles of processed compounds are compared to the SEM images of their unprocessed precursors in Figure 2–4.

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CYTOLOGICAL STUDY OF WOUND HEALING PROCESS AT USE OF CHITIN-CONTAINING PREPARATION MYCOTON

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Key words: chitin-containing material, wound treatment, cytological study

The chitin-containing preparation Mycoton has shown many valuable properties at treatment of wounds, burns and ulcers. The laboratory and clinical research of the Mycoton properties have shown that as a dressing material it possesses a large complex of properties: stops bleedings; anesthetizes wounds; depresses an inflammatory process; prevents suppuration; accelerates the healing of wounds; biodecomposes in wounds without scars; is an atraumatic material; has its own therapeutic action relative to trophic ulcers; enhances local and total immunity in patients with purulent wounds; has high hygroscopicity and good capillarity (1–4). The research of Mycoton action on laboratory animals has shown absence of a toxicity even in large doses. In parallel with studing of wound healing capacities of Mycoton was studied its action on a state of internal organs of patients by standard clinical methods. These researches have shown absence of negative action on a liver, kidneys, pancreas, peripheric blood and system of a blood coagulation. Mycoton is safe for application throughout. On the establishment of these researches the Pharmacological Committee of the Ministry of Public Health Services of Ukraine has given the sertificate on nontoxicity of a dressing material Mycoton.

Main aim of our research was analysis of the chitin-containing material Mycoton behavior in a wound and processes of the wounds healing connected by it. The researches were made in clinic of Department of Obstetrics and Gynecology of Kiev State Medical University. Women with postnatal traumas were the patients in clinic investigations. The wound healing in two groups have been investigated by cytological methods. At group 1 (42 persons) applied Mycoton as a dressing material for treatment inflammatory process. Comparison carried out with a group of patients (27 persons) at which treatment by Mycoton was not applied (group 2). Standard techniques of selection, fixing and painting of cytological preparations (prints with wound surfaces) was used.

To estimation of cytogramms we used existing classification of wound process stages: necrosis, degenerate-inflammatory, inflammatory, inflammatory-re-

generative and regenerative stages. In cytogramms we took into account quantity of leukocytes, hystocytes, phagocytes, cells of a connecting fibers, epithelium, microflora and morphological changes of Mycoton fibres.

As result it is shown that in the first phase of a wound healing process fibroblasts and multinuclear cells are full absent. Many neutrophiles are in a condition of a degeneration and desruction with displays of caryopicnosis, caryorecsis and cytolysis. Attributes of neutrophyles phagocytic activity are absent or is very poor. Microorganismes are among cellular detritus. Fibrin in wound exudate is submitted by single fibres and shows the weak tendency to formation of mesh structures. Fibres of Mycoton in the first days of a presence in a wound look without changes (Fig. 1, 2).

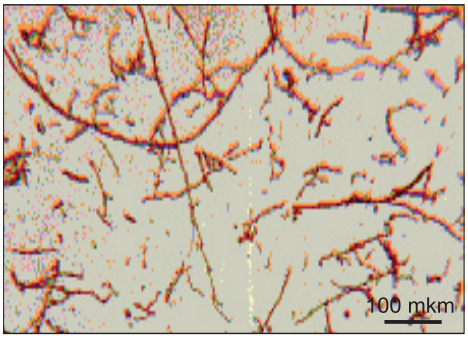

Figure 1. Mycoton's fibers under microscope

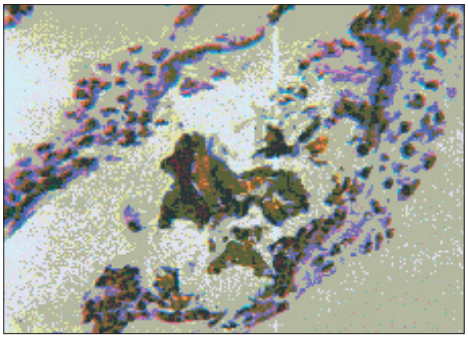

Figure 2. Mycoton in tissue detritus in wound (the dark brown incorporation). The second day

Essential distinctions in cytologic prints appear on the third day. In the first group with use of Mycoton purification of wounds happens on the second or third days, granulation begins and process passes to a inflammatory-regenerative phase (Fig. 3). The similar condition in the second group is observed only on the seventh or ninth days. Mycoton possesses strong antibacterial properties. During its application the titers of main bacterial agents in a purulent wound are already on the third day of treatment reduced from 10^7–10^9 microorganisms per 1 g of biopsy to 10–100 bacteria per 1 g. In cytogramms of the 1st group the quantity of neutrophyles decreased by 20.2%, and in the 2nd – by 6.7%. The regenerative type of cytogramm at the first group of patients is observed already on the fourth or fifth days, and at the second groop only on the tenth or twelfth days. Striking differences are observed in behaviour of leukocytes. In 1-st group destruction of leukocytes has decreased and in the second has increased up to 71%. We connect this phenomenon with adsorption properties of chitin in Mycoton due to which there was a purification of a wound off microorganisms and various toxins. Changes of Mycoton structure in wounds become appreciable for the seventh day. In places of active granulation and collegenesys its fibers lose the color, and then a fragmentation and destruction are observed (Fig. 4). In places of a congestion of Mycoton the recovery of injured tissues passes much faster (Fig. 5, 6).

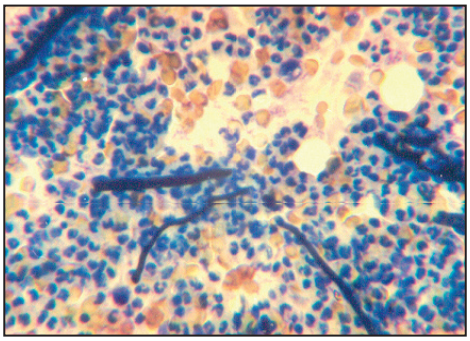

Figure 3. Mycoton's fibers in field of neutrophil leucocytes. The third day

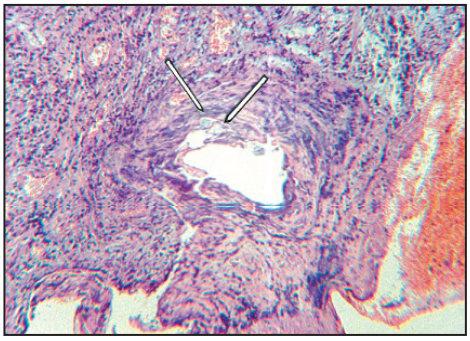

Figure 4. Losses by Mycotont's fibers of the coloring. The seventh day

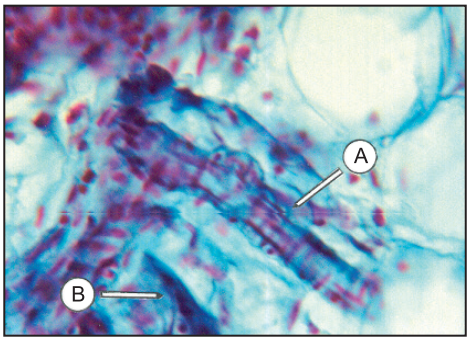

Figure 5. Recovery of capillary tubes in places of a Mycoton congestion. The seventh day

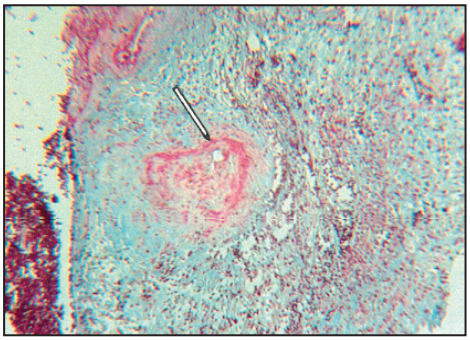

Figure 6. Fibrinogen formation and tissue granulation in places of a Mycoton congestion. The ninth day

The use of Mycoton improves the main indices of local immunity in the wound by 1.5–2 times. The traditional treatment with the use of antibiotics practically does not intensify the immune response of an organism. Resolving in a wound Mycoton also influences on many parameters of common immunity of an organism and outputs them on a level of a control (patients without the infection process). Especially Mycoton has significant influences on amount of circulating immune complexes and on phagocytic number of patients with purulent wounds.

Mycoton has properties very important for the dressing material: atraumaticity and biodecomposity. When using Mycoton, the quantity of dressings reduces. During a dressing it does not cause the secondary damage of a wound surface and bleeding. Thus the repeated infection is excluded. The remains of Mycoton in tissues are resolved without traces and no scars remain on the wound site.

Mycoton shows multivector influence on wound healing process. Strong bactericidal properties, the ability to adsorb pathogenic bacteria and their toxins and immunomodulating properties of Mycoton accelerate the healing of purulent wounds. The study of the wound healing process has shown that Mycoton has a favourable effect on all stages of this process. The complex of these properties allow on 4–5 days to reduce terms of treatment as compared to traditional treatment.

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INVESTIGATION OF EQUILIBRIUM IN LACTIC ACID COMPLEXES WITH CHITOSAN

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Key words: lactic acid, chitosan, vaginal pH, complex

INTRODUCTION

Previous research studies on the application of lactic acid for gynaecological purposes involved gels, globules, tablets used in the treatment of vaginal pH disturbances [1–6].

The above drug forms were positively assessed as to their usefulness and effectiveness in the clinical and ambulatory management of the above mentioned disorders.

Purpose: The aim of the present study was to investigate the state of equilibrium of lactic acid complexed with chitosan used in the above mentioned drug forms.

EXPERIMENTAL METHODS

Materials

Lactic acid – P.Z.F. Cefarm (Wrocław, Poland). Chitosan – deacetylation degree of 93.5% – Sea Fisheries Institute (Gdynia, Poland). Glycerol pro analysis – POCH (Gliwice, Poland). 1,2-propylene glycol, – POCH (Gliwice, Poland). Polyoxyethylene glycol 200, LOBA – Chemie, Wien – Fishamend (Wien, Austria).

Apparatus

The dialysis vessel consisting of a donor compartment and acceptor compartment separated by a semi-permeable dialysis membrane.

Shaker universal – WU-4.

pH – meter Hanna Instruments – 302, compact elektrode EPS – 2ZM.

INVESTIGATIONS AND RESULTS

The results of investigations of equilibrium in lactic acid complexes with chitosan indicated that the concentration of free lactic acid increases with time until a state of equilibrium is achieved.

The measurements indicated that the concentration of lactic acid in a state of equilibrium with the complex depends on the acid to polymer molar ratio and reaches the maximum value when the molar ratio is 4:1, being lower at 2:1 ratio and the lowest at 1:1 ratio (Fig. 1). This is a directly proportional relationship, thus equilibrium concentration of lactic acid increases with the increase of the acid to polymer molar ratio.

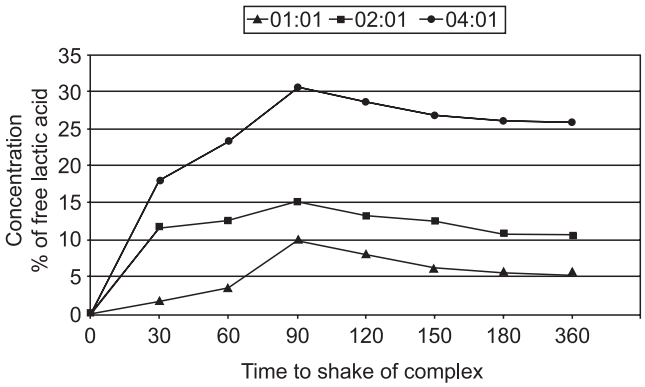

Figure 1. Course of equilibrium setting in lactic acid complex with chitosan in which the acid to polymer molar ratio is 1:1, 2:1, 4:1 without the addition of hydrophilizing substances

The addition of hydrophilizing substances affects the increase or decrease of lactic acid equilibrium concentration in the lactic acid and chitosan complex. Glycerol and 1,2-propylene glycol at individual, investigated concentrations result in an increase of the concentration of lactic acid being in equilibrium with the complex in relation to the reference preparation at all the investigated acid to polymer molar ratios. The highest increase of the concentration was observed at acid to chitosan molar ratio of 2:1 and 4:1. Lower increase was observed at 1:1 molar ratio. The only exception was observed in the case of 15% PEG-200, which decreased the equilibrium concentration of the acid in all the investigated cases.

In complexes in which the lactic acid to polymer ratio was 1:1, the highest increase of equilibrium concentration was observed after addition of 25% PEG-200 and 25% 1,2-propylene glycol, while in case of glycerol, the same effect was observed after 5% addition of this substance.

For complexes with 2:1 lactic acid to polymer molar ratio, the highest increase of lactic acid equilibrium concentration was observed after addition of 25% PEG-200 and 5% 1,2-propylene glycol as well as after 5% glycerol.

In complexes with 4:1 molar ratio, the highest increase of the concentration occurred after addition of 5% PEG-200, 5% of 1,2-propylene glycol (Fig. 2) and 5% glycerol.

15% addition of the hydrophilizing substance had a lower effect on the increase of the equilibrium concentration, while the lowest effect was observed after 25% addition of the substances (Fig. 3).

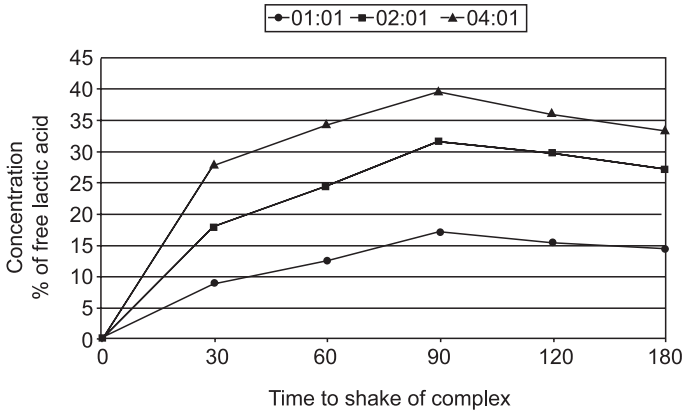

Figure 2. Course of equilibrium setting in lactic acid complex with chitosan in which the acid to polymer molar ratio is 1:1, 2:1, 4:1 with the addition of 5% 1,2 propylene glycol

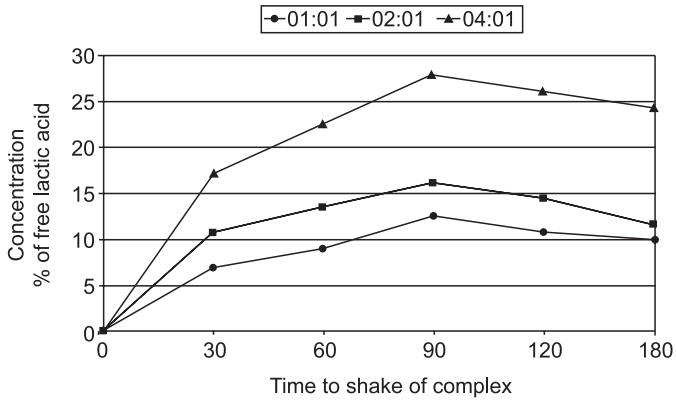

Figure 3. Course of equilibrium setting in lactic acid complex with chitosan in which the acid to polymer molar ratio is 1:1, 2:1, 4:1 with the addition of 25% glycerol

DISCUSSION AND SUMMARY

Analysis of the results may lead to a conclusion that in the majority of cases, the highest effect on the increase of lactic acid concentration being in equilibrium with its complex was observed following addition of 1,2-propylene glycol in 5–25% concentration.

Fifteen percent addition of the hydrophilizing substances in majority of cases had a lower effect on lactic acid equilibrium concentration, while the lowest effect was observed after 25% addition.

CONCLUSIONS

1. Equilibrium concentration of lactic acid increases with the increase of the acid to polymer molar ratio.

2. The addition of hydrophilizing substances affects the increase or decrease of lactic acid equilibrium concentration in the lactic acid and chitosan complex.

ACKNOWLEDGMENTS

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DETERTION OF LIPASE INHIBITION BY MEANS OF PREPARATIONS CONTAINING CHITOSAN AND XENICAL PATTERN IN THE BIOPHARMACEUTICAL MODEL OF THE DIGESTIVE TRACT IN VITRO

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Key words: chitosans, lipase, orlistat

Numerous preparations containing natural multimolecular compounds the action of which is to support weight loss are used in the clinical practice in treatment of obesity. In the study, Xenical® preparation was used as a pattern (the only slimming preparation that does not reveal central effect) which was treated as a selective inhibitor of gastric and pancreatic lipase.

MATERIALS

Chitosan capsules manufactured by different companies: Vitana, Hitec Nutrition, Chromdiet as well as pure chitosan manufactured Vanson submitted to radiation in the amount of 5, 15, 30 kGy were used in the study. The applied adjuvant substances, sodium estolate (Sigma 2003), Kollidon 25 (Sigma 2003), hydrochloric acid (POCH 2003), sodium carbonate (POCH 2003) fulfilled the requirement of pharmaceutical purity according to FP VI. Xenical® (Orlistat) preparation was used as pattern selective inhibitor of gastric and pancreatic lipase.

Lipase colorimetric (LIPC) contains an in vitro diagnostic reagent system intended for use on COBAS INTEGRA systems for the quantitative determination of the catalytic activity of lipase (EC 3.1.1.3; triacylglycerol acyl-hydrolase) in serum and solutions (test LIPC, 0–100).

METHODS

Chitosan samples of 120.00 mg exact to 0.01 mg were dissolved in 7.00 ml centrifugal tubes. Next 3 ml of 0.05 M HCl were added. Thus prepared solutions contained 1 mg of the preparation in 25 µl of the solution. Next the portions of 0.0125; 0.025 ; 0.250 ; 2.5 ml were collected to separate tubes and diluted to 0.05 M HCl to 0.2 ml (physiological conditions in the stomach). Next the mixture was stirred in

a shaker (300 rpm) at 37°C until dissolution and formation of a homogenous liquid. The process of shaking (300 rpm) lasted 2 hours. Next the pH was adjusted at 6.4–0.2 M Na_2CO_3 (duodenum). The samples were centrifuged (15000 rpm) and filtered through a membrane filter with the pore size of 0.45 μm. Next solution containing an enzyme, pancreatic lipase with a known activity was added. The mixture was supplemented with aqua purificata to obtain 1 ml of liquid each time. The control sample was prepared in the same way adding 0.3 ml of purified water to 0.7 ml of the pattern pancreatic lipase. The samples were collected after 30, 45 and 60 minutes, all the time maintaining the temperature at 37°C in order to imitate the conditions in the gastric tube. The measurements were taken 3 times from every solution and the results were analyzed statistically calculating standard deviation and variability coefficient.

Test principle

The chromogenic lipase substrate l,2-*O*-dilauryl-rac-glyero-3-glutaric acid-(6-methylresorufin) ester is cleaved by the catalytic action of alkaline lipase solution to form 1,2-*O*-dilauryl-rac-glycerol and an unstable intermediate, glutaric acid (6-methylresorufin) ester. This decomposes spontaneously in alkaline solution to form glutaric acid and methylresorufin. Addition of detergent and colipase increases the specificity of the assay for pancreatic lipase. Virtually no lipase activity is detected in the absence of colipase. Colipase only activates pancreatic lipase, but not other lipolytic enzymes found in solutions. The high amount of cholates ensure that the esterases present in the solutions do not react with the chromogenic substrate due to highly negative surface charge.

1,2-*O*-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester

↓

1,2-*O*-dilauryl-rac-glycerol + glutaric acid-(6-methylresorufin) ester

↓

glutaric acid + methylresorufin

The color intensity of the red dye formed is directly proportional to the lipase activity. It is determined by measuring the increase in absorbance at 583 nm.

RESULTS AND DISCUSSION

Table 1 presents the composition of the investigated samples of the commercially available slimming preparations.

Table 1. Composition of the investigated samples

Sample	Preparation
Chitosan A	Vitana capsules
Chitosan B	Hitec Nutrition capsules
Chitosan C	Chromdiet capsules
Chitosan D	Pure chitosan
Chitosan D(L)	Pure chitosan + Sodium estolate (0.5 mg)
Chitosan D(L,K)	Pure chitosan +Sodium estolate (0.5 mg), Kollidon 25 (0.5 mg)
Chitosan E	Pure chitosan degraded with 5 kGy
Chitosan F	Pure chitosan degraded with 15 kGy
Chitosan G	Pure chitosan degraded with 30 kGy
Xenical	Xenical (orlistat) capsules

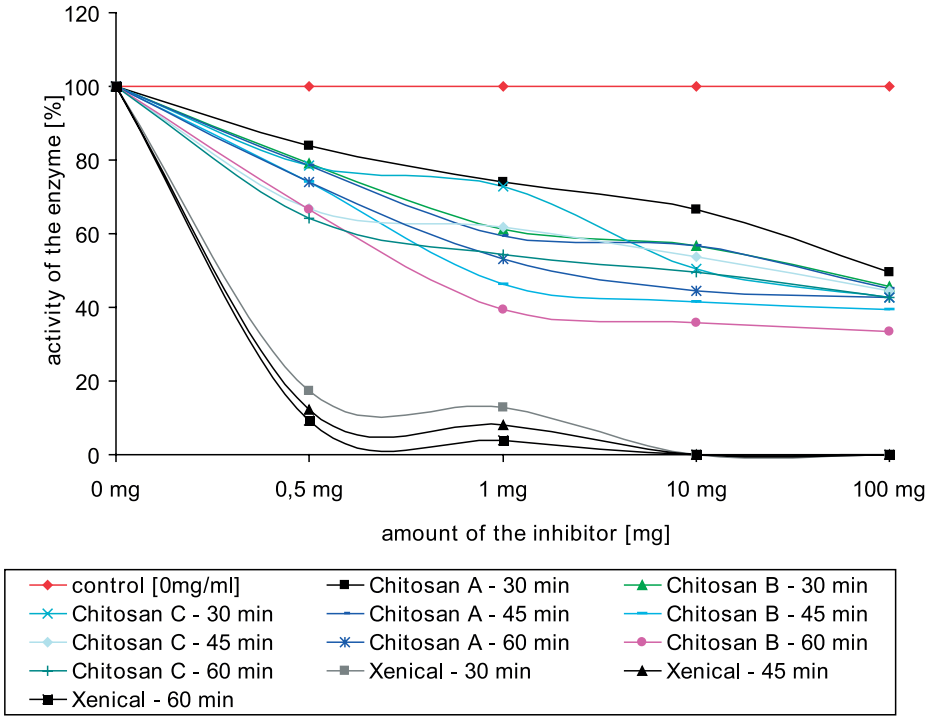

Figure 1. The effect of pancreatic lipase inhibition by chitosan and Xenical as pattern (to control sample 0 mg/ml of the inhibitor) at 37°C in the biopharmaceutical model of the digestive tract in vitro in the time of 30, 45, 60 min

Fig. 1. presents the effect of pancreatic lipase inhibition by preparations containing chitosan in relation to Xenical® which was used as a pattern with known

effect on lipase. Standard deviation was in the range from 0.13 U/L to 2.54 U/L while variability coefficient W_z from 0.084 to 2.28%, what confirms high precision of the results.

As indicated by the presented data, inhibition of the lipase activity by extracts obtained after passage through the digestive tract, depends on the dose of the preparation and the time of exposure. Only a preparation containing chromium (Chromdiet®) behaved differently. Decrease of the inhibition results from a presumed formation of a complex between the element and chitosan.

The activity of lipase depends on the administered preparation and its dose and it ranges from 78.6 to 83.8 of the primary activity at the dose of 0.5 mg /ml.

The highest decrease of pancreatic lipase activity was observed in case of Hitec Nutrition® preparation, in which case the activity was decreased to about 40% of the initial value.

The addition of adjuvant substances to the drug form, such as sodium estolate used as a solubilizer in the production of capsules confirmed the presumption that the substance exert an effect on the activity of the lipase enzymatic system.

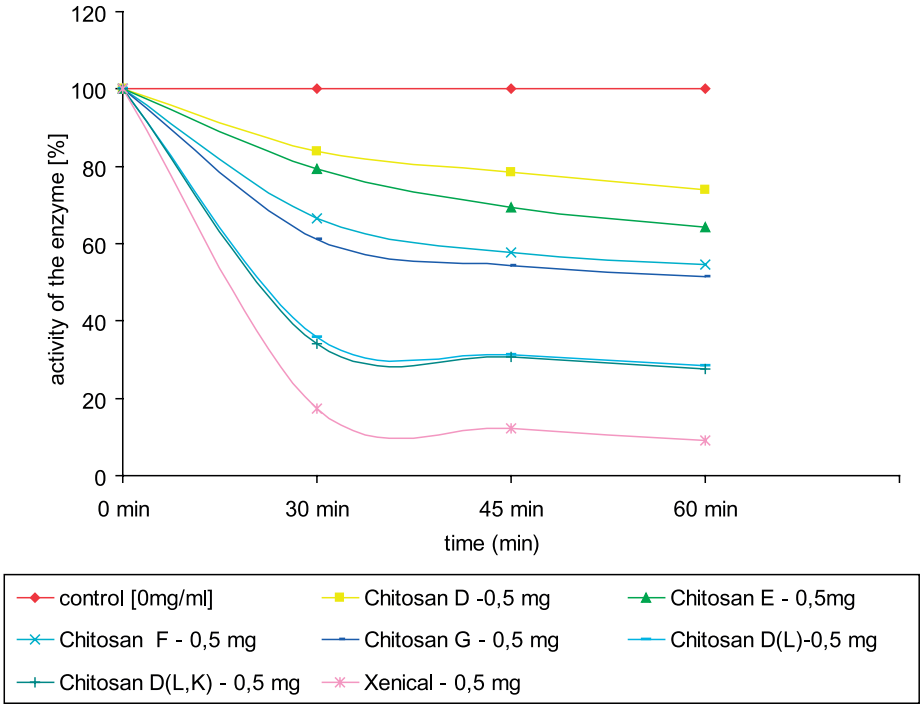

Figure 2. The effect of pancreatic lipase inhibition by chitosan, adjuvant substances and Xenical as pattern (for control sample 0 mg/ml of the inhibitor) at 37°C in biopharmaceutical model of the digestive tract in vitro in 30, 45, 60 min

Fig. 2. presents the effect of pancreatic lipase inhibition by the investigated preparations containing chitosan and adjuvant substances.

CONCLUSIONS

The addition of sodium estolate decreases the enzymatic activity of lipase to from 40 to 50% of the initial value. On the other hand, the addition of Kollidon 25 at applied doses exerts an insignificant effect on the activity (decrease by about 1%).

Ionizing radiation, producing chitosans with a low molecular weight, increases lipase inhibition.

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EFFECT OF CHITIN AND ITS DERIVATIVES ON SKIN REGENERATION

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In general, the process of wound healing is divided into 4 stages, such as hemostatic, inflammatory, proliferating and remodeling stages [1]. From the results of our clinical or *in vivo* studies, each stage of wound healing is enhanced by administration of chitin and its derivatives to a wound. Furthermore, from the results of our *in vitro* studies, effects of chitin and its derivatives on the former three stages were clearly demonstrated, namely, chitin and chitosan activate platelets, inflammatory leucocytes, fibroblasts and vascular endothelium. However, the effect on the final remodeling stage, especially mechanism of re-epithelization is yet unknown. In the present study, we demonstrate direct effect of chitin and its derivatives on proliferating of normal human keratinocytes.

MATERIALS AND METHODS

Keratinocytes

Neonatal human preputial epithelial keratinocytes (NHEK) were purchased from Kurabo Biomedical (Osaka, Japan). NHEK were incubated manually in HuMedia-KB2 (Kurabo Biomedical, Osaka, Japan) for cell maintain and HuMedia-KG2 for cell proliferation (Kurabo Biomedical, Osaka, Japan).

Chitin and its derivatives

Chitin fine particles (MW 300,000, squid origin, Sunfive Co. Ltd, mean particle size: ca 30 micron-meter), chitosan fine particles (MW 80,000, crab origin, Sunfive Co. Ltd., mean particle size: ca 30 micron-meter), *N*-acetyl-chito-oligo- saccharide mixture (NACOS, Yaizu-suisankagaku Industry Co. Ltd., Shizuoka, Japan), chito-oligosaccharide mixture (COS, Yaizu-suisankagaku Industry Co. Ltd., Shizuoka, Japan), GlcNAc (Koyo Chemical Co. Ltd., Osaka, Japan), and GlcN (Koyo Chemical Co. Ltd., Osaka, Japan). We call chitin, NACOS and GlcNAc as Chitinous group, chitosan, COS and GlcN as Chitosanous group.

NHEK proliferation test

Proliferation of NHEK was determined by a modified MTT assay (WST-8 method). In brief, the chitin and chitosan particles were sterilized by ethylene ox-

ide gas and were suspended in sterile saline at a concentration of 1, 10, 100, 1000 mg/ml, respectively. Other materials were dissolved in saline at same concentrations as described above, respectively and were sterilized by filtration before use. NHEK were pre-incubated in 96 wells micro-plate at a concentration of 3000 cells per well for 12 hr. Thereafter, each sample was added 0.01 ml to the well and the plate was incubated for 24 and 48 hrs at 37 degree of C under 5% CO_2 with moisture. After incubation, 0.01 ml of Cell Counting Kit – 8 (Dojin Kagaku, Tokyo, Japan) was added to each well, and then the plate was kept quietly for 1~4 hr at 37 degree of C under 5% CO_2 with moisture. Chromometry for each well was performed using micro-plate reader (Easy reader EAR340, Medi-Con Co.Ltd., Osaka, Japan) at 420 nm. Cell counting was estimated from the each absorbance using a standard equation making in a preliminary experiment.

RESULTS AND DISCUSSION

Results of correlation between cell counting and absorbance were shown in Table 1.

Table 1. Correlation between number of viable cells and absorbance

Number of cells (Cells per well)	0	1875	3750	7500	15000
Absorbance* ($x10^{-2}$ AU)	5.5 ± 0.4	14.2 ± 2.0	25.2 ± 5.1	51.1 ± 6.0	86.8 ± 4.0

* Mean absorbance ± standard deviation

From the results of Table 1, the equation between absorbance and number of cells is $y=178.08x - 900.12$ ($R^2=0.9848$).

Results of the Chitinous and the Chitosanous groups were shown in Table 2 and 3, respectively.

Table 2. Cell proliferating effect in the Chitinous group after 48 hr incubation

Concentration	Control	Chitin	NACOS	GlcNAc
0.1 μg/ml	1629 ± 247	1616 ± 277	1318 ± 388	1312 ± 169
1 μg/ml		1724 ± 149	1955 ± 241	2372 ± 415*
10 μg/ml		1977 ± 224	1727 ± 135	2155 ± 175*
100μg/ml		1798 ± 122	1852 ± 172	2128 ± 165*

* Cells significantly increased ($p<0.05$)

Table 3. Cell proliferating effect in the Chitosanous group after 48 hr incubation

Concentration	Control	Chitosan	COS	GlcN
0.1μg/ml	1629 ± 247	1309 ± 149	1364 ± 659	1616 ± 244
1μg/ml		930 ± 238$^{\phi}$	961 ± 782	2108 ± 344*
10μg/ml		363 ± 105 $^{\phi}$	1714 ± 61	1839 ± 160
100μg/ml		908 ± 188 $^{\phi}$	1709 ± 134	1758 ± 424

* Cells significantly increased ($p<0.05$). $^{\phi}$ Cells significantly decreased ($p<0.05$)

With 24 hr incubation, GlcNAc and GlcN significantly increased the number of NHEK compare to that by the other materials. However, chitosan significantly decreased that of NHEK. Chitin and its oligomer increased the NHEK compare to that by chitosan oligomer. After 48 hr incubation, only 1 μg/ml of GlcN increased cell number, and chitosan yet inhibited cell proliferation (Table 3). In the Chitinous group, GlcNAc increased cell number except for the concentration of 0.1 μg/ml (Table 2). GlcNAc was most effective material for NHEK proliferation.

From the results of basic examination for cutaneous regeneration, effects of chitin and GlcNAc to skin regeneration were confirmed in a clinical case with burn of third degree in 90% of the face.

Clinical Case: Miniature dachshund, 2 years old, female, 3.8 kg of B.W., encountered with a fire of her breeder's hut. She was just nursing up her puppies in a metal cage, so she could not run away from the fire. A courageous neighbor saved her from the fire, but all puppies were already dead. She also contracted with severe burn on the face, left chest, right abdominal wall and all pads of her feet. Degree of burn was most severe in the face. The neighbor brought her to a veterinary hospital soon. A veterinarian saved her life temporary by his treatment. The dog was brought to Tottori University Veterinary Hospital, because of difficulty of controlling of the wounds. Fig. 1 showed macroscopic findings of the dog's face at first examination (day 1) in the University Hospital (7 days after the accident).

Figure 1. Macroscopic findings of the dog at the first examination (day 1) in the Tottori University Hospital Ninety percent of the head skin had 3rd grade burn. Purulence of wounds was already observed.

Figure 2. Administration of GlcNAc to the wound. The wound surface was covering with cotton-type of chitin, and additionally 10 mg/ml GlcNAc solution was scattered over the chitin covering.

Figure 3. Ten days after the treatment. Skin was regenerated around eyes. Skin contraction was little. No purulence was observed

Figure 4. One month after the treatment Good skin regeneration was observed. Little scar formation was observed in the top head skin

Necrotic skin was removed as much as possible, and wounds were rinsing with sterile saline. The wounds were covered with a cotton-type chitin (Fig. 2). Chitosan suspension (100 μg/ml) and 10 mg/ml GlcNAc solution (ca. 2 ml) was scattered over the chitin covering. From the day 2, additional covering by chitin and administration of 10 mg/ml GlcNAc solution (twice or thrice a day) were continued until off hospital.

Almost 1 week from the beginning of the treat, regenerated skin was observed around eyes. The wound surface was good covered by the cotton-type chitin for long time, and after skin regeneration completed, attached chitin cotton was naturally dropped off. The severe burn was cured within one month and the dog recovered her normal skin with minimum scar formation.

It is well known that chitosan quickly regenerates granulating-tissue in the wound, however, excessive granulating tissue prevents skin regeneration. From the present results, clinical evidence on granulating tissue and skin-regeneration by chitosan or chitin administration on wounded surface was clearly recognized. Most recommended procedure on skin defect treatment especially for contaminated wound is final administration of GlcNAc with chitin to the wound following granulating tissue formation by an initial administration of chitosan.

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DETERMINATION OF HYDROGEL CHITOSAN MEMBRANE STRUCTURE

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Key words: chitosan, hydrogel, powder X-ray diffraction method

A structural characteristic of hydrogel chitosan membranes is given in this study. The membranes were formed by the phase inversion method from chitosan of molecular mass 500 kD and deacetylation degree 90%. The membrane-forming solutions were chitosan salts in the form of acetate with different polymer concentrations. The amount of solvent was chosen stoichiometrically to the content of amino groups in a chitosan molecule. Coagulation was carried out up to 5% NaOH.

The effect of polymer concentration on rheological properties of chitosan acetate and on the structure of hydrogel formed after coagulation was determined. The structural characteristics were based on the analysis of DSC and XRD spectra. The character of water present in the polymer structure was determined from DSC curves, and hydrogel crystallinity from XRD patterns.

Formation of resistant membranes requires that they are made from solutions with a relatively high polymer concentration at sufficiently low viscosity retained. Rheological properties were determined by specifying changes in the shear stress as a function of shear rate. Results are given in Fig. 1a and 1b. Concentrations in film-forming solutions were selected on the basis of rheological studies. Chitosan acetate solutions are stable shear-thinned liquids. Apparent viscosity of the tested media increases with an increase of molecular mass and polymer concentration. Particularly interesting are the solutions that contain more than 8% polymer. In these solutions the flow curve shifts downwards, i.e. a rapid decrease of apparent viscosity is observed. Most probably, such behaviour of chitosan salt solutions provides an evidence that in highly viscous solutions stable dipole-dipole cohesion is observed which is destroyed by shearing forces. In parallel, the flow curve drawn in the direction of increasing shearing forces overlaps the curve made in the opposite direction and these runs do not reveal hysteresis which confirms that the tested solutions are not thixotropic, hence their rheological properties do

not depend on the shearing time, which suggests the lack of polymer degradation due to shearing forces.

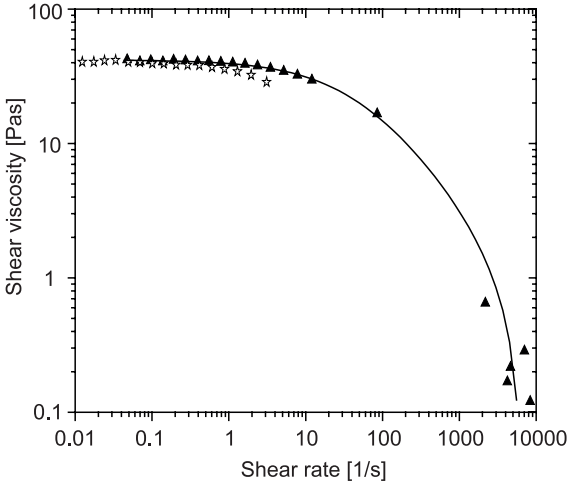

Figure 1a. Shear stress τ versus shear rate γ

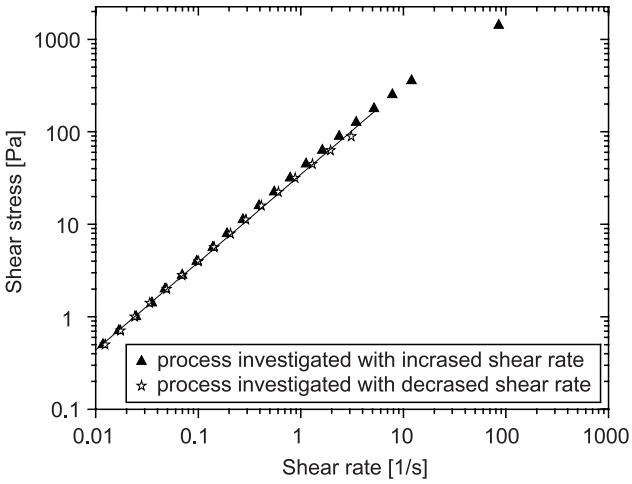

Figure 1b. Shear viscosity η versus shear rate γ

The structure of hydrogels obtained from polymers of varying concentrations is different.

The character of water present in the polymer structure was specified on the basis of DSC curves. An example of the DSC curve for membranes formed from 8% chitosan acetate is shown in Fig. 2.

Membranes contain in their structure water that is bound with the polymer in a different way. The presence of water is confirmed by a peak that appears at the temperature 2.3°C (related to the water that is frozen, i.e. unbound or very weakly bound with the polymer molecule) and a number of peaks at the temperature

80 to 120°C (most probably related to water evaporation from particular granule layers). On the basis of ice melting enthalpy, using the Mansor and Malcolm method, the amount of water bound in the hydrogel structure was calculated from the equation

$$W_b\ (\%) = W_t - (W_f + W_{fb}) = W_t - (Q_{endo} / Q_f) \cdot 100$$

where:
W_t – total amount of water in hydrogel,
W_b – water bound in hydrogel that does not freeze,
W_f and W_{fb} – water unbound or partly bound that can freeze.

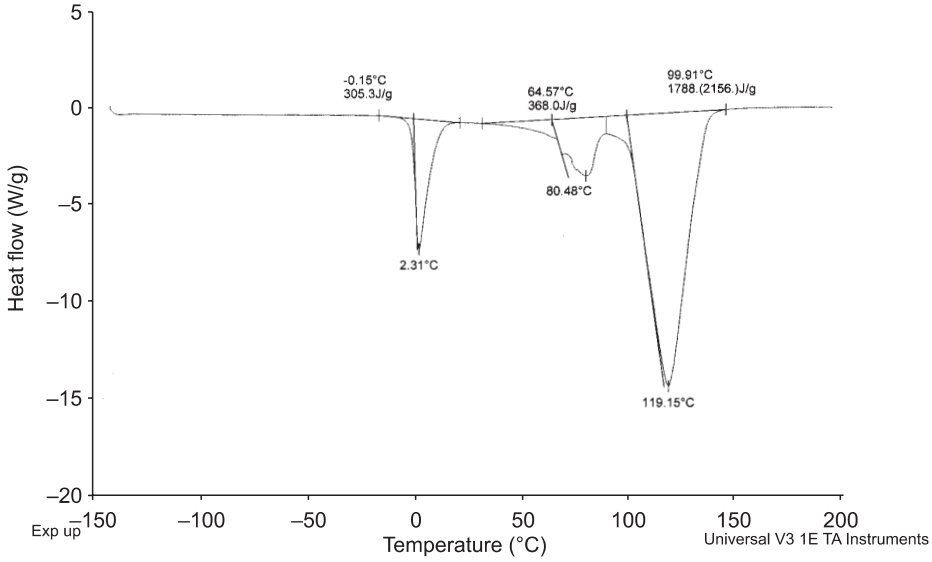

Figure 2. DSC curve

Table 1 gives spectral characteristics of particular membranes.

Table 1. Analysis of endothermal peaks for membranes

Solvent type	Concentration	Ice melting enthalpy		Amount of bound water [%]	Enthalpy of water evaporation			
		Temp. [°C]	Enthalpy [J/g]		Temp. [°C]	Enthalpy [J/g]	Temp. [°C]	Enthalpy [J/g]
Acetic acid	6%	3.09	286.0	5.4	93.30	781.7	110.14	1176
	8	2.31	305.3	0.3	80.48	368.0	119.15	1788
	10	2.86	264.2	7.1	78.25	400	107.67	1483
	12	2.93	250.6	8.2	78.59	289.6	113.75	1367

These results show that in the case of membranes formed from chitosan acetate, with an increase of polymer content in the membrane-forming solution, the amount of the so-called bound water does not grow and the total amount of water in the structure does not decrease (peaks around 100°C). This departure from the rule is observed for the membranes formed from chitosan acetate with 8% polymer concentration. Lack of bound water in the structure and the highest enthalpy most probably related to partly bound water in the structure, may suggest ordering of the chitosan molecules already in the solution.

Untypical behaviour of the membranes formed from chitosan acetate with 8% polymer concentration were confirmed by X-ray studies of crystallographic properties. The X-ray diffraction patterns were determined using a wide-angle X-ray Simens D5000 diffractometer and the $K_{\alpha,Cu}$ radiation. X-ray diffractograms for particular membranes are illustrated in diagram 3.

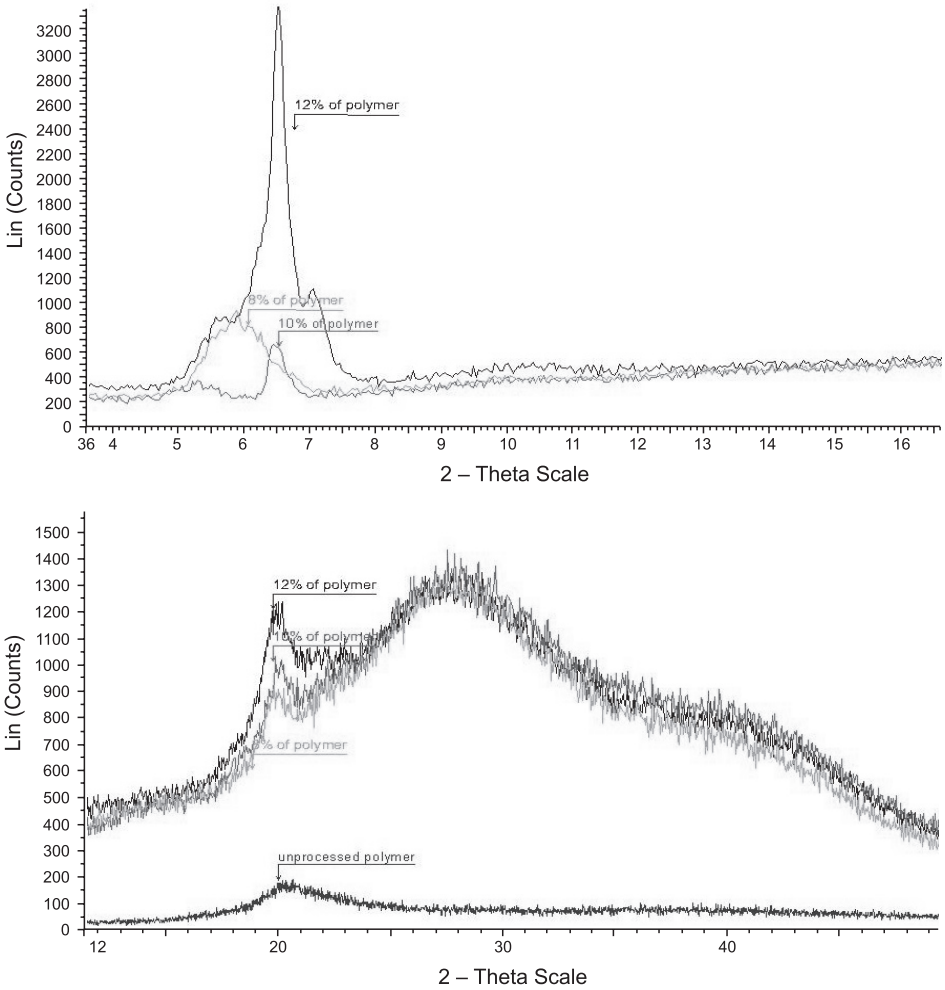

Figure 3. X-ray diffractograms depend on polymer concentration in the solution

The XRD patterns showed that crystallinity of the membranes depends on polymer concentration in the solution. Hydrogel chitosan membranes reveal better ordering than the initial polymer. Pure chitosan used in this study exhibits rather amorphous properties. Membranes formed from a polymer of concentration below 6% are amorphic, like pure chitosan. This is revealed by a broad peak with a maximum at the angle $2\theta = 28°$ (in comparison to the diffractogram of pure chitosan, shifting towards higher angles is observed). For the membranes formed from solutions of concentrations exceeding 6%, the XRD patterns are characterized by broad peaks around $2\theta = 5–8°$ and $2\theta = 19–20°$. Membranes formed from 8% solution have untypical properties, namely they are characterised by higher peak intensity in the low angle range than the membranes formed from 10% polymer solution. Moreover, this peak is shifted towards lower angles.

The above study seems to confirm that membranes with more crystalline structure can be obtained from 8% solutions.

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CHITOSAN-GENTAMYCIN MEMBRANES AS DRESSINGS – TESTING THEIR BACTERICIDAL ACTION

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INTRODUCTION

Local antibiotic treatment is a method for ensuring proper concentration of a therapeutic agent and control of infection in morbid changes. This leads to restriction of the systemic action of an antibiotic and to decrease of its side effects.

Efficiency of this method is determined by the choice of an appropriate non-toxic, biocompatible and biodegradable antibiotic carrier. Analysis of the literature shows that chitosan is one of the most interesting polymers that can be used for this purpose [1–4].

The authors present a method for the formation of chitosan membranes containing gentamycin in their structure.

MATERIALS AND METHODS

Tested material

The membranes were formed by the phase inversion method from chitosan of molecular weight 500 kD, using acetic and lactic acids as solvents. The acids were used in a stoichiometric ratio to the amount of D-glucosamine monomers in chitosan.

Investigations were made on membranes into which gentamycin was introduced in the following way:

- during membrane formation, 100 mg of gentamycin per 1 gram of chitosan was introduced into the structure of membranes which were then coagulated with gaseous ammonia or 98% ethanol solution;
- during adsorption – the membranes were prepared from 10% solutions of acetate and chitosan lactate, coagulated with 10% NaOH solution or gaseous ammonia.

Identical membranes without gentamycin were used as control samples.

Analysis of bactericidal action of chitosan-gentamycin membranes

Bactericidal properties of chitosan-gentamycin membranes were estimated in relation to the tested bacterial species *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442 obtained from the ŁOCK 105 Collection in the Institute of Fermentation Technology and Microbiology, Łódź Technical University. A suspension of these bacteria of density 10^8 cells in 1 cm^3 was prepared in the physiologic sodium chloride solution. One cubic centimetre of the suspension was added to 100 cm^3 of Mueller-Hinton agar and poured in the amount of 15 cm^3 onto Petri dishes. Afterwards, on the agar surface 2 cm × 2 cm membrane samples were placed. After incubation at the temperature 37°C for 16 to 24 hours, the bactericidal action of the tested membranes was estimated on the basis of criteria described in the standards SN 195921 and SN 195922, i.e. on the basis of:

- the presence and size of the zone inhibition the growth of tested bacteria around the tested membrane,
- growth of bacteria or its lack in the membrane-culture medium contact zone.

RESULTS

Chitosan-gentamycin membranes revealed different bactericidal properties against *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442. Results are shown in Table 1 and illustrated in Figs 1 and 2. The zone of inhibited bacterial growth was from 6 to 14.5 mm. The strongest bactericidal action revealed the membrane prepared from 10% chitosan acetate solutions coagulated with 10% NaOH solution, to which gentamycin was introduced by means of adsorption. The membranes that did not contain gentamycin, inhibited the growth of bacteria *Pseudomonas aeruginosa* in the region of contact with a culture medium, however no bacteriostatic action of chitosan against *Staphylococcus aureus* was observed.

Bactericidal properties of chitosan-gentamycin membranes after desorption were also tested. The samples stayed for 3 and 24 hours in double distilled water, to which the antibiotic was desorbed. After removal from the desorbing medium, the samples were placed again in the pure, double distilled water for several seconds and then the bacteriostatic action of the membranes was determined. The aim of these tests was to check up durability of the bactericidal action of the membranes. It was of primary importance because the process of gentamycin

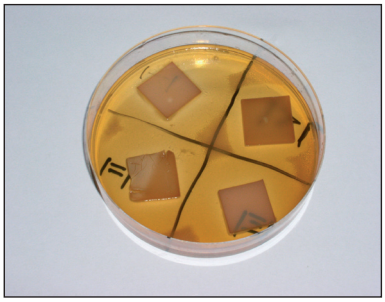

Figure 1. Zone of inhibited *Pseudomonas aeruginosa* growth

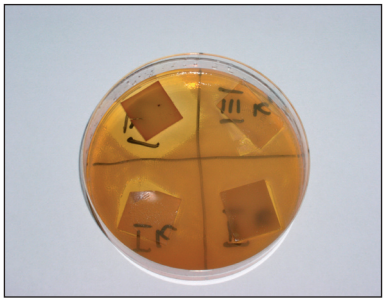

Figure 2. Zone of inhibited *Staphylococcus aureus* growth

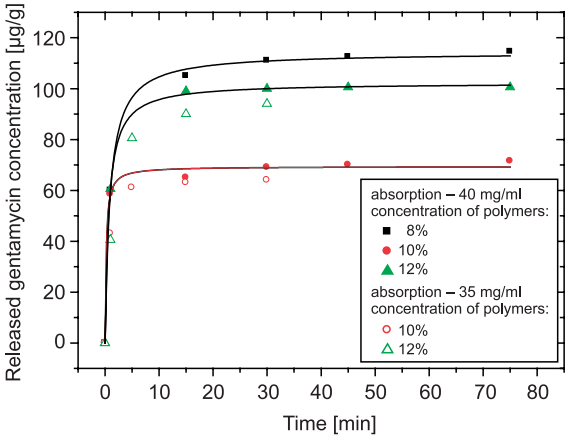

Figure 3. Release of gentamycin from hydrogel membranes formed by the method of phase inversion – gentamycin is introduced during the adsorption process

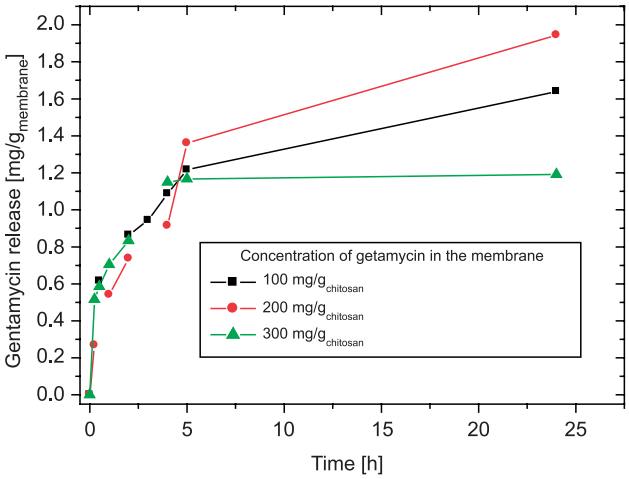

Figure 4. Release of gentamycin from hydrogel membranes formed by the method of phase inversion – gentamycin is included in the structure during membrane formation

release is characterised by a so-called "burst" known from literature, as presented in diagrams in Figs. 3 (gentamycin was introduced by means of adsorption) and 4 (gentamycin introduced during membrane formation) and there were doubts if relatively small amounts of the released gentamycin would retain the bactericidal properties during the release. The results show that after desorption, the chitosan-gentamycin membranes still preserved them. These results confirm stable bactericidal properties of the tested membranes.

Table 1. Zone of inhibited *Pseudomonas aeruginosa* and *Staphylococcus aureus* growth

Samples	Pseudomonas aeruginosa (mm)	*Staphylococcus aureus* (mm)		
	0	0	3h	24h
10% of polymer in acetic acid – coagulation in NaOH, gentamycin – adsorption	11	14.5	2.5	3.5
10% of polymer in acetic acid coagulation in NH_3, gentamycin – adsorption	12	11	4	2.5
10% of polymer in lactic acid coagulation in NaOH, gentamycin – adsorption	11	13	x	x
10% of polymer in acetic acid coagulation in ethanol, gentamycin in structure	8	8	0	0
10% of polymer in acetic acid coagulation in NH3 gentamycin in structure	6	7	0	0
10% of polymer in acetic acid coagulation in NaOH – control	1	0	x	x
10% of polymer in acetic acid coagulation in NH_3 – control	0	+	x	x
10% of polymer in lactic acid coagulation in NaOH – control	0	+	x	x

0 – no zone, but no bacterial growth reported under the sample
+ – no protective action, growth of bacteria under the sample

CONCLUSIONS

1. Chitosan membranes without gentamycin reveal bacteriostatic action against *Pseudomonas aeruginosa*, however they have no protective action against *Staphylococcus aureus.*
2. Gentamycin release from chitosan-gentamycin membranes in the case when the antibiotic is included during adsorption, is characterised by a so-called "burst", however, the membranes retain their bacteriostatic properties against both *Pseudomonas aeruginosa* and *Staphylococcus aureus* even 24 h after the gentamycin release.
3. Chitosan-gentamycin membranes containing an antibiotic in their structure reveal weaker bactericidal properties despite the fact that the burst result is limited, nevertheless, they hampered development of *Staphylococcus aureus* in the region of contact with agar, which was not observed in the case of membranes formed from pure chitosan.
4. Chitosan-gentamycin membranes can be a good dressing material to apply on infected wounds.

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STUDIES ON PHOTODEGRADATION OF CHITOSAN BLENDS

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Abstract: Products formed on the basis of chitosan find many applications in medicine and veterinary, agriculture, horticulture and packaging industry. Searching for new applications gives rise to the need of developing modern methods of production of chitosan products, e.g. blends that contain chitosan and investigation of their structure and degradability. Beside important applicative aspects, the estimation of chitosan chain stability in different process conditions (e.g. in the atmosphere of oxygen or in UV irradiation) is a very significant problem to study. The studies we carried out allowed us to estimate the photochemical stability of chitosan (CH) and its blends with polyvinyl alcohol (PVAL), starch (S) and hydroxypropylcellulose (HPC) in the form of transparent, homogeneous films. Molecular changes that take place in the blends during photodegradation were recorded by a FTIR spectrophotometer. On the basis of analysis, distinct structural changes of chitosan were observed. They were related first of all to chain breaking and destruction of amino group and also detaching of pendant groups: methylene and hydroxyl, and oxidation. The effect of PVAL, S and HPC on chitosan stability was also determined. To specify the average number of polymer chain breaking, the viscosity of diluted chitosan solutions and blends was investigated. The experimental results were analysed on the basis of a kinetic model of chitosan photodegradation in the tested blends as well. Decay and oxidation rate constants of functional groups were determined and their dependence on weight fraction of chitosan in the blend was established.

Key words: chitosan, blends, photodegradation, viscosity, FTIR, kinetics

INTRODUCTION

Owing to numerous applications of chitosan in various branches of life [1–5], it is very important to find out a possibility for improving its physical properties and resistance to degradation by mixing with other polymers. So, a growing interest in chitosan blends has been observed [6–11].

As there are also requirements concerning blend stability and safe application, studies on the decomposition of such materials exposed to different agents become significant.

The kinetics of photodegradation of chitosan itself was described previously [12]. The aim of this work was to study the kinetics of photodegradation of poly-

mer blends obtained on the basis of chitosan with special reference to the impact of PVAL, HPC and S. Good miscibility of chitosan with both polysaccharides and polyvinyl alcohol is a result of possible interactions of functional groups of components. The kinetics of chitosan blend photodegradation was analysed on the basis of viscosimetric and spectrophotometric studies.

EXPERIMENTAL

Materials

- Chitosan (CH) was produced in the Sea Fishery Institute in Gdynia; its deacetylation degree was DD = 78.5% and molecular weight $M_w = 4.3 \cdot 10^5$ g/mol.
- Polyvinyl alcohol (PVAL) was a commercial product purchased in the "Polish Chemical Reagents" company (Gliwice) at $M_w = 7.2 \cdot 10^4$ g/mol.
- Hydroxypropylcellulose (HPC) at $M_w = 1 \cdot 10^5$ g/mol was purchased at SIGMA-ALDRICH.
- Starch (S) was a commercial product with particle size ca. 20 μm.

Blends preparation

Blends of chitosan with PVAL, HPC and S of different compositions were obtained by mixing appropriate portions of water solutions of the polymer with CH solution in 1% acetic acid. Next, the solutions were casting onto glass plates at the temperature 25°C. The samples were predried for about 1 h at the temperature 45°C. Films dozen μm thick were obtained as a result of solvent evaporation at room temperature. Then, the films were dried additionally in order to remove thoroughly acetate residues at the temperature of about 80°C for one week. Spectroscopic studies revealed that this period was long enough to eliminate residuals of acetic acid and unbound water.

Measuring methods

Polymer films were exposed to UV irradiation at λ=253.7 nm, I=700 Lux. The photodegradation of polymer blends was analysed on the basis of the results of viscometric and spectrophotometric studies. Measurements of the viscosity of diluted polymer solutions were carried out at the temperature 25°C using Ubbelohde's viscometer.

Spectrophotometric studies were made using FTIR "Genesis II" spectrophotometer (Mattson). Functional groups present in macromolecules of the tested polysaccharides and PVAL and the corresponding absorption bands of defined wave number were identified. The spectra for tested polymer films 10 μm thick were collected at ambient temperature at the resolution 4 cm^{-1} and 16-times scanning.

RESULTS AND DISCUSSION

Viscosity studies of diluted solutions of chitosan and its blends

The viscosity of diluted solutions of chitosan and its blends with PVAL and HPC (in the case of chitosan blended with starch, viscosity was not investigated because films exposed to photodegradation were not wholly soluble) was mea-

sured after different periods of photodegradation. A significant decrease of reduced viscosity η_{zr} was a result of the main chain breaking and decrease of the polymer molecular weight.

Through the extrapolation of reduced viscosity to zero concentration the boundary value of the specific viscosity [η] was determined. An average number of molecular chain scissions S was calculated according to the following formula:

$$S = \left(\frac{[\eta_o]}{[\eta_t]} \right)^{1/\alpha} - 1 \tag{1}$$

where $[\eta]_o$, $[\eta]_t$ – specific viscosity: before and after photodegradation, $\alpha = 0.9$.

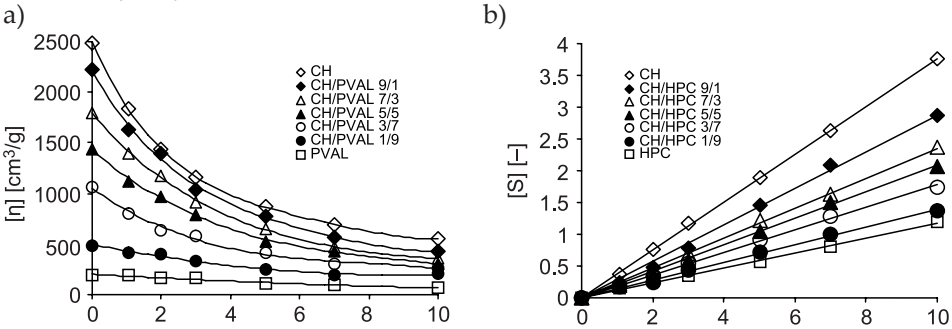

Figure 1. Dependence of: a) specific viscosity, b) molecular chain scissions on the time of exposure of chitosan and its blends with PVAL and HPC

Figure 1 shows an example of the dependence of the specific viscosity and molecular chain scissions on the time of photodegradation of chitosan blends with PVAL and HPC, which increases linearly with the time of exposure and depends on the weight fraction of chitosan in the blend. From the slopes of straight lines S = f(t), photodegradation rate constants k_f for the tested systems were determined. Figure 4a shows their dependence on the weight fraction of chitosan in the blends with PVAL and HPC. It was found that chitosan had the highest value of constant k_f. In the case of chitosan blends with HPC, an increase of the weight fraction of HPC has a slight stabilising effect on the process of chitosan decomposition in the blend – negative deviation from additivity.

Spectroscopic studies of chitosan and its blends

To determine structural changes that occur in chitosan and tested blends due to photodegradation, spectroscopic FTIR studies were carried out.

The effect of PVAL, HPC and S fraction in chitosan blends on the kinetics of photodegradation of characteristic functional groups of this polymer was investigated. The order of reaction and process rate constants, among the other things breaking of the main chain (C-O-C), destruction of amino group (NH_2), and detaching of pendant groups (CH_2, OH) were determined.

Selected FTIR spectra of chitosan, and CH/PVAL blends with weight fraction of chitosan $w_f = 0.7$ and CH/HPC blends with chitosan weight fraction $w_f = 0.3$ after different time of photodegradation are shown in Fig. 2.

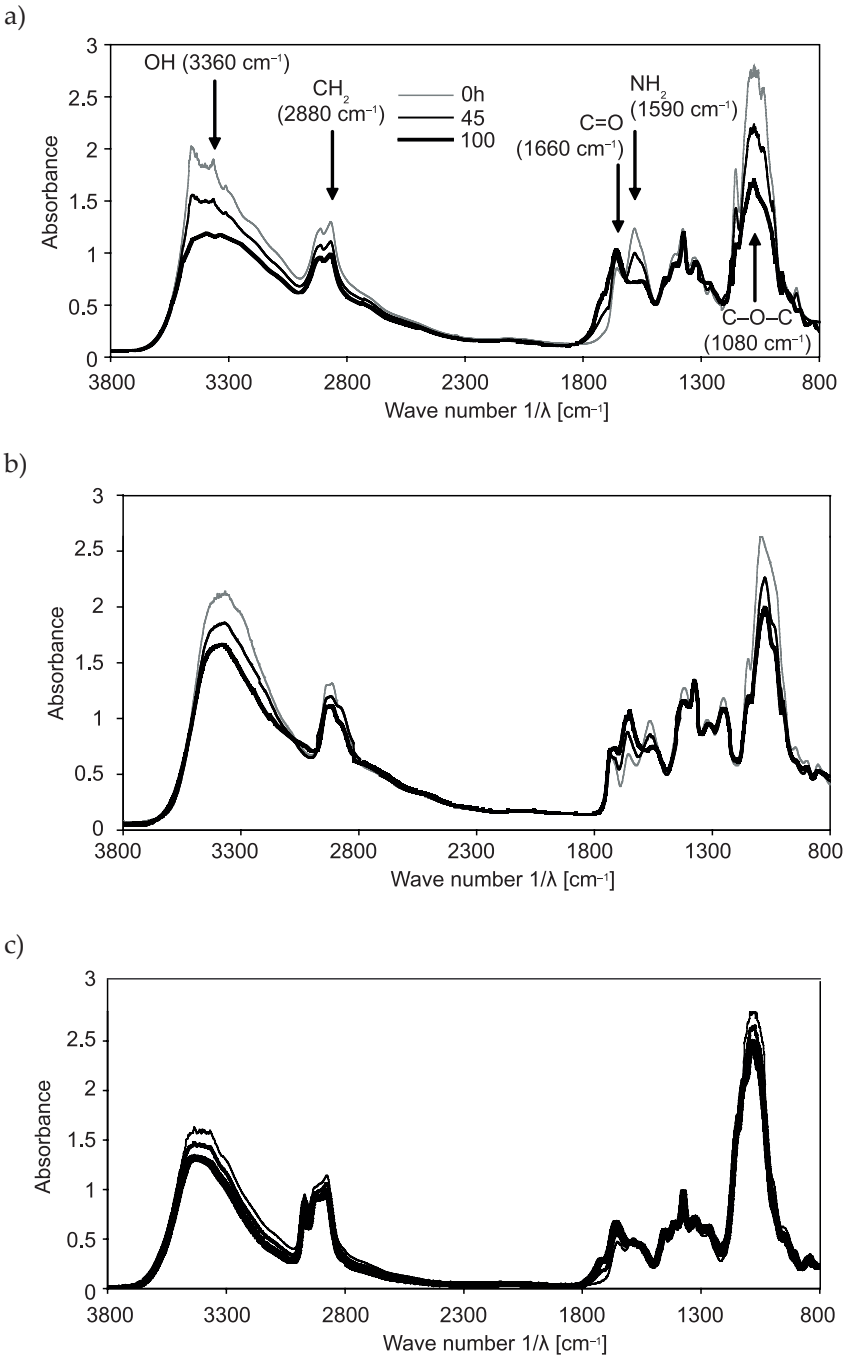

Figure 2. Comparison of FTIR spectra obtained for: a) chitosan and b) CH/PVAL blends with $w_f = 0.7$, c) CH/HPC blends with $w_f = 0.3$ after different times of photozdegradation (time: 0, 45, 100 hours)

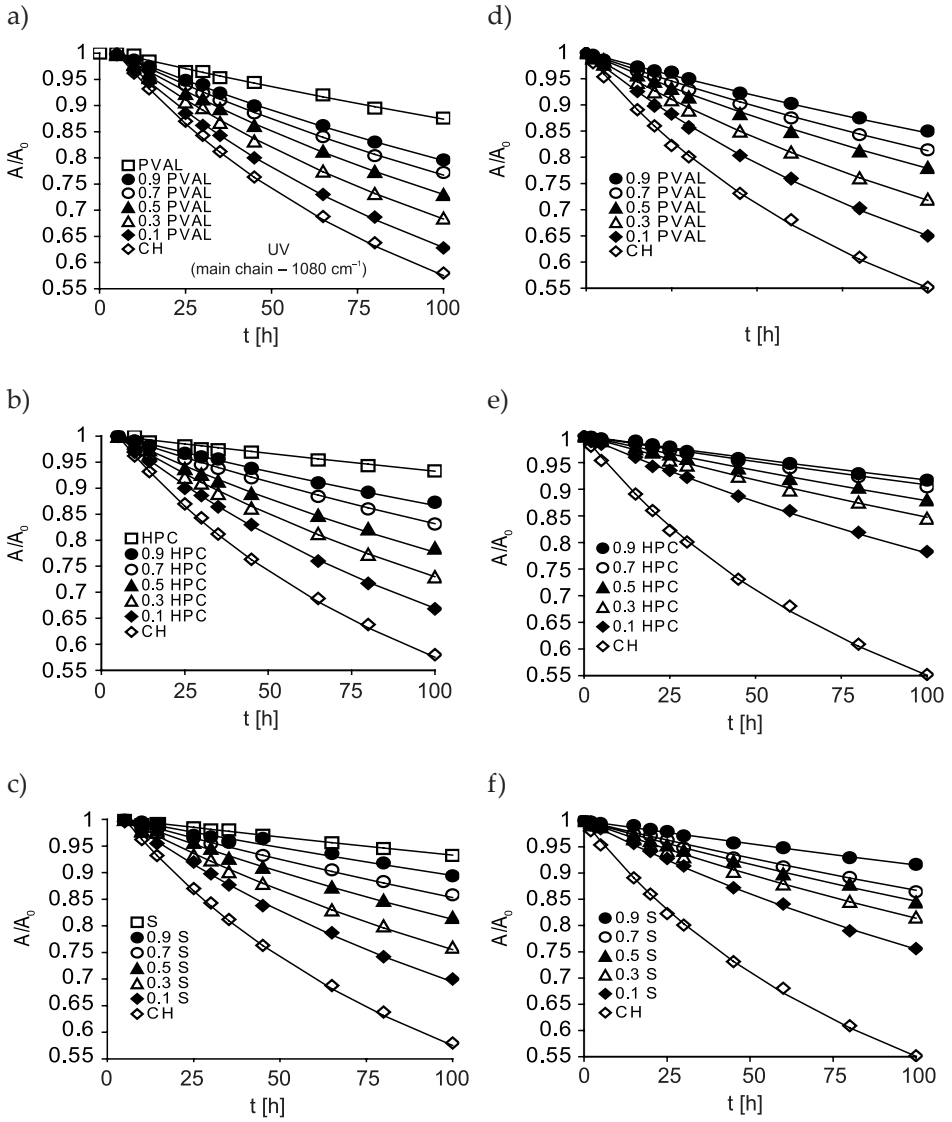

Figure 3. Dependence of relative absorbance change in the region of selected functional group: (a), b), c)- main chain (1080 cm^{-1}), d), e), f) amino group -NH_2-(1590cm^{-1}) on the time of photodegradation obtained for chitosan and its blends

Figure 3 illustrates the dependence of relative absorbance change in the region of selected functional groups on the time of photodegradation of CH/PVAL, CH/HPC and CH/S blends. Points denote experimental data and lines – theoretical fitted curves determined on the basis of the second-order kinetic equation at a fitted value of the degradation rate constant for given functional groups.

Diagrams representing the dependence of rate constants of amino group destruction on weight fraction of CH blends are shown in Fig. 4b. Dependence of

destruction rate constants of other functional groups is linear in relation to CH weight fraction.

A non-linear dependence of the rate constant of amino group destruction on the composition (weight fraction of chitosan w_f) can be described by the equation:

$$k_F = Ae^{-B(1-w_f)^{0.5}} \tag{2}$$

where experimentally determined constant values for the tested blend are: A=0.007/h, and B=1.3, 1.6, 1.45 for CH/PVAL, CH/HPC and CH/S system, respectively.

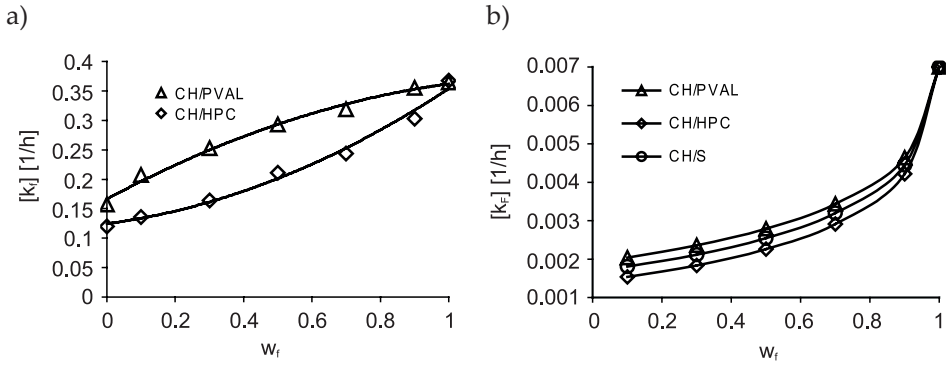

Figure 4. Dependence of a) photodegradation rate constant k_I, b) destruction rate constant k_F of amino group (NH_2) on weight fraction of chitosan w_f in PVAL, HPC and S blends.

CONCLUSIONS

When analysing results of the blends studies a significant decrease of the specific viscosity and absorbance drop induced by scission of the main chain and a decrease of molecular weight of the polymer as a result of photodegradation were observed. The number of scissions S increases linearly with time of exposure and depends on the weight fraction of chitosan in the blend. On the basis of changes in the destruction rate constants of functional groups with blend composition, it can be found that with an increase of PVAL, HPC and S fraction, the photochemical stability of chitosan in blends is improved. First of all, the destruction rate of the most active and sensitive amino group present in the chitosan is slower in blends than it might results from the weight fraction of chitosan.

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APPLICATION OF CHITOSAN FILMS IN SULCOPLASTY OPERATIONS

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Key words: chitosan film, sulcoplasty operation, chlorhexidine gluconate, antimicrobial

INTRODUCTION

Preprosthetic sulcoplasty surgery, by deepening the sulci surrounding the jaws (i.e. the vestibule, the floor of the mouth and the perituberosity region) can improve the anatomical conditions so the prosthesis becomes serviceable to satisfaction of the patient [1]. Following supra-periosteal dissection of the mucosa and the muscles attached to the alveolar bones, fixation of a prefabricated splint carrying a tissue-conditioning agent to protect the operation site is performed [2]. Generally, the wound heals with re-epithelialization of the periosteum. During the re-epithelialization period, infectious reactions may occur due to the presence of activated oral micro-flora because of the retentive effect of the fixed surgical splints that lead delayed wound healing. An ideal tissue-conditioning agent would promote healing, cause minimal pain to the patient, prevent infection, result in minimal scarring, and easy to use [3]

Chitosan has been claimed to act both as a bioadhesive and permeabilizer, making it a candidate system for oral mucosal drug delivery. In addition to its lack of toxicity and allergenicity and its biocompatibility, biodegradability and bioactivity (such as antimicrobial, wound healing and hemostatic) make it a very attractive substance for diverse applications as a biomaterial in dental field, where it has been used for systemic and local delivery of drugs. [4, 5, 6]

In this study, chitosan in film form as well as chitosan film incorporated with chlorhexidine gluconate were examined *in vivo* as an alternative tissue-conditioning systems in patients who have undergone sulcoplasty surgeries for oral rehabilitation, in comparison to a commercially available tissue-conditioning agent, Visco-gel®.

EXPERIMENTAL

Preparation of formulations

The chitosan (Protasan CL 213, FMC Biopolymers, Norway) films were prepared by solvent casting method, and cross-linked with tripolyphosphate [7]. Chlorhexidine gluconate (Chx), which is widely used antimicrobial, antiseptic agent in clinical dental practice, was incorporated into the films at 0.1% (w/w) concentration.

Visco-gel was purchased from De Trey Division, Dentsply Limited, Weybridge, Surrey, England). The liquid and powder components of the visco-gel were mixed at the proposed ratio before applying to the inner surface of the splint.

Patient groups and study protocol

In this study, 21 patients with a mean age 64 ± 8 years referred for sulcoplasty operations were included. Patients were divided into three groups and microbial activity of the formulations on the healing tissue and the splint was evaluated:

1. Chitosan film applied group: Chito+
2. Chitosan film incorporated with chlorhexidine applied group: Chito/Chx+
3. Visco-gel® applied group: Control

The films were applied only on one half of the inner side of the splints (Chito+ and Chito/Chx+) and no application on the other neighboring half (Chito- and Chito/Chx-) (Fig. 1). Then the splints were fixated to the operation site using posteriorly placed two mini titanium screws in order to avoid irritations that would occur because of the splint's mobility (Fig. 2).

Figure 1. Application of chitosan film onto one half of the inner side of the surgical

Figure 2. Fixation of surgical splint with two titanium mini screws placed posteriorly

Microbiological evaluations

Microbiological evaluations were carried out on the samples taken from the surface of the healing tissue and the surgical splints on postoperative days 1, 2, 5 and 10 by removing the surgical splint. The preoperative normal oral flora elements of 21 patients were also determined for comparison to the postoperative microbiological results. The samples were taken with a swab and transferred into 1 ml liquid medium. They were plated on Sabouraud's dextrose agar, Eo-

sine Methylene Blue and blood agar for; *Candida species (spp), Staphylococcus spp, Streptococcus spp, Diphteroid basilles, Escherichia coli, and Neisseria spp* using a loop, which can hold 0.01 ml in volume. Plates were incubated for 24 to 48 hours at 35°C and colonies were evaluated macroscopically by three observers. The results were given in colony forming unit (cfu). Statistical analyses of the variables were done using the t- test.

RESULTS AND DISCUSSION

There was a significant decrease in colonization of *Candida species (spp), Staphylococcus spp, Streptococcus spp, Difteroid basilies, Escherichia coli, Neisseria spp* on both the healing tissue and the surgical splint after application of the chitosan formulations (Chito+ and Chito/Chx+). The antimicrobial activity of chitosan films incorporated with chlorhexidine (Chito/Chx+) was found to be significantly higher than that of chitosan alone (Chito+) ($p<0.05$) (Fig.3a,c and Fig. 4a, c).

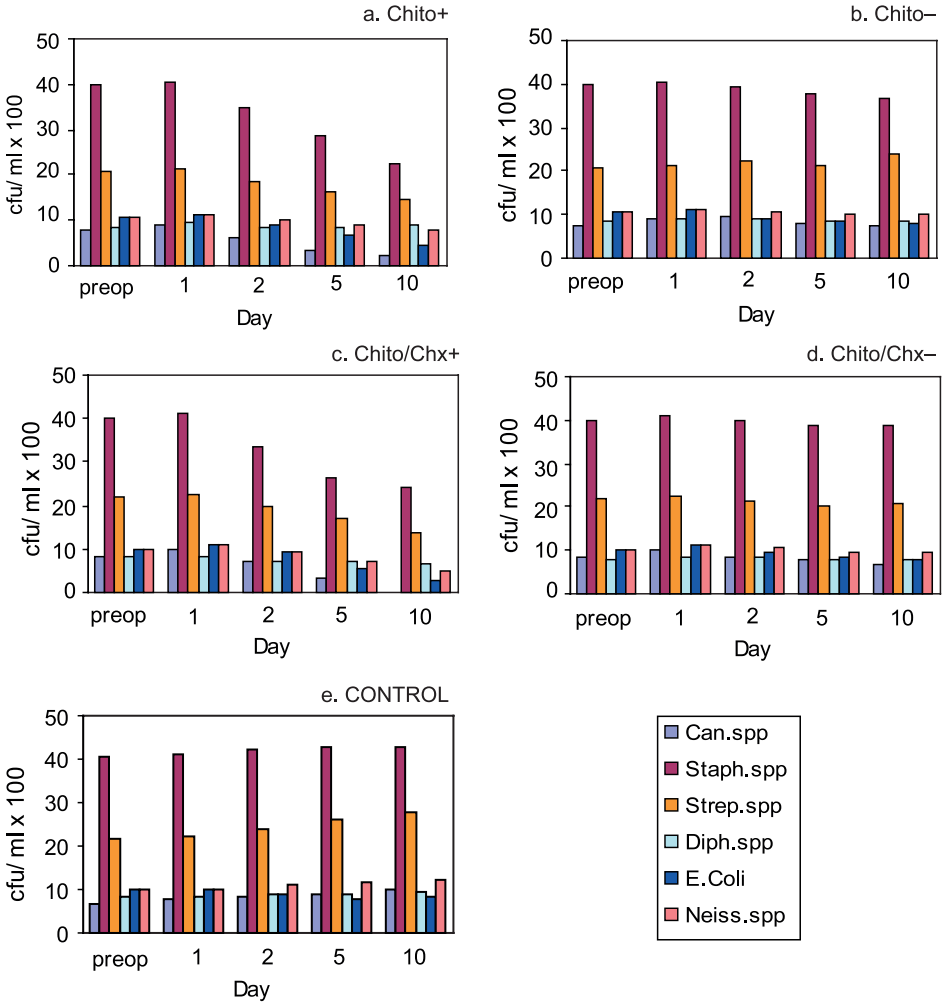

Figure 3. The microbial activity of chitosan films on the healing tissue

No significant change in colonization was observed in Chito- and Chito/Chx- groups over the study period ($p>0.05$) (Fig. 3b, d and Fig. 4b, d).

This can be explained by a possible penetration of chlorhexidine as well as dissolved chitosan from the formulation applied half to the neighboring half.

When compared to the formulation applied groups, significantly higher microbial growth was observed in the control group (only visco-gel applied) during the study period ($p<0.05$) (Fig. 3e and Fig. 4e).

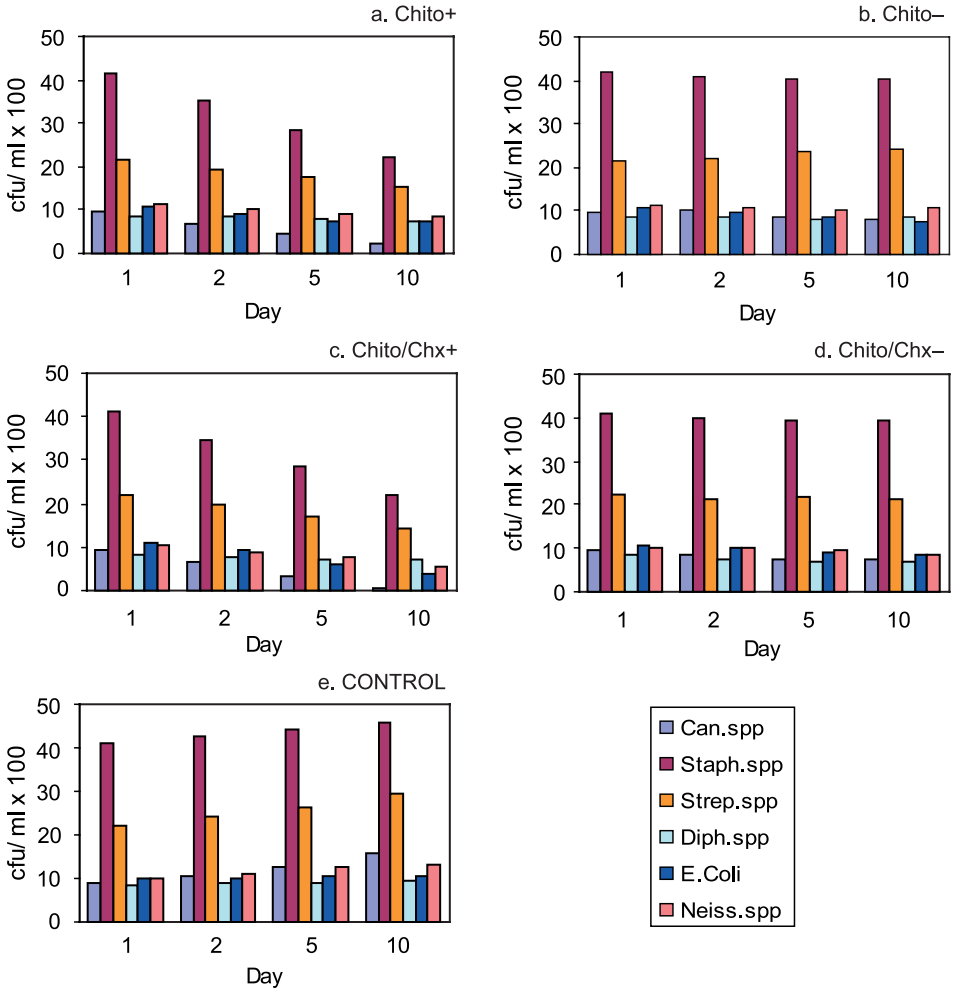

Figure 4. The microbial activity of chitosan films on the surgical splint

The process of re-epithelialization that leads to healing of oral mucosal wounds results from a carefully orchestrated proliferation and migration of epithelial cells. The wound surface should be kept just damp enough to obtain the benefits of accelerated healing, but there should be no bacterial invasion that may cause delayed wound healing. Such protection can be accomplished by covering

the wound with an agent during the healing period [8]. With its bioadhesive property and its biocompatibility, and its antimicrobial property, which is well demonstrated in this study, chitosan is an excellent candidate for tissue conditioning [9, 10]. It can be used in film form to protect ulcerated areas of the mucosa by moistening and coating it. The occlusion and lubrication of the bioadhesive film will reduce the discomfort of the lesion [3].

CONCLUSIONS

Our results suggest that the chitosan films can be used as an alternative effective tissue-conditioning agent as well as a delivery system for the antimicrobials in preprosthetic sulcoplasty operations for oral rehabilitation.

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ELICITOR ACTIVITY OF MF3-PROTEIN FROM *PSEUDOMONAS FLUORESCENS* AND COMBINATION OF MF3-PROTEIN WITH CHITOSAN IN DIFFERENT HOST - PATHOGEN PAIRS

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Screening of various microorganisms for the ability to induce defensive reactions in plants resulted in the finding that extracts from *Pseudomonas fluorescens* are capable to induce tobacco plant resistance to tobacco mosaic virus (TMV). Later it was found that the active substance of the bacterial extract was a low-molecular (16.929 kDa) thermostable protein named Microbial Factor 3 (MF3). After the identification of MF3-protein gene structure, it was shown that the protein had a high level of homology with peptidyl-prolyl *cis-trans* isomerases of FKBP-type. [5]

Before this work in all our experiments, we used carborundum for treatment of plant leaves with MF3-protein. Rubbing with carborundum damaged cell membranes of tobacco leaves and allowed the protein to penetrate into plant cells. Treatment of experimental plants with MF3-protein water solution by spraying did not induce resistance since the protein was not capable of penetrating to intact plant cells. So, searching for a carrier to provide MF3-protein transport into plant cells was the main purpose of our investigation.

Chitosan is well known to be able to enhance the transmembrane absorption of peptides. [2] Therefore, chitosans with different molecular weights (6; 10; 17 kDa) were used as a possible MF3-protein carrier in the next series of experiments.

Concentrations of MF3-protein and chitosans were selected so, that the separate use of these compounds did not influence the virus and *Septoria nodorum* development.

METHODS

Chitosan hydrolysis procedure

Chitosan produced from Kamchatsky crab shell chitin with a degree of deacetylation of 85% and molecular weight 700 kDa was a product of "Bioprogress" (Moscow region, Russia). For obtain low-molecular weight chitosan the industri-

al preparation Celloviridine G20x (Berdsk, Russia) is used. Enzyme preparation obtained from *Trichoderma reesei* included glucanase, xylanase and cellulase activities. Hydrolysis of chitosan was performed by the following method [1]. The samples of low-molecular weight chitosans with average viscosimetric weights (6, 10 and 17 kDa) were prepared.

***Nicotiana tabacum* - Tobacco Mosaic Virus**

Tobacco plants were grown to the sixth leaf stage (for about 3 weeks) in pots with soil in a climatic chamber at 60% relative humidity and temperature of 24°C during light and dark periods (12 h each). Undersides of five detached tobacco leaves were treated by spraying with MF3 and chitosan (6 kDa) preparations, in concentrations: 0.01 μg/ml + 0 mg/ml, 0 μg/ml + 0.1 mg/ml, 0.01 μg/ml + 0.1 mg/ml of MF3 and chitosan, respectively. Control leaves were treated with water. The next day, the face sides of treated leaves were inoculated with the TMV suspension by rubbing with carborundum. Treatment of one leaf side with preparations and another side with TMV helped avoid possible influence of MF3-protein and/or chitosans on TMV. The number of infective lesions on each leaf was counted on the 3-rd day. Disease development (%) was calculated as a ratio of number of lesions in test to their number in control.

Triticum aestivum – Septoria nodorum

Plants of wheat (cv. Mironovskaya 808) were grown up to the second leaf stage (about 13–15 days) in pots with soil in a climatic chamber. Ten detached leaves were put on Petri dishes with 1% agar contained 40mg/l of benzimidasol. The following mixtures of MF3-protein and chitosans were used in the experiment:

1. MF3 (1 mg/ml) + chitosan 6 kDa (0.1 mg/ml);
2. MF3 (1 mg/ml) + chitosan 10 kDa (0.1 mg/ml);
3. MF3 (1 mg/ml) + chitosan 17 kDa (0.1 mg/ml).

Foregoing combinations were dripped on upper halves of leaves, and water or suitable chitosan (0.01%) was dripped on lower halves of the same leaves. After 24 hours, all drops were removed and 10 μl of *S. nodorum* spore suspension ($1x10^6$ spores per ml) was dripped on treated and control sites. Dishes were placed in dark room. In 24 hours they were placed under 10kW xenon lamp (DKsT-10000), 8–10 Klux, and incubated at the temperatures of 20–22°C during 16 h light period. Symptoms were watched for next 7 days.(4)

RESULTS AND DISCUSSION

It was shown in our experiments, that development of phytopathogens such as TMV and *S. nodorum* was weaker if their host plants were pretreated with combination of MF3-protein and chitosan. So, the number of lesions developed after inoculation with TMV on tobacco leaves treated with the MF3-protein and chitosan mixture was around 71% less than on the control leaves. It is necessary to note, that direct interaction between components of the mixture and TMV was excluded, therefore treatment with combination of MF3-protein and chitosan was capable to induce tobacco leaf resistance. Similar results were obtained after

treatment of wheat leaves parts with the mixtures of MF3-protein and chitosans. The highest disease development on the treated parts was around 57% lower than on the control ones. Furthermore, it was shown that preparations on basis of MF3-protein and chitosans of different molecular weights protected plants from the disease equally effectively, while individual components of the mixture in the same concentrations did not induce plant resistance. Probably, low molecular weights chitosans and MF3-protein possessed synergistic action that enhanced their protective efficacy against plant pathogens.

Thus we showed that it was possible to induce nonspecific resistance of plants by using the MF3-protein and chitosans being environmental safety substances. Inasmuch as aforesaid substances in working concentrations influence on the development of the diseases through induction of defense mechanisms of plants, the probability of appearance of resistant mutants of pathogens become lower if these compounds will be applied in agricultural practice.

Present investigation showed that the use of combination of MF3-protein and chitosans for plant protection from pathogens was effective and significantly enlarged the sphere of application of each component. For example, there were carried out many trials of chitosans for the ability to protect tobacco plants against TMV but they were unsuccessful, while application of chitosans together with MF3-protein effectively induced the resistance of tobacco to TMV.

Table 1. Protective properties of MF3-protein mixture with chitosan against TMV on tobacco leaves

Variants of experience	Number of lesions on leaf ($X \pm ts/\sqrt{n}$)*	Development of disease, %
Water (control)	113 ± 27	100
MF3 (0.01mg/ml)	100 ± 46	88
Chitosan 6 kDa (0.1mg/ml)	108 ± 53	96
MF3 (0.01 mg/ml) + chitosan 6 kDa (0.1 mg/ml)	33 ± 22	29

* ($X \pm ts/\sqrt{n}$) – confidence interval limits were calculated with use t-distribution of Student. (3) significance level = 0,10 (or 90%)

X – mean value, t – t-distribution of Student, s – quadratic mean for sample, n – sample size

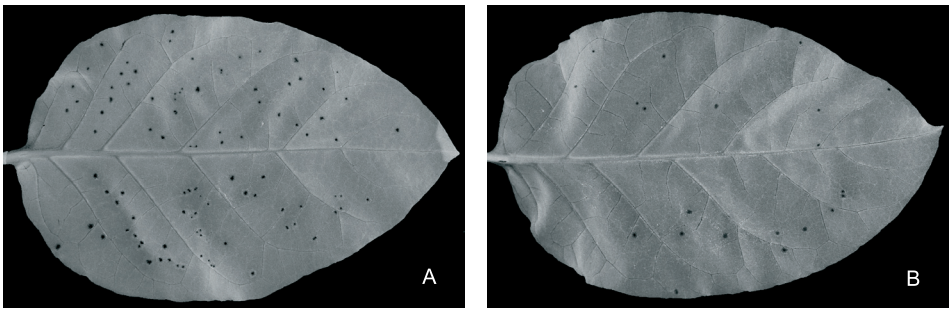

Figure 1. Development of TMV on leaves of tobacco pretreated with water (A) and pretreated with mixture of MF3-protein and chitosan (0.01 mg/l+0.1 mg/ml) (B)

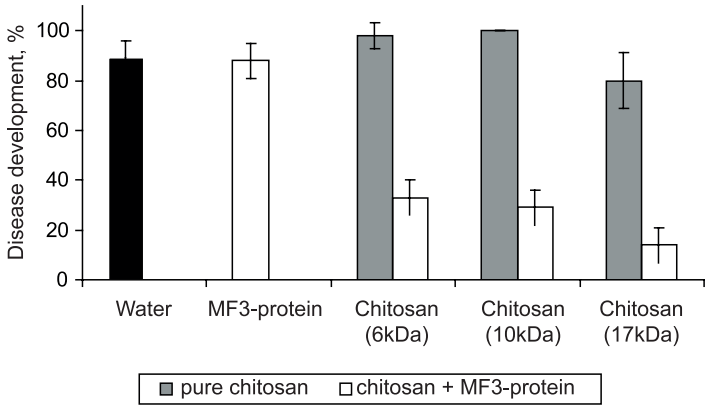

Figure 2. Protective properties of MF3-protein and chitosan against *Septoria nodorum* on wheat leaves

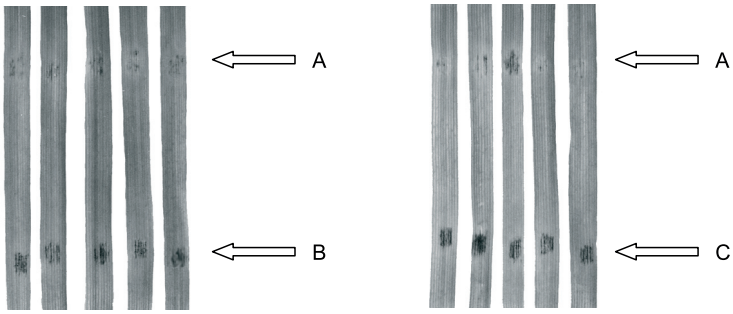

Figure 3. Development of *S. nodorum* on leaves of wheat treated with mixture MF3 (1mg/ml) and chitosan 17kDa (0.1 mg/ml) [A], water [B] and with chitosan 17 kDa (0.1mg/ml) [C]

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SOME PROPERTIES OF DIBUTYRYLCHITIN FIBRES

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INTRODUCTION

The principal objective of the research was the development of the spinning technology of dibutyrylchitin (DBC) fibres, multifilament yarns from DBC, followed by the elaboration of the production method of textile materials for wound dressings and implants as well as estimation of their biological properties.

The application of chitin is limited due to low solubility in most common organic solvents. Therefore, chemically modified chitin derivative – dibutyrylchitin characterized by high solubility seems to be a valuable polymer for fibres manufacture and their processing to textile medical products [1, 2]. DMF, methylpyrrolidone, EtOH and DMSO were used as solvents for preparing the DBC spinning solutions.

Spinning process for the production of DBC fibres in semi-technical scale was optimized at Institute of Chemical Fibres (IWCh), Poland. DBC fibres were manufactured in the process of wet spinning carried out on an apparatus commonly used for preparation of rayon fibres. Technologies for the formation of fleece from DBC fibres, manufacturing of flat non-woven DBC materials as well as manufacturing of DBC knitted goods have been elaborated at TUL, Poland.

MATERIALS AND METHODS

Following materials were used in the research work:

Two types of DBC produced and delivered by Institute of Dyes and Organic Products (IDOP) and Technical University of Lodz (TUL) with specification presented in Table 1.

Table 1. Some properties of polymer used for spinning of DBC fibres

Symbol of the sample	Intrinsic viscosity [η]	Moisture
	dL/g	%
IDOP/DBC4	1.75	1.60
TUL/DBC4	3.06	5.40

Preparation of DBC spinning solutions

Organic solvents: DMF, methylpyrrolidone, EtOH and DMSO were used as solvents of DBC for preparing spinning solutions.

Suitable amounts of IDOP/DBC were introduced into the reactor equipped with jacket and agitator (100 rpm) and next the solvent was added. The dissolving was continued for next 60 min. Spinning solution was deareated at temperature 20 ± 2°C for 24 h, then the solution was filtered on a plate filter and used for the manufacture of fibres.

Spinning of DBC fibres

The spinning process of DBC fibres in semi-technical scale was optimized at Institute of Chemical Fibres (IWCh), Poland. DBC fibres were manufactured in the process of wet spinning carried out on a spinning line commonly used for preparation of rayon fibres. Solutions of DBC in the organic solvents were used as a dope. The dope was extruded into a coagulation bath through a Pt-Au spinneret with 300 holes with a holes diameter of 80 micrometer. Filaments were washed and drawn in the next two bathes and collected on a roller with a rate of up to 30 m/min and dried. Within the optimization of DBC fibres spinning conditions, samples of DBC fibres were prepared in the form of staple fibres and multifilament yarns. Staple fibres were used for the production of non-wovens.

Manufacturing of DBC non-woven textile

Technologies of fleece formation from DBC fibres, manufacturing of flat non-wovens DBC materials as well as manufacturing of DBC knitted materials have been elaborated at Department of Textile Metrology, Technical University of Lodz, Poland.

Determination of intrinsic viscosity of DBC solutions.

Intrinsic viscosity of DBC solutions in DMAc was determined at 25°C using viscometry method (concentration of DBC in DMAc were in range of 0.4–0.1 g/100 ml) .

Determination of dynamic viscosity of DBC solutions

The dynamic viscosity of the DBC solutions was measured using Brookfield Dial Viscometer. The method consists in the measurement of the torque, necessary to overcome the resistance of viscouse fluid at 6, 12, 30, or 60 rpm using adequate spindle [3].

Determination of mechanical properties of DBC fibres.

Mechanical properties of obtained DBC fibres were determined, according to Stantards PN-ISO 1973:1997 and PN-EN ISO5079:1999 [4, 5].

Microscopic assessment of DBC fibres

The cross-section of DBC fibres was assessed using Scanning Electron Microscope JSM-35C, Joel Co. Japan.

RESULTS AND DISCUSSION

Within the research the following solvents were used for dissolution of DBC and preparation of spinning bathes: dimethyl sulfoxide (DMSO), ethyl alcohol (EtOH), dimethyl formamide (DMF), *N*-methyl-2-pyrrolidone (MP). The spinning solutions were characterised by assessment of polymer content and dynamic viscosity. Prepared DBC fibres were characterised by determination of their mechanical properties and morphological parameters, mainly by SEM. The properties of these solutions as well as mechanical parameters of obtained fibres are presented in Table 2. SEM photos of the cross – section of selected fibres are shown on Fig. 1–3.

Table 2. Spinning solutions of DBC and mechanical properties of DBC fibres

Symbol of fibres	Solvent	Spinning solutions		Fibres properties		
		Polymer content wt %	Dynamic viscosity/ temp.*,cP	Titre dtex	Tenacity cN/tex	Elongation at break %
IWCh/FDBC – 55/56	DMSO	12.11	8 000 (20°C)	3.50	10.5	22
IWCh/FDBC – 57/58**	DMSO	10.03	> 100 000 (24°C)	3.19	12.0	13
IWCh/FDBC – 6E	EtOH	13.2	16 300 (16.5°C)	2.03	7.29	10.0
IWCh/FDBC – 8E/1	EtOH	14.0	17 500 (18.5°C)	2.96	5.47	6.4
IWCh/FDBC – 2M/4	MP	15.57	40 000 (16.0°C)	2.74	10.1	13.2
IWCh/FDBC – 35/1	MP	14.22	18 250 (18.5°C)	2.80	11.9	14.0
IWCh/DBC – 30/3	DMF	18.30	29 000 (20°C)	2.08	15.8	7.5
IWCh/FDBC – 32/1	DMF	18.19	33 500 (20°C)	2.26	14.6	8.6

* dynamic viscosity measured at temp. of fibres spinning
** DBC from Technical University of Lodz (TUL), symbol TUL/DBC4

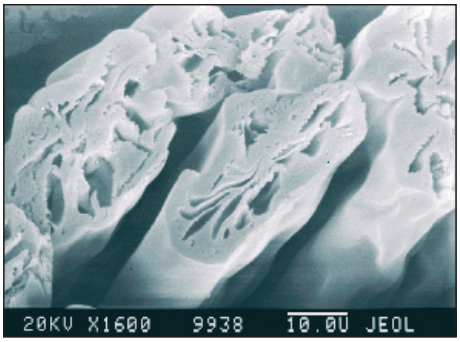

Figure 1. SEM photos of DBC fibres IWCh/FDBC - 6E (solvent-EtOH), cross-section

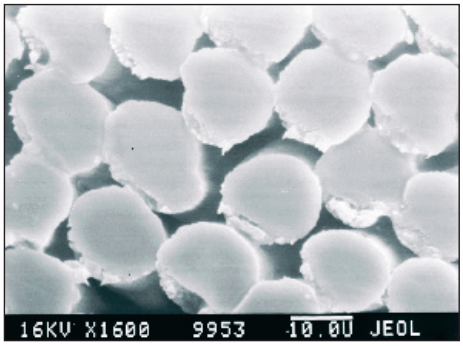

Figure 2. SEM photos of DBC fibres IWCh/FDBC-32/1 (solvent-DMF), cross-section

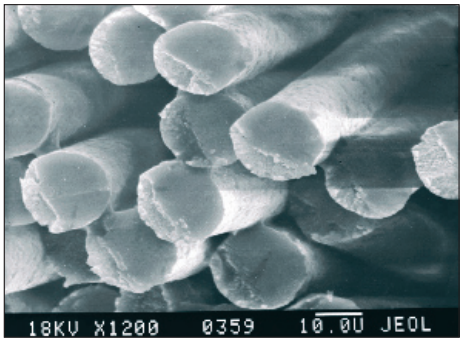

Figure 3. SEM photos of DBC fibres IWCh/FDBC-55/56 (solvent-DMSO), cross-section

Prepared samples of DBC fibres in form of staple fibres and multifilament yarns were transferred to Technical University of Lodz for the assessment of their processability into non-woven and knitted materials. Some selected properties of non-wovens manufactured from various DBC fibres are presented in Table 3.

Table 3. Characteristic of DBC non-wovens

Symbol of nonwoven	Symbol of fibres	Solvent type	Surface mass, m_p g/m^2	Thickness mm	Apparent density g/cm^3	Air permeability dm^3/m^{2}s
W1ak	IWCh/ DBC-8E/1	EtOH	40.4	0.28	0.144	159.5
W5	IWCh/ DBC-32/1	DMF	54.5	0.69	0.082	506.9
W8 chitin of France Chitin, No 4	IWCh/ DBC-57/58	DMSO	35.5	0.45	0.079	982.2
W4	IWCh/ DBC-35/1	MP	51.5	0.69	0.075	545.9

Type of preparation: Span/Tween

CONCLUSIONS

1. It is possible to spin DBC fibres using various solvents such as: DMF, DMAc, MP, DMSO, EtOH. Obtained fibres demonstrate different mechanical properties depending on type of solvent used and spinning conditions.
2. DBC solution in DMF was characterized by the highest polymer content with viscosity suitable for fibres forming, ability to dissolve at temperature 20 ± °C and good filterability. Fibres spinning process was stable at take-up speed 30 m/min.
3. The DBC fibres spun from DMF solutions have shown the best tenacity. It is possible to spin both staple and multifilament yarns from the DMF solutions.
4. Within the optimization of DBC fibres spinning conditions, samples of DBC fibres in the form of staple fibres and multifilament yarns for the production of knitted materials: bandages, dressing gauze, nets for supporting and/or isolating internal organs under surgery were prepared and transferred to Department of Textile Metrology, Technical University of Lodz, Poland.
5. The best results in the production of the webs were obtained with fibres made form DBC solution in DMSO. The increase of DBC intrinsic viscosity results in obtaining fibres better suited to web formation, compared to fibres with lower values of viscosity.

ACKNOWLEDGMENTS

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CHITOSAN APPLICATION FOR THE MANUFACTURE OF CELLULOSE/CHITOSAN THREE DIMENSIONAL OBJECTS

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INTRODUCTION

Objective of the research was to elaborate an advanced ecological technology for the manufacture of three dimensional (3D) cellulose-chitosan objects such as sponges, wound healing dressings, filtering material. Investigations included modification of the properties of cellulose sponges to enhance their biological properties like antibacterial and antifungal and to improve water imbibition and to confer filtration capacity by introducing chitosan in adequate forms to the structure of cellulosic products obtained according to NMMO technique or by surface modification with proper chitosan preparations.

Chitosan, a natural polymer featured by controlled bioactivity, biodegradability, biocompatibility and ability to chelate metal ions is a valuable material for the modification of cellulose in the lyocell process. Ability to control its structure by modification process resulting in assumed bioactivity, including antibacterial and antifungal action, as well as its miscibility with NMMO/cellulose blends and high adhesion to the surface of different products allows to use this polymer to produce high value blended materials for applications, among other in household, sanitary, cosmetic, medicine and waste water treatment.

EXPERIMENTAL

Materials

Microcrystalline chitosan (MCCh), IWCh, Poland
Modified chitosan lactate salt, IWCh, Poland
Chitosan fibres, IWCh, Poland
Aqueous NMMO conc. 49.4% , Lenzing, Austria
Cellulose pulp and sponges of cellulose/PES (70% /30%), SPONTEX, France
Some properties of materials used are presented in Tables 1 and 2.

Table 1. Some properties of selected chitosan forms

Chitosan preparation	$\overline{M}_v$ kD	DD %	Polymer content %	WRV %	GPC results		
					$\overline{M}_n$ kD	$\overline{M}_w$ kD	Pd
MCCh	70.0	82.2	3.30	1270	14.6	49.8	3.40
Modified chitosan lactate	109.0	84.3	1.91	–	17.7	61.8	3.49

Table 2. Some properties of chitosan fibres

Chitosan fibres	$\overline{M}_v$ kD	DD %	Polymer content %	WRV %	Mechanical properties		
					Titre, dtex	Tenacity, cN/tex	Elongation at break, %
	296.0	82.2	100	680	2.64	10.9	26.3

Methods

The average molecular weight $\overline{M}_v$ of chitosan was determined viscometrically by measuring the intrinsic viscosity (Standard Procedure GLP SPR/BB/5). The distribution of molecular weight and polydispersity of chitosan was estimated by gel permeation chromatography (GPC) using the module chromatographer HP 1050 Hewlett-Packard (Standard Procedure GLP SPR/BLF/10). The deacetylation degree of chitosan was determined by potentiometric titration (Standard Procedure SPR/BLF/5). Water Retention Value of chitosan preparations was determined according to Standard Procedure GLP SPR/BB/16. The content of polymer in chitosan preparations was determined according to Standard Procedure GLP SPR/BB/11–13. The surface and cross section of cellulose sponges impregnated with chitosan preparations and cellulose/chitosan blends were assessed using the scanning electron microscope JSM-35C, Joel Co. Japan (Standard Procedure GLP SPR/BB/22). The mechanical properties of chitosan fibres were estimated according to standard PN-EN-ISO-527-1:1998. Biomedical tests (in vitro and in vivo) of modified cellulose sponges were carried out on the Department of Experimental Surgery and Studies of Medical Academy in Wroclaw using the standards PN-EN ISO 10993-5 and PN-EN ISO 10993-10

RESULTS AND DISCUSSION

Surface modification of cellulose sponges with selected chitosan forms

In the research sponges made of cellulose/PES were used with different surface weight in the range of 280–420 g/m^2. The coating with MCCh and chitosan lactate salt was performed using Foulard machine made by E. Benz and the drying using an air-circulation dryer type KBC-125W. For the coating a padding bath was applied, containing 1.5wt% chitosan. The cellulosic sponges were immersed in the bath for 10 minutes. Excess of the bath was next removed in the squeezer of the Foulard machine at a roller pressure of 2 kG/cm and a speed of 1 m/min. The coated sponge sheets were dried at 40°C for 4–6 hours depending upon the used chitosan form. Selected SEM surface and cross-section pictures are presented in Fig. 1.

Blending of cellulose / chitosan forms based on NMMO process

The chitosan preparations as chitosan lactate salts, MCCh and staple fibres (6 mm staple length) were mixed with aqueous NMMO pre-concentrated to 70wt% applying a high speed mixer Ultra-Turrax T50 Basic (Ika-Werke). The process was performed using 500 g as standard amount of 70wt% NMMO. The amount of chitosan preparations used for preparing the blends was within 1–6 g limits (in the limits of 5–30wt% on polysaccharides) at chitosan concentration of 1–2 wt%. After 10–25 min. of mixing a sample was taken for microscopic inspection. For selected charges the pH of the blends was measured. Then the NMMO process to dissolve cellulose in such blends was carried out in a special vacuum autoclave, 5 dcm^3 volume with heating jacket. Next the liquid cellulose/chitosan/NMMO blends were poured on teflon plates, regenerated in water and dried at 105°C under thrust. The selected SEM photos of surface and cross-section are presented in Fig. 2.

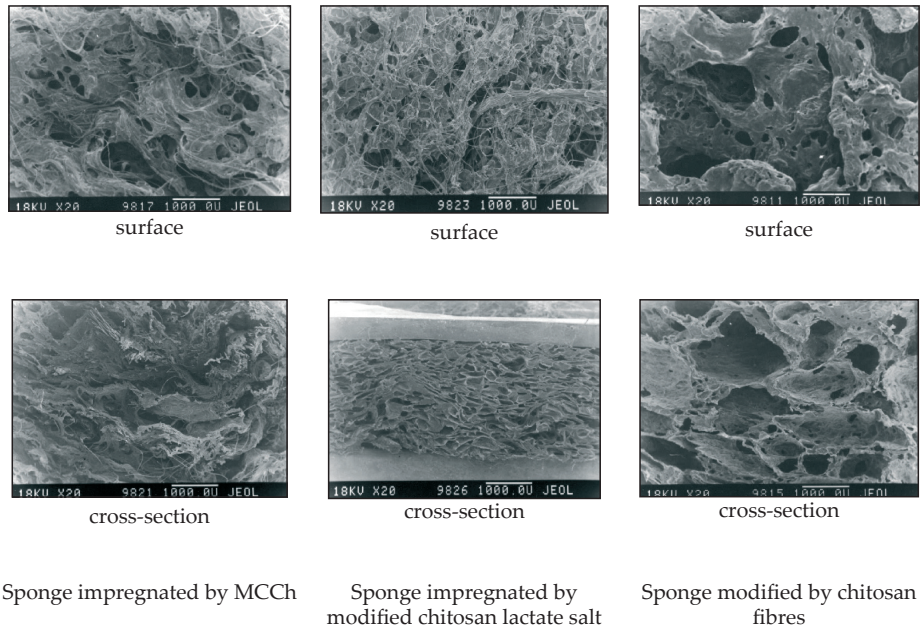
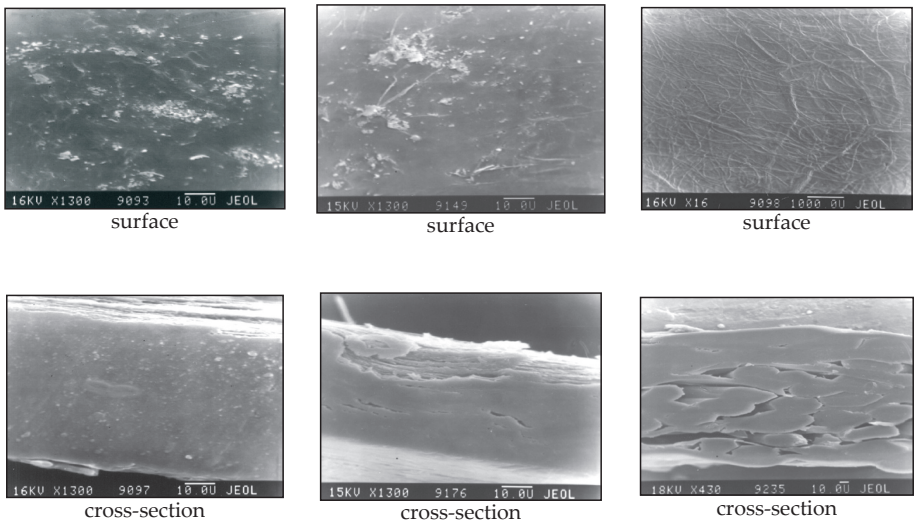


Figure 1. Scanning electron microscopy photographs of modified cellulose sponges



Cellulose/modified chitosan lactate (30 wt%) blend | Cellulose/MCCh (30 wt%) blend | Cellulose/chitosan fibres (30 wt%) blend

Figure 2. Scanning electron microscopy photographs of cellulose/chitosan blends

Biodegradation tests of chitosan/cellulose sponges

Sponges modified with various chitosan forms were subjected to degradation in a soil medium. A garden silica undersoil named Perlit was added to the soil to assure air-permeability and to improve water equilibrium. The soil RH was kept at 65wt% and temperature at 23°C. The selected examples of results are presented in Fig. 3.

a) initial | b) after 8 weeks | c) after 12 weeks

Figure 3. Biodegradation test in soil of cellulose sponge impregnated by modified chitosan lactate

The biomedical tests of modified cellulose sponges

The cellulose/chitosan fibres sponges were used for both medical tests and an additional cellulose sponge modified with MCCh for in vitro testing. Cellulose

sponge without chitosan was a control. The selected examples of results are presented in Fig. 4, 5 and 6.

Figure 4. Photo of the wounds coated with:

a) cellulose/chitosan fibres sponge dressing
b) control gauze pad

Figure 5. Microscopic picture 14 days after operation. Image of the wound after removal of the cellulose/chitosan fibres sponge dressing

Figure 6. Microscopic picture 14 days after operation. Image of the wound after removal of the gauze pad

CONCLUSIONS

1. Chitosan forms were manufactured and selected for modification of cellulose sponges surface and for preparing stable cellulose/NMMO/chitosan blends.
2. A method for the impregnation of cellulose sponge surface with selected chitosan forms was elaborated.
3. A method to blend the selected chitosan forms with cellulose/NMMO process was elaborated.
4. Modified sponges demonstrated susceptibility to biodegradation in soil after 12 weeks of testing.
5. Medical tests proved that cellulose sponges modified with chitosan:
   – do not cause cytotoxicity effects
   – stimulate the division of mouse's fibroblasts
   – do not cause intradermal irritating action
   – advantageously affect the wound healing process: accelerate the wound healing process
   – cause faster regeneration of injured animal skin

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NOVEL CHITOSAN-ALGINATE COMPOSITE FIBRES FOR MEDICAL APPLICATIONS

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INTRODUCTION

Only few companies in the world produce chitosan and alginate fibres for medical applications as healing dressings [1, 2]. The aim of research was to elaborate novel chitosan-alginate fibres useful as effective biological dressing materials. New bi-component chitosan-alginate fibres were elaborated characterized by the skin-core structure:

skin-sodium alginate and/or calcium alginate, core-chitosan

The obtained composite chitosan-alginate fibres combine advantageous properties of the two biodegradable natural polymers yielding unique wound dressing materials.

Spinning solutions were prepared for a series of spinning trials to obtain the chitosan-alginate fibres. The impact was studied of the polymer composition on the fibre mechanical properties.

MATERIALS AND METHODS

The following polymers were used:

- Chitosan characterized by $\overline{M}_v$ 285 kD and DD 81.3%, (Primex, Norway)
- Sodium alginates characterized by high amount of guluronic acid-Protanal LF 5/60 and Protanal LF 10/60, (FMC Biopolymer, Norway)

Chitosan spinning dope

was prepared by dissolving the polymer in 3 wt% of aqueous acetic acid at 20°C. Chitosan spinning solutions stored at temp. ~5°C has shown good stability (dynamical viscosity at 20°C of the solution 17 000 cP, $K^*_w = 85$).

Alginate solution

with concentration of 6 wt% was prepared by dissolution of sodium alginate (Protanal LF 5/60 or Protanal LF 10/60) in demineralized water.

The chitosan-alginate composite fibres formation

was carried out with the use of an experimental wet spinning machine. To obtain the bi-component fibres with skin-core structure two basic methods were applied:

a) **One-step spinning procedure**
Chitosan spinning dope was extruded under pressure through a spinneret into a coagulation bath containing sodium alginate.
b) **Two-step spinning procedure [3]**
In the first stage one-component chitosan fibres were spun, then these fibres were passed through a water bath and treated with sodium alginate solution. The fibres were then squeezed between rollers to remove excess liquid, and dried. Finally, the fibres were conditioned 24 h at 20 °C temperature.

Determination of Water Retention Value (WRV) of composite fibres

The WRV of fibres was measured gravimetrically by submerging the fibres sample in distilled water for 20 hours, next centrifuging for 10 min at 4000 rpm. The weight of the sample was determined after centrifuging and after drying to constant weight [4].

Determination of mechanical properties of chitosan/alginate fibres

Mechanical properties of obtained fibres were tested according to ISO Standards: PN-ISO 1973:1997 and PN-EN ISO5079:1999 in Metrological Laboratory IWCh having the Certificate of Analysis PCA AB 388 [5,6].

Microscopic assessment of chitosan/alginate fibres

The cross-section of chitosan/alginate fibres was assessed using Scanning Electron Microscope JSM-35C, Joel Co. Japan.

RESULTS AND DISCUSSION

The process of chitosan-alginate composite fibres forming was carried out using two methods: one-step and two-step procedure. Both methods allowed to obtain bi-component chitosan-alginate fibres characterized by skin-core structure, where the skin is sodium alginate and the core constitutes chitosan. Some chitosan-sodium alginate fibres were additionally treated with aqueous calcium chloride solution, then skin as a calcium alginate was created.

Within the optimization studies the impact of composition of the fibres on their mechanical properties was determined. Some mechanical properties of bi-component fibres are presented in Tables 1–2.

SEM photos of cross-section of selected fibres are shown in Fig. 1–2.

It was found that, in the one-step method, bi-component fibres can be obtained with calcium alginate content up to 2.5% in the skin.

The two-step method is more advantageous for two reasons:

– possibility of introducing a higher amount of alginate to composite fibres up to 28 wt%
– decrease of fibres tenacity in comparison to control sample is insignificant

Table 1. Some properties of the chitosan/calcium alginate fibres (one-step spinning procedure)

Symbol of fibres	Chitosan content in composite fibres	Titre	Tenacity at conditioned state	Elongation at break at conditioned state	WRV
	wt%	dtex	cN/tex	%	%
CH-8/0	100.0	3.49	10.5	21.0	194
CH-8/1	98.9	4.01	7.9	25.0	185
CH-8/2	97.5	5.24	6.9	26.5	172

Table 2. Some properties of the chitosan/sodium alginate fibres (two-step spinning procedure)

Symbol of fibres	Chitosan content in composite fibres	Titre	Tenacity at conditioned state	Elongation at break at conditioned state	WRV
	wt%	dtex	cN/tex	%	%
CH-13/0	100.0	3.25	10.8	21.0	182
CH-13/3	86.3	3.62	11.0	22.5	202
CH-13/4*)	85.1	3.64	10.7	23.0	178
CH-1/8	75.4	4.70	9.4	31.0	270
CH-2/5	71.7	3.89	9.6	28.0	193

* – skin: calcium alginate

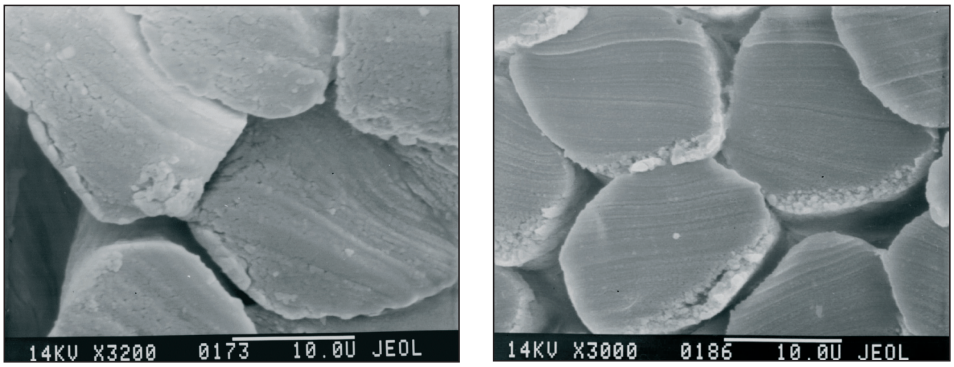

Figure 1. SEM photos of chitosan/alginate fibres. CH-13/3 (skin-sodium alginate)

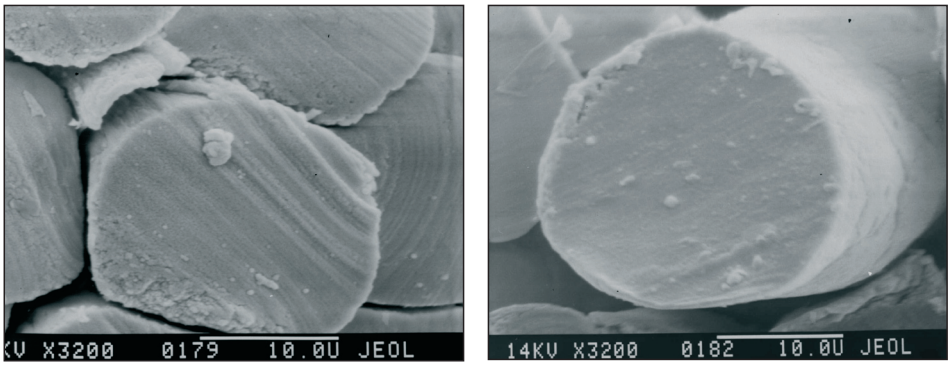

Figure 2. SEM photos of chitosan/alginate fibres. CH-13/4 (skin-calcium alginate)

CONCLUSIONS

1. The method of one-step composite fibres manufacture allowed to obtain chitosan/alginate fibres with 2.5 wt% of sodium alginate and/or calcium alginate in the skin.
2. The two-stage method of composite fibres forming appeared to be more effective and the alginate content in chitosan/alginate fibres was up to 28 wt%. No change of strength was observed and the fibres were characterized by pleasant soft and smooth touch.

ACKNOWLEDGMENTS

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INFLUENCE OF CHITOSAN ON NEMATOPHAGOUS FUNGI AND ROOT-KNOT NEMATODES (*MELOIDOGYNE* SPP.)

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Key words: chitosan, nematophagous fungi, root-knot nematodes

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are widely distributed throughout the world. In Poland they are the most economically important quarantine pest of various vegetable crops in glasshouses and cause significant yield losses [1]. The use of chemical nematicides in control on these nematodes is expensive and dangerous for environment. Hence, there is much interest in alternative methods of control. Biological control methods for the management of root-knot nematodes in vegetable crops are the most attractive from the environmental standpoint. The nematophagous fungi have been investigated extensively and shown to be promising agents for the biological control of plant parasitic nematodes [2, 3 ,4, 5]. Chitin derivatives were reported to have various biological activities such as induction of plant resistance to pathogens, stimulation of plant growth, antimicrobial activity, microbial antinematodes activity and so on. There is no data about influence of chitosan with nematophagous fungi on nematode population. This paper focuses on the influence of chitosan of nemathophagus fungi and root-knot nematodes with respects to joint use of chitosan in the control of nematodes.

The objectives of the research reported here were:

1. determination of the influence of chitosan on nematophagous fungi,
2. determination of the influence of chitosan on *Meloidogyne hapla* population,
3. determination of the the efficacy of combinations of different isolates of *Pochonia chlamydosporia*, *Paecilomyces lilacinus* and chitosan for control of *Meloidogyne arenaria* and *M. hapla* on tomato plants under growth chamber conditions.

MATERIALS AND METHODS

The Polish isolates of Vc-1 *Pochonia chlamydosporia* (Goddard) Zare et Gams (10^3 chlamydospores per g of soil) and Pl *Paecilomyces lilacinus* (10^7 conidia per g of soil) were collected from eggs of cyst beet nematode (*Heterodera schachtii*

Schmidt). Solution of chitosan (pH 5.5) was prepared in acetic acid (MW-150kDa; DD-85%).

Laboratory experiments

Agar plugs (7 mm diam.) with different fungi were placed in the middle of 9 cm diam. Petri dishes containing medium with chitosan in different concentrations: 0.1%; 0.5% and 1%. SNA medium was control treatment. Daily fungal colony growth was measured and number of spores/1 cm^2 of the colony was estimated using haemocytometer.

Experiments in controlled chamber conditions

Chlamydospores and spores were produced on a solid medium and extracted using standard techniques. Suspension from the medium was mixed with non-sterile soil. Five replicate pots (15-cm diameter) were used for each fungal isolate. One-week-old seedlings of the tomato cultivar Slonka F1 were transplanted into the inoculated soil. Next week 1700-second stage juveniles of *M. arenaria* or *M. hapla* were pipetted in aqueous suspension into 5-cm deep holes made in the soil around the plants. Chitosan was used separately by wattering of plants (50 ml per pot), and in combination with fungi. The plants were maintained in the chamber with artificial light at 25^0C for 7 weeks.

There were following treatments: Treatment 1: a) Vc-1+*M. hapla*, b) Pl+*M. hapla,* c) Chitosan (0.5%)+*M. hapla,* d) Control (*M. hapla* only); Treatment 2: a) Vc-1+*M.arenaria*, b) Chitosan 1%+*M. arenaria*, c) Chitosan 1%+Vc-1+*M. arenaria,* d) Chitosan 0.5%+*M. arenaria,* e) Chitosan 0.5%+Vc-1+*M. arenaria,* f) Control (*M. arenaria* only), g) Control (without fungi, chitosan and nematode).

Nematode egg masses were picked from 1 g of samples of roots and counted. Samples of 10 egg masses were taken at random from those picked from roots and treated with 5% sodium chlorite for 5 min to dissolve the gelatinous matrices and release the eggs so that the numbers of eggs per egg mass could be counted using standard techniques [6].

RESULTS AND DISCUSSION

Chitosan influenced daily growth of *P. chlamydosporia* and *P. lilacinus*. The increase of chitosan concentration from 0.1% to 1% led to significant decrease of fungi growth. The growth rate of *P. chlamydosporia* at 0.1% chitosan concentration increased from 0.5 mm to 2.5 mm daily, at 0.5% chitosan concentration the growth rate increased from 0.8 mm to 2.5 mm daily while at 1% chitosan concentration fungus colony growth increased from 0.2 mm to 1.8 mm daily only (Fig. 1). On the control medium (SNA) the growth of this fungus was the greatest and reached 4.3 mm daily. The same tendency showed *P. lilacinus*. The growth rate of this fungus at 0.1% chitosan concentration increased from 0.3 mm to 4.2 mm daily, at 0.5% chitosan concentration fungus colony growth increased from 0.3 mm to 4 mm daily and at 1% chitosan concentration was the smallest and reached 2.5 mm daily only (Fig. 2). On the control medium (SNA) daily growth of *P. lilacinus* was the greatest and reached maximum 7 mm.

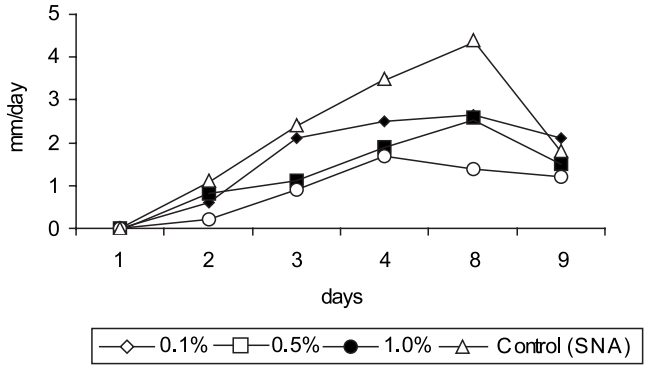

Figure 1. Daily growth of *P. chlamydosporia* on midium with different chitosan concentrations

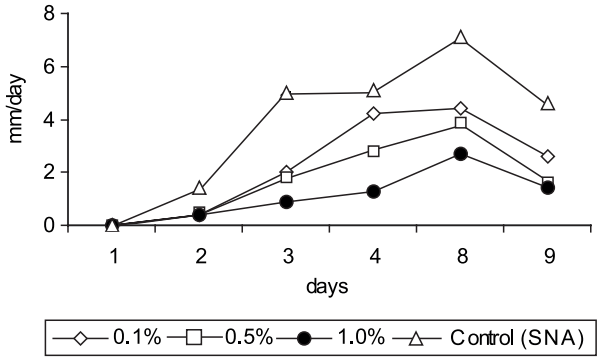

Figure 2. Daily growth of *P. lilacinus* on midium with different chitosan concentrations

The increase of chitosan concentration from 0.1% to 1% led to increase of nematophagous fungi sporulation. The greatest number of *P. chlamydosporia* and *P. lilacinus* spores was observed on 1% chitosan concentration (Fig. 3). We observed 420 x 10^5 *P. lilacinus* spores on 1% chitosan concentration, and 180 x 10^5 spores of *P. chlamydosporia* per 1 cm^2 of fungi colony. At the 0.1% and 0.5% chitosan concentration we observed the smallest number of fungi spores.

The effect of chitosan on fungi sporulation could be useful in practice, because more fungi spore concentration in soil conditions should give better nematode control, but negative influence of chitosan on daily fungi growth can be impractical and delay fungi development.

Experiments in controlled chamber conditions showed that chitosan at 0.5% concentration reduced *M. hapla* population as the same as nematophagous fungi. The greatest control of *M. hapla* eggs was achieved when chitosan was added (1040 eggs per egg mass), but the greatest reduction of egg masses per g of tomato root was achieved when *P. lilacinus* was added (Fig. 4). There is no data in literature about joint use of chitosan with nematophagous fungi. Our experiment showed that the joint use of chitosan with *P. chlamydosporia* was effective against *M. arenaria* population. The smallest number of eggs per g of tomato root was achieved when chitosan at 0.5% and 1% concentration was applied to the soil together with fungus, but it was not significant differences between these treat-

ments and *P. chlamydosporia* Vc-1 treatment (Fig. 5). Synergetic effect was not observed. Chitosan in 1% and 0.5% concentration used separately was not effective against *M. arenaria* population. In Russia Guskova at all. [7] observed greatest *M. incognita* control on cucumber plant when chitosan in 0.25–1% concentration was applied. Different species of nematodes can influence efficacy of chitosan as well as plant species. In our experiment tomato plants were used while in Russia cucumber plants and different species of root-knot nematode. Maybe this is reason that we can not compare results of these different experiments. The greatest number of healthy J1J2 *M. arenaria* was observed when chitosan in concentration of 0.5% was used, but combination of chitosan 0.5% with Vc-1 fungus significantly decreased J1J2 population. The number of healthy *M. arenaria* eggs per g of tomato root also significantly decreased when chitosan (0.5%) in combination with Vc-1 fungus was used.

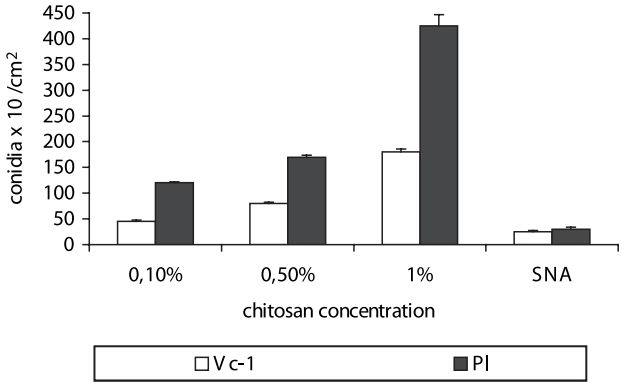

Figure 3. Influence of chitosan on *P. chlamydosporia* and *P. lilacinus* sporulation

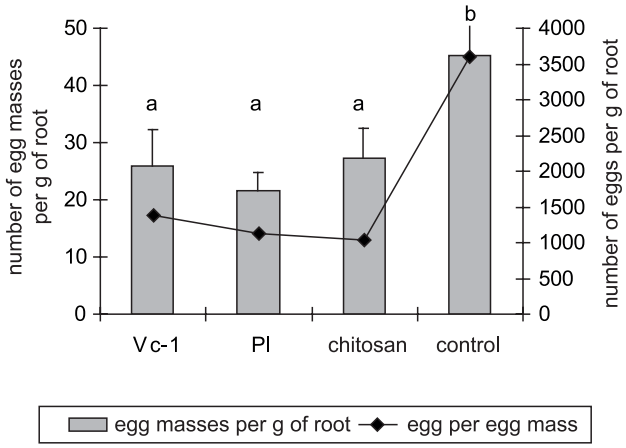

Figure 4. Effect of fungi and chitosan (0.5%) on number of egg masses and eggs per egg mass of *M. hapla* on tomato roots

The examined nematophagous fungi mixed with chitosan (0.5%) should be considered as potential candidates for implementation into programs of environmentally safe biological and integrated control of root-knot nematodes in glasshouse conditions. However, futher studies are needed.

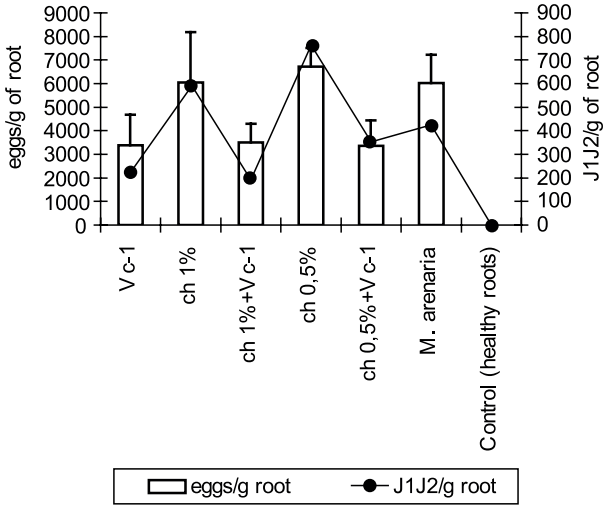

Figure 5. Effect of fungi and chitosan on numbers of *M. arenaria* J1J2 stages and eggs on tomato root

CONCLUSIONS

1. Chitosan influenced growth and sporulation of *Pochonia chlamydoaporia* and *Paecilomyces lilacinus*. The increase of chitosan concentration from 0.1% to 1% led to significant decrease of fungi growth on the medium, while the number of spores increased.
2. Chitosan in 0.5% concentration reduced *M. hapla* population as the same as nematophagous fungi.
3. Chitosan in 1% and 0.5% concentration was not effective against *M. arenaria* population. The joint use of chitosan with *P. chlamydosporia* was effective against *M. arenaria* population.

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BIOCHIKOL 020 PC (CHITOSAN) IN THE CONTROL OF SOME ORNAMENTAL FOLIAGE DISEASES

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INTRODUCTION

The occurrence of ornamental foliage diseases causes a decrease in the quality of plants and in some cases even a complete loss of yield. The most frequently occurring diseases on plant foliage are powdery mildew, downy mildew, rust and leaf spot [2, 4]. Fungicides used to spray plants are still the main method of disease control [3]. Chemical control of plant diseases carries a risk of pathogens developing resistance to chemicals and decreasing their effectiveness. The use of new compounds (to protect plants against pathogens) which work differently in comparison with the currently used fungicides could reduce the possibility of pathogens developing resistance to the compounds used so far or provide an opportunity to fight the resistant isolates of pathogens. This kind of possibility is offered by the new product chitosan (as Biochikol 020 PC). Chitosan is not only a potential elicitor of plant defence responses, but also an active inhibitor of fungal growth [11]. In plant tissues, chitosan induces a multitude of biological processes (stimulation of chitinases, accumulation of phytoalexins, synthesis of proteinase inhibitors, and increased lignification) [1]. In plant protection, chitosan (Biochikol 020 PC) has given good results in the control of viral [9], bacterial [10] and fungal diseases [5, 12, 14]. Also, in past experiments, chitosan gave good results in the control of foliage diseases [13] and some soil borne pathogens [6, 7, 8].

The aim of the experiments was to evaluate the effectiveness of Biochikol 020 PC used as a plant spray against some ornamental foliage diseases (*Sphaerotheca pannosa* var. *rosae*, *Peronospora sparsa* and *Diplocarpon rosae* on rose, *Erysiphe polyphaga* on gerbera, *Puccinia pelargonii-zonalis* on pelargonium, *Melampsora epitea* on willow and *Colletotrichum violae-tricoloris* on pansy).

MATERIAL AND METHODS

The following compound was used in the experiments

Biochicol 020 PC (2% microcrystalline chitosan, obtained by the Institute of Chemical Fibers, Łódź, and produced by Gumitex Poli-Farm Sp. z o.o., Łowicz, Poland).

Control of *Sphaerotheca pannosa* var. *rosae*

The experiment was carried out on rose bushes cv. "Madelon" cultivated in beds in a polytunnel. The bushes were sprayed with Biochikol 020 PC 4 times at weekly intervals using concentrations from 0.5% to 2.5% (Tab. 1). Spray treatments started as soon as the first disease symptoms had been noted. The effectiveness of the tested solutions against the pathogen was evaluated after 2 and 4 weeks of the experiment. Saprol 190 EC (190 g triforine per dm^3) at 0.15% was used as the standard fungicide.

Control of *Erysiphe polyphaga*

The experiment was carried out on gerbera grown on benches in a greenhouse. After the disease symptoms had appeared, the plants were sprayed with Biochikol 020 PC at 0.5 to 2.0% 4 times at 7-day intervals, making sure that both sides of leaves are thoroughly covered (Tab. 2). The effectiveness of the tested solutions against *E. polyphaga* was evaluated after 2 and 4 weeks of the experiment using a 6-point scale (see *S. pannosa* var. *rosae*). Nimrod 250 EC (250 g bupirimate per dm^3) at 0.15% was used as the standard fungicide.

Control of *Peronospora sparsa*

The experiment was carried out on rose bushes cv. "Madelon" cultivated in beds in a polytunnel. Biochikol 020 PC was applied as a curative treatment (Tab. 3). The degree of the infection was assessed before and after 2 and 4 weeks of the experiment. Sandofan Manco 64 WP (8% oxadixyl + 56% mancozeb) at 0.2% was used as the standard fungicide.

Control of *Puccinia pelargonii-zonalis*

The experiment was carried out on pelargonium cv. Pulsar F1 Salmon grown in pots on benches in a greenhouse. After the first disease symptoms, plants were sprayed 4 times at weekly intervals. After 4 weeks from the beginning of the treatment, the number of uredinia per leaf and the height of plants were determined. After 2 weeks from the end of the experiment, the percentage of uredinia that had germinated on a potato dextrose agar was calculated (Tab. 4). Score 250 EC (250g difenoconazole per dm^3) at 0.05% was used as the standard fungicide.

Control of *Melampsora epitea*

The experiment in controlling rust was carried out on willow cv. "Iwa" grown in open field conditions. After some time, when the first symptoms of the disease (uredinia on leaves) had been noted, the plants were sprayed with Biochikol 020 PC at a concentration of 2.5%. After 2 treatments at 7-day intervals, the number of uredinia per leaf, the percentage of diseased leaves and the percentage of destroyed uredinia were determined (Tab. 5). Folicur BT 225 EC (100g triadimefon + 125g tebuconazole per dm^3) at 0.1% was used as the standard fungicide.

Control of *Diplocarpon rosae*

The experiment in controlling black spot was carried out on roses cv. "Red Berolina" grown in an open field. After the first symptoms of the disease, the rose bushes were sprayed with Biochikol 020 PC 10 times at 7-day intervals (Tab. 6).

The effectiveness of the tested solutions was evaluated after 3, 6 and 9 weeks of the experiment. Saprol 190 EC at 0.15% was used as the standard fungicide.

Control of *Colletotrichum violae-tricoloris*

The experiment in controlling leaf spot was carried out on pansy cv. "Delta F1 Yellow with Blotch". After the appearance of visible disease symptoms, plants were sprayed with Biochikol 020 PC or Folicur BT 225 EC 4 times at 7-day intervals (Tab. 7). The effectiveness of the tested solutions was evaluated before and after 2 and 4 weeks of the experiment.

In the case of gerbera and pelargonium, the experiment was carried out on plants grown in pots filled with peat + composted pine bark and sand at a ratio of 1 : 1 : 0.5 and pH 6.5 with a supplement of 2 g/dm^3 of the fertilizer Azofoska. The pots were placed on greenhouse benches cushioned with fibre mats. During the experiment, the air temperature ranged from 23 to 26°C and the relative humidity was kept above 90%. Water was applied directly to the pots or onto the fibre mats.

About 1 dm^3 of fungicide suspension per 10 m^2 was used in all the experiments. Citowett AL (100% alkylarylopolyglycol ether) was added to the spray mixtures at a concentration of 0.02%.

The experiments were set up in a randomised block design with 4 replications, where each replication included 5 plants or shoots (willow).

RESULTS AND DISCUSSION

Control of *Sphaerotheca pannosa* var. *rosae*

After 2 weeks of the experiment, Biochikol 020 PC at concentrations of 0.5 to 2.5%, used curatively after the appearance of abundant powdery mildew symptoms, had an effectiveness of about 39 to 63% (Tab. 1). Two weeks later the effectiveness of the compound was 56 to 64% depending on the concentration used. An increase in the concentration of chitosan resulted in an increase in its effectiveness. Saprol 190 EC at 0.15% gave a significantly better result than chitosan. The results obtained in this experiment agree with the results of earlier experiments by Wojdyła et al. [15].

Table 1. Effectiveness of Biochikol 020 PC in the control of *Sphaeroteca pannosa* var. *rosae* on rose: mean degree of rose bush infection.
Beginning of the experiment and initial infection index: 2004. 06. 24 = 0.5

Treatment	Conc. in %	After weeks	
		2	4
Control	–	2.30 e	3.20 c
Folicur BT 225 EC	0.1	0.00 a	0.05 a
Biochikol 020 PC	0.5	1.40 d	1.40 b
Biochikol 020 PC	1	1.20 c	1.30 b
Biochikol 020 PC	2.5	0.85 b	1.15 b

Note: Means followed by the same letter within the columns are not significantly different at p=0.05.
Disease index: 0 – no symptoms, 1 – up to 1% of plant area covered with mycelium, 2 – 1.1 to 5%, 3 – 5.1 to 10%, 4 – 10.1 to 20%, 5 – over 20% of shoot area covered with fungus

Control of *Erysiphe polygoni*

After 2 weeks of the experiment, the effectiveness of Biochikol 020 PC at concentrations of 0.5 to 2.0%, used curatively against abundant powdery mildew symptoms, ranged from 57 to 74% (Tab. 2). After 4 weeks of the experiment, the effectiveness of chitosan was in the range 74 to 90%. Saprol 190 EC (standard) gave significantly better results than the tested solutions.

Table 2. Effectiveness of Biochikol 020 PC in the control of *Erysiphe polyphaga* on gerbera: mean degree of shoot infection
Beginning of the experiment and initial infection index: 2004. 05.14 = 5.0

Treatment	Conc. in %	After weeks	
		2	4
Control	–	5.00 e	5.00 e
Nimrod 250 EC	0.2	0.30 a	0.00 a
Biochikol 020 PC	0.5	2.15 d	1.30 d
Biochikol 020 PC	1	1.80 c	0.90 c
Biochikol 020 PC	2	1.30 b	0.50 b

Note and disease index: see Table 1

Control of *Peronospora sparsa*

After 4 weeks of the experiment, the tested compound at concentrations of 0.25% to 1.0% was 54 to 31% effective depending on the concentration used (Tab. 3). An increase in the concentration of Biochikol 020 PC caused a decrease in its effectiveness. The high effectiveness of chitosan in the control of *P. sparsa* on roses had been determined earlier by Wojdyła [13].

Table 3. Effectiveness of Biochikol 020 PC in the control of *Peronospora sparsa* on roses: mean degree of rose bush infection
Beginning of the experiment and initial disease index: 2004.05.06 = 1.3

Treatment	Conc. in %	After weeks	
		2	4
Control	–	3.10 d	5.00 e
Sandofan Manco 64 WP	0.15	1.70 a	1.44 a
Biochikol 020 PC	0.25	2.05 b	2.30 b
Biochikol 020 PC	0.5	2.35 c	3.20 c
Biochikol 020 PC	1	2.40 a	3.45 d

Note: see Table 1
Disease index: 0 – no disease symptoms, 1 – from 0.1 to 25% of leaves with disease symptoms, 2 – over 25% of leaves with disease symptoms, 3 – up to 25% of fallen leaves and the rest with disease symptoms, 4 – from 25 to 50% of fallen leaves, 5 – over 50% of fallen leaves

Control of *Puccinia pelargonii-zonalis*

After 4 weeks of the experiment, Biochikol 020 PC used curatively at a concentration of 2.5% was 83% effective in reducing the number of uredinia (Tab. 4). Score 250 EC at 0.05% had a significantly lower effect in controlling uredinia for-

mation compared with Biochikol 020 PC. Also, after 2 weeks from the end of the experiment, germination of uredospores taken from plants treated with Biochikol 020 PC was suppressed by more than 66%.

Table 4. Effectiveness of Biochikol 020 PC applied curatively to control *Puccinia pelargoni-zonalis* on pelargonium First spraying: 2004.02. 25.
Initial disease index: mean of invaded leaves – 20.0. Mean number of pustules per leaf – 6.0

Treatment	Conc. in %	Mean number of pustules/leaf	% of germinated spores after 14 days from end of experiment	Height of plant in cm
Control	–	24.21 c	76.9 b	17.9 b
Score 250 EC	0.05	8.79 b	20.9 a	19.1 b
Biochikol 020 PC	2.5	4.26 a	25.6 a	15.7 a

Note: see Table 1

Control of *Melampsora epitea*

After 2 weeks, the effectiveness of the tested compound used curatively at a concentration of 2.5% was about 67%, and 81% of uredinia were browned and had dried up (Tab. 5). Also, about 77% of leaves showed disease symptoms, compared to 92% in the case of the control plants.

Table 5. Effectiveness of Biochikol 020 PC applied curatively to control *Melampsora epitea* on willow First spraying: 2004.07. 28.
Initial disease index: mean of invaded leaves – 65. Mean number of pustules per leaf – 7.5

Treatment	Conc. in %	Mean number of pustules/leaf	% of uredinia dried up	% of diseased leaves
Control	–	31.8 c	0.0 a	92.4 a
Folicur BT 225 EC	0.05	4.7 a	99.3 c	77.7 a
Biochikol 020 PC	2.5	10.6 b	81.3 b	77.7 a

Note: see Table 1

Control of *Diplocarpon rosae*

After 3 weeks of treatment, the effectiveness of Biochikol 020 PC ranged from 52 to 82% depending on the concentration used (Tab. 6). After 9 weeks of protection, the effectiveness of the tested product ranged from 37 to 44% depending on the concentration used. Higher concentrations of Biochikol 020 PC gave better results than lower concentrations. Saprol 190 EC at 0.15% was significantly better than the tested solutions in controlling black spot. Similar results of the effectiveness of Biochikol 020 PC in the control of this pathogen had been obtained in an earlier experiment [13].

Table 6. Effectiveness of Biochikol 020 PC in the control of *Diplocarpon rosae*: mean degree of rose bush infection
Beginning of the experiment and initial infection index: 2004.06.01 = 0.1

Treatment	Conc. in %	After weeks of experiment		
		3	6	9
Control	–	1.35 d	3.20 e	5.00 e
Saprol 190 EC	0.15	0.11 a	0.40 a	1.00 a
Biochikol 020 PC	0.5	0.65 c	2.00 d	3.15 d
Biochikol 020 PC	1.0	0.40 b	1.80 c	3.00 c
Biochikol 020 PC	2.5	0.24 ab	1.65 b	2.80 b

Note: see Table 1
Disease index: 0 – no disease symptoms, 1 – from 0.1 to 25% of leaves with disease symptoms, 2 – over 25% of leaves with disease symptoms, 3 – up to 25% of fallen leaves and the rest with disease symptoms, 4 – from 25 to 50% of fallen leaves, 5 – from 50 to 90% of fallen leaves, 6 – over 90% of fallen leaves

Control of *Colletotrichum violae*

After 2 weeks of treatment, the effectiveness of Biochikol 020 PC ranged from 36.5 to 46.2% depending on the concentration used (Tab. 7). After 4 weeks of protection, the effectiveness of the tested product was in the range 61 to 65% depending on the concentration used. Higher concentrations of Biochikol 020 PC gave better results than lower concentrations. Folicur BT 225 EC at 0.1% gave similar results as Biochikol 020 PC at 2.5%.

Table 7. Effectiveness of Biochikol 020 PC in the control of leaf spot on pansy
Beginning of the experiment and initial disease index: 2004.07. 01 = 2.1

Treatment	Conc. in %	After weeks	
		2	4
Control	–	2.60 d	4.60 c
Folicur BT 225 EC	0.1	1.35 a	1.60 a
Biochikol 020 PC	0.5	1.65 c	1.80 b
Biochikol 020 PC	1	1.50 b	1.70 ab
Biochikol 020 PC	2.5	1.40 ab	1.60 a

Note: see Table 1
Disease index: 0 – no disease symptoms, 1 – from 0.1 to 25% of leaves with disease symptoms, 2 – 25 to 50% of leaves with disease symptoms, 3 – more than 50% of leaves with disease symptoms, 4 – from 0.1 to 25% of dead leaves and others with spots, 5 – from 25 to 50% of dead leaves.

CONCLUSIONS

1. Biochikol 020 PC at 0.5 to 2.5%, used soon after the first visible powdery mildew symptoms had appeared on rose, was 56 to 64% effective in controlling the disease. An increase in the concentration of chitosan resulted in an increase in its effectiveness.

2. In the control of *Erysiphe polygoni* on gerbera, the effectiveness of Biochikol 020 PC at concentrations of 0.5 to 2.0% ranged from 74 to 90%.
3. In the control of *Peronospora sparsa* on rose, Biochikol 020 PC used at concentrations of 0.25 to 1.0% controlled pathogen development with an effectiveness of 31 to 54%. However, an increase in the concentration of Biochikol 020 PC resulted in a decrease in its effectiveness.
4. In the control of *Puccinia pelargonii-zonalis* on pelargonium, Biochikol 020 PC at a concentration of 2.5% was 83% effective in controlling the number of uredinia formed.
5. In controlling *Melampsora epitea* on willow, the effectiveness of Biochikol 020 PC at 2.5% was 66.8%, with about 81% of uredinia browned and destroyed.
6. In the control of *Diplocarpon rosae* on roses, the effectiveness of Biochikol 020 PC at concentrations of 0.5 to 2.5% ranged from 37 to 82% depending on the concentration used. Higher Biochikol 020 PC concentrations resulted in higher effectiveness.
7. In controlling *Colletotrichum violae-tricoloris* on pansy, the effectiveness of Biochikol 020 PC at 0.5 to 2.5% ranged from 61 to 65%.

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USE OF FOOD CHITOSAN FOR MANUFACTURING OF PROTECTIVE COATINGS FOR FROZEN FISH PRODUCTS

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Abstract: Food chitosan is used as basic material for manufacturing of protective coatings for frozen fish products (whole, gutted, dressed, filleted fish) with purpose to preserve quality of products, to prevent loss on drying, to reduce oxidation of lipids and microbial seeding and to prolong shelf life. During development of protective coatings such advantages of chitosan have been taken into consideration as its non-toxity, bactericidal and biodegradable properties, that present special value from the point of human health and environment protection, as well as appropriate rheological characteristics (viscosity, adhesive properties, stickiness, etc.) necessary for preparation of solutions and spreading them over products [1, 2]. The method of creating thin film on the surface of frozen products has been performed by variety of methods with further formation of thin film on frozen surface. Both chitosan and complex coatings by successive application of chitosan, sodium alginate, pectin and copolymer of polyvinilpirrolidon with crotonic acid (catanol) have been tested. Formation of complex coatings makes it possible to combine the properties of different polymers, to vary adhesive characteristics of solutions, to intensify strength and biodegradability of coatings. The physical-and chemical properties of solutions have been studied, optimal concentrations and order and method of their application upon products have been defined. It was revealed that the described method of frozen fish products protection with use of chitosan permits to preserve good quality of fish and to prolong shelf life for 2–3 months.

Key words: chitosan, protective coatings, fish products

INTRODUCTION

Analysis of new materials used for manufacturing of packing material for food products shows great perceptiveness of polysaccharides application – on one hand they are eco-friendly, on the other hand they are of nature origin (renewable resources).

Use of polysaccharides permits development of packing material that has property of biodegradability and does not disharmonise environment.

Amylose starch, zein, methylcellulose, polyvinylpyrrolidone, pectin, wax compositions are often used in biodegradable and edible packages. They are able to decrease rate of water loss from the product, they create a barrier for ingress of atmospheric oxygen and other substances, and they have high sorption capacity. Nevertheless majority of them are hydrolabile and are unfit for extended storage period.

Natural biopolymer chitosan that is extracted from crusts of marine and freshwater crustaceans has bactericidical and fungicidical properties required for packaging material, has respective rheological characteristics that permit preparation of solutions and formation on their base of coatings and packaging films. It is biocompatible, biodegradable; it has the property of heavy metals and radionuclides sorption and cholesterol fixation. By virtue of enumerated advantages chitosan has been used as basic material for manufacturing of protective coatings for preservation of frozen fish products.

MATERIALS AND METHODS

Food chitosan was extracted from crusts of crab, shrimp and krill by method of cold alkali deacetylation [1, 2]. Choice and specification of chitosan for protective coatings and packing materials have been performed by main qualitative indicators, such as solvability, deacetylation degree, viscosity, molecular mass. Samples of chitosan with tailor-made functional properties were obtained [3, 4].

Several batches of food chitosan have been tested that are characterized by the following indicators:

Humidity – 9.5 – 11.5%

Ash content – 0.25 – 1.1%

Kinematic viscosity – 400 – 1600 CP

Deacetylation degree – 83.5 – 90.1%.

It was demonstrated that ash content of chitosan should be less than 1,5%, deacetylation degree should be within range of 0.80–0.90, kinematic viscosity – within range from 400 up to 1500 CP during preparation of solutions for protective coatings [5].

Organoleptic determination and physicochemical properties of food chitosan are presented in the table:

Name of indicator	Specification and its value
Appearance	Powder, flakes, scales
Colour	From white to cream or pink with pearly gloss or without it. It can be of greyish or yellowish tinge.
Flavour	Appropriate to this product, without foreign flavour
Odour	Appropriate to this product, without foreign odour
Moisture content, mass%, less than	10.0
Mineral substances content, mass%, less than	0.7
pH of 1% chitosan solution in 2% acetic acid, less than	7.5
Insoluble substances content, mass%, less than	0.2

Content of toxic elements, pesticides, radionuclides, microbiological values of used chitosan complied with current sanitary requirements and norms.
The following substances besides chitosan were used for complex coatings:

- Pectin – polysaccharide that has good gelling properties, excellent resistivity to shear load and stability in acid spectrum of pH. Combined galacturonic acid is its main component. It forms three-dimensional chain stabilized by connecting zones between chains in such a way so that water is bound.
- Sodium alginate extracted out of brown algae. It is widely known due to its bactericidal action (it suppresses staphylococcus and fungi growth) and also due to its ability to sorb toxic substances. Alginates are widely used in food industry, biotechnology, medicine.
- Copolymer – vinylpyrrollidone – crotonic acid that comes into composition of antiseptic catanol; it has wide spectrum as disinfectant agent; it is permitted for use in food and fish industries.

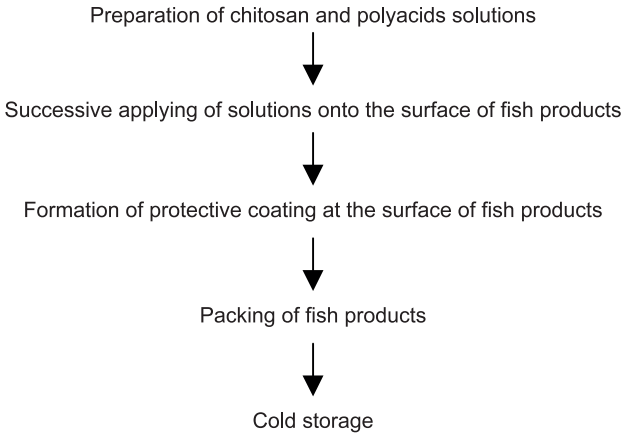
Frozen fish of different species (cod, pike perch, redfish, mackerel and others), whole, gutted as well as filleted have been objects of study during application of chitosan based protective coatings.

Quality evaluation of fish products has been performed by determination of water content, nitrogen of volatile substances, acid and peroxide numbers, moisture retaining quality, microbial seeding and organoleptic properties by standard methods.

Solutions of chitosan in 2% acetic acid, copolymer, solution of apple pectin, solution of sodium alginate of definite concentrations have been used for processing of frozen fish products.

Preparation of chitosan and polyacid solutions has included the following procedures: weighing of certain quantity of material; dispersion of chitosan or polyacid shot in water by means of mechanical mixer; dilution of chitosan in acetic acid and dilution of polyacids in water; aging of solutions; filtration; storage of solutions; conditioning to required concentration before usage.

Process flowsheet of applying chitosan and polyacids solutions onto fish products can be presented in the following way:



Solutions have been conditioned to optimal concentrations and temperatures before application on the surface of fish products.

During application of complex coatings with usage of copolymer, pectin and sodium alginate solutions it is important to observe processing sequence of fish products by them.

Temperature of treated frozen fish products should be less than 18°C.

Fish products with applied coatings are sent to refrigeration chamber for final formation of coating and further cold storage.

It was revealed that the coatings on the base of chitosan and other used polysaccharides retain moisture in product; its content does not practically change during extended period of storage. Content of nitrogen of volatile substances also does not practically change; this indicates good protective properties of these coatings that inhibit hydrolysis process in product.

Insignificant increase of acid and peroxide numbers is observed; it indicates some growth of hydrolysis and lipids oxidation. Nevertheless in control group (coating with water glaze) these changes are more significant.

Changes of moisture retaining ability during several (more than 6) months of storage are insignificant. Microbiological values during the whole period of storage, organoleptic properties (colour, flavour, odour) and consistence complied with requirements to these types of production of high quality.

Biological testing of protective coatings to natural bacterial community showed their full biodegradation in natural conditions.

CONCLUSIONS

Film-forming properties of natural polysaccharide, food chitosan have been discovered, components of complex coatings on the base of chitosan have been determined with usage of apple pectin, sodium alginate, copolymer of vinylpyrrolidone with crotonic acid.

Complexing of chitosan with these components has been studied, conditions of complex coating formation have been developed, and their functional characteristics have been defined.

Optimal specifications of polysaccharides solutions have been set up; methods of their application – by dipping and by spraying – have been determined.

Biological properties of coating on the base of chitosan, their bactericidal action and biodegradation, have been studied.

It was demonstrated that packing on the base of chitosan permits to preserve good quality of fish and to prolong shelf life for 2–3 months.

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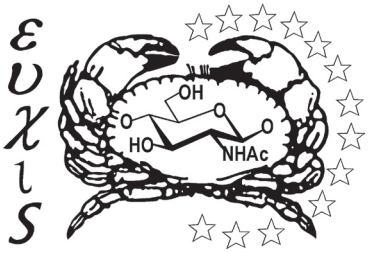
CHAPTER VI

BIOSYNTHESIS, BIODEGRADATION AND BIOACTIVITY



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SOME FACTORS AFFECTING ANTIMICROBIAL PROPERTIES OF CHITOSAN

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Abstract: Chitosan and its salts were tested for antimicrobial properties in the form of aqueous solutions, air-dried membranes and film coatings applied to polypropylene (PP) spunbond nonwoven [1]. Solutions of chitosan salts were found to be active against Gram-negative and Gram-positive bacteria as well as against yeast organism *Candida albicans*. The effectiveness of these salts depended markedly on their acid component. In the solid film form, chitosan acetate was found to reduce the population of *Staphylococcus aureus* cells whereas chitosan sulfate and the chitosan film obtained by regeneration from chitosan acetate had both neutral effects. The same three substrates were also tested in the form of thin coatings on the PP fabric. The obtained results suggest that the regenerated chitosan coatings were particularly active and significantly diminished the number of cells of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans.* Chitosan-coated surface of the nonwoven material eliminated practically all cells in each experiment after 6 hours whereas the system based on chitosan acetate was quite efficient only in the case of *Escherichia coli* but at a relatively faster rate. The data obtained in this work clearly imply that the physical and/or chemical form of a chitosan-based material may have a profound effect on its antimicrobial characteristics.

INTRODUCTION

The ultimate goal of this work was to develop antimicrobial polypropylene nonwoven material, which would be useful for medical and personal hygiene applications. Our approach was based on the known fact that various polyionic polymers, both natural and synthetic can kill or inhibit the growth of numerous microorganisms [2–5]. Chitosan was one of more important candidates for investigation due to its proven cell sequestering capability and skin wellness-related benefits [6, 7]. In the course of the project it was quickly realized that various techniques of preparation of the prototype chitosan samples had an effect on their antimicrobial efficacy. Therefore, it became apparent that a better understanding of this finding would be helpful to maximize the desired practical outcome of the study.

EXPERIMENTAL

Solutions of polyionic polymers. Aqueous solutions of chitosan salts, *i.e*. hydrochloride and acetate, were prepared by dissolving chitosan (Vanson, USA,

M_w=11,000,000, DA=0.14) in dilute aqueous solutions of HCl and acetic acid, respectively. The concentration of chitosan in these solutions was 2% and the final pH in both cases was in a range from 5 to 5.5. A 2% aqueous solution of sodium carboxymethylcellulose (Hercules, USA, M_w=1,000,000, DS=0.7) served for comparative bioassays with various microorganisms.

Chitosan-based membranes. Chitosan acetate, chitosan sulfate and regenerated chitosan films were prepared in the following ways. Chitosan (10 g, same material as above) was suspended in 2 L distilled water and mixed with acetic acid at a molar ratio of chitosan (based on primary amino groups) to CH_3COOH of 1.1:1. This solution was used both in film casting and in the nonwoven coating treatments (see below). To cast the film the solution was poured onto a surface-treated (non-sticky) pan and air-dried in ambient conditions for 2 days. The dried film as further heat-treated 80°C for 30 minutes in order to make it insoluble in water. The thickness of the membrane was about 0.3 mm. Part of the chitosan acetate membrane prepared as described above was immersed in large amount of 1% aqueous H_2SO_4 solution for 4 hours, washed thoroughly with distilled water and dried at 60°C. Another part of the chitosan acetate film was treated with excess 1% aqueous NaOH solution for 4 hours, then washed thoroughly with distilled water and dried at 60°C.

PP nonwoven coated with chitosan-based films. Commercial spunbond nonwoven (Kimberly-Clark) having basis weight of 17 g/m^2 and composed of PP fibers (2.2 dtex) was dipped into the 0.5% chitosan solution described above, then dried in ambient conditions and heat-treated at 80°C for 30 minutes. The add-on of chitosan acetate was 3% by weight of the nonwoven. Part of the obtained sample was treated with 1% aqueous H_2SO_4 solution for 4 hours and dried in order to convert the coating into chitosan sulfate. Another part of the nonwoven with chitosan-acetate application was used for regeneration of the polymer salt into chitosan by neutralizing it with 1% aqueous NaOH solution, thorough washing with distilled water and drying at 60°C.

Challenge microorganisms. Three kinds of bacteria and one species of fungus were selected as challenge organisms for the study. These were: Gram-negative *Escherichia coli*, Gram-positive *Staphylococcus aureus*, Gram-negative *Pseudomonas aeruginosa*, and *Candida albicans* yeast culture.

Antimicrobial activity of polymer solutions. The tests were performed by mixing the polymer solutions with the challenge organisms, incubating at ambient temperature for 24 hours and periodically sampling the mixture to determine the number of viable organisms remaining in the sample. Enumeration of these cells in the mixtures enabled quantitative measurement of antimicrobial activity, as measured by Colony Forming Units (CFU).

Antimicrobial activity of membrane samples. The test films were cut in two sample weights in each case, *i.e.* 25 mg and 50 mg, and placed in individual wells of Falcon tissue culture plates. *S. aureus* inoculum was prepared in physiological saline such that the bacterial concentration was fixed at approximately $5x10^6$ CFU/mL. Inoculum volumes added to pre-weighed samples were: 10 mL for chitosan acetate film, due to its highly absorbent nature, and 5 mL for membranes of chitosan sulfate and of regenerated chitosan. Inoculated samples were covered and incubated on a rotating platform. Serial dilutions of the samples were pre-

pared in letheen neutralizing broth at different time intervals. Viable *S. aureus* recovery was determined by plating the dilutions onto nutrient agar. Controls of straight inoculum (5 mL) were simultaneously evaluated at the designated time points. Antimicrobial activity was determined by sample recovery relative to control recovery.

Antimicrobial activity of coated nonwoven material. The test materials were cut into 3.8 cm disks and weighed. Each challenge organism (*E. coli, S. aureus, C. albicans*) was washed and resuspended in pH 5 acetate buffer. The organisms were applied to the test material in a volume of 2 μL of inoculum per mg of material. Inoculated organisms were allowed contact incubation for various periods of time. The zero time point samples were processed immediately, and the rest of the samples were incubated in a sterile, humidified, enclosed 31°C chamber. The samples were processed by placing inoculated disks into 25 mL of letheen and vortexing vigorously for 30 seconds to remove adhering cells into surrounding fluid. Serial dilutions of this solution were spread plated onto nutrient agar to recover viable organisms and count them.

RESULTS AND DISCUSSION

Microbial growth inhibition by soluble polyionic polymers. The antimicrobial activity is understood here as sequestering or immobilization of microorganisms such that their population within a suspension medium is reduced, although the microbes may not be killed. For example, some positively charged polymers can attract negatively charged cells and form aggregates, which cease to multiply. Some results of the bioassays conducted with polymer solutions are summarized in Table 1.

Table 1. Evaluation of antimicrobial activity (as change of CFU) of polymer solutions

	Chitosan HCl		Chitosan CH_3COOH		CMC Na	
	t=0	t=24 h	t=0	t=24 h	t=0	t=24 h
E. coli	2.1×10^5	0	3.8×10^5	6.4×10^3	4.2×10^7	6.4×10^8
S. aureus	1.9×10^7	0	1.7×10^8	1.7×10^7	7.2×10^7	1.8×10^8
P. aeruginosa	4.9×10^6	1.4×10^5	4.0×10^5	0	5.0×10^7	6.5×10^8
C. albicans	0	0	0	0	5.5×10^5	9.4×10^6

The data clearly suggest that aqueous solutions of chitosan hydrochloride and chitosan acetate exhibited generally high antimicrobial activity. In particular, these compounds were remarkably active against *Candida albicans*, the yeast, which is a pathogen sometimes difficult to fight and may cause for instance severe irritation of infant's skin. Even at time "zero" no viable cells of this organism were found in the aqueous media of chitosan hydrochloride and of chitosan acetate. On the other hand, sodium carboxymethylcellulose did not show any bioactivity in the tests conditions.

Bioactivity of chitosan-based films. Film samples of chitosan acetate, chitosan sulfate and regenerated chitosan were evaluated in culture plates with *S. au-*

reus. The results were compared to the control assays without any antimicrobial agent (Table 2). In view of the encouraging information contained in Table 1, it was interesting to realize that only chitosan acetate film exhibited some biologic activity in this part of the study.

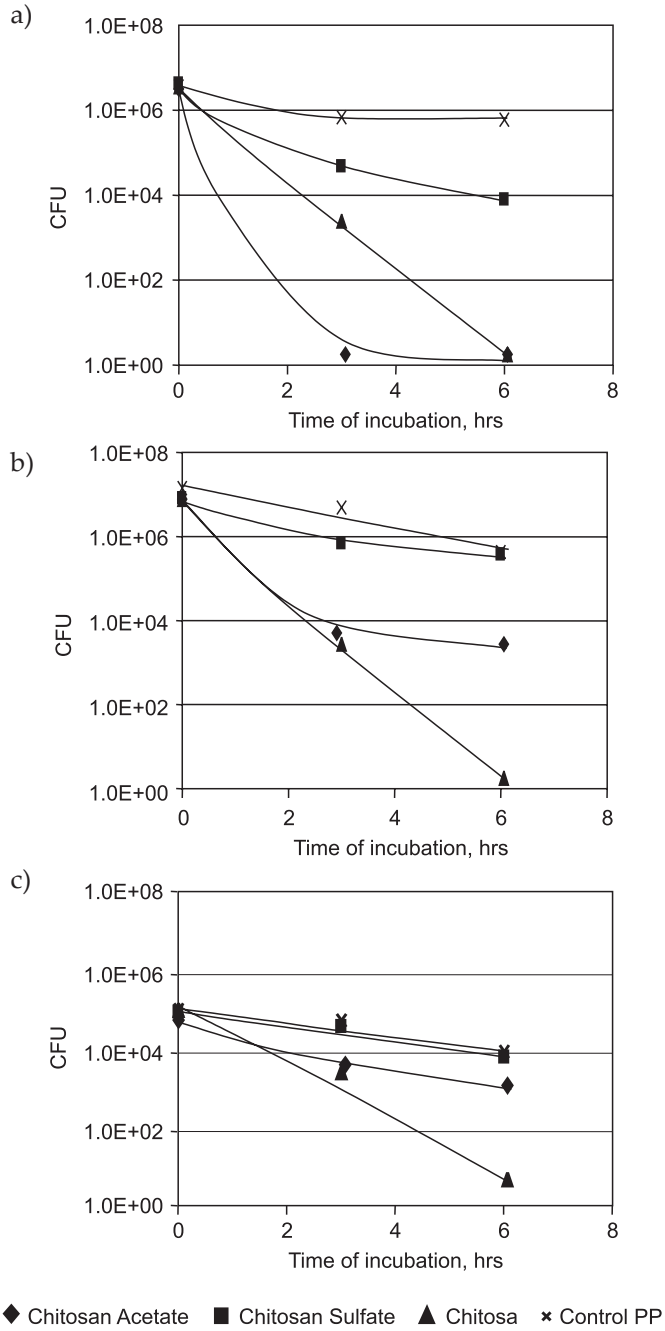

Figure 1. Antimicrobial activities of various chitosan coatings on PP nonwoven:
a) *Escherichia coli,* b) *Staphylococcus aureus,* c) *Candida albicans*

Table 2. Evaluation of antimicrobial activity (as CFU change) of polymer films

Time, hours	Chitosan acetate		Chitosan sulfate		Regenerated chitosan		Control
	25 mg	50 mg	25 mg	50 mg	25 mg	50 mg	
0	3.9×10^6	5.9×10^6	6.1×10^6	5.9×10^6	7.7×10^6	4.9×10^6	7.9×10^6
2	1.3×10^4	7.1×10^3	6.4×10^8	5.1×10^6	5.2×10^6	3.6×10^6	1.2×10^6
4	6.9×10^4	1.4×10^3	7.9×10^8	5.4×10^6	2.2×10^6	3.5×10^6	1.7×10^5

Antimicrobial properties of treated PP nonwoven. In this phase of the study chitosan acetate solution was applied onto the polypropylene nonwoven and then treated in various ways to insolubilize the resultant coating. The techniques used in this case were similar to those employed for the preparation of insoluble chitosan-based membranes. This procedure led to three types of materials: with chitosan acetate, chitosan sulfate and with regenerated chitosan coating. The antimicrobial assay data, illustrated in Fig. 1, suggest clearly that regenerated chitosan was most universal and generally effective against all the tested organisms. The finding may seem somewhat surprising when compared to the data obtained for corresponding individual membranes (Table 2), which suggested poor antimicrobial activity of the regenerated chitosan film. Although such a difference in the behavior of apparently the same type of material is not quite clear to us it seems to be related to the polarity of the environment in which the polymer film solidified. It can be speculated that when coating a chitosan material onto a hydrophobic surface of a substrate such as polypropylene, the hydrophobic polymer attracts more hydrophobic segments of chitosan and repels the more polar sites. This results in a structure in which most of the bioactive sites of chitosan are outwardly directed at the surface of the product. As the coating becomes thicker, the interaction between the hydrophobic sites of chitosan and the hydrophobic surface will decrease, thereby reducing the otherwise preferential outward orientation of the polar segments.

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GLYCOPEPTIDE SYNTHESIS: CHITOSAN OLIGOSACCHARIDES AS MODULATORS OF BIOLOGICAL ACTIVITY OF HORMONAL PEPTIDES

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Abstract: Glucosamine oligosaccharide (tetra to heptamer) made from highly deacetylated chitosan by endochitosanase enzymatic hydrolysis was derivatized at *N*-sites with *t*-butyloxycarbonyl (Boc) protecting group. This amino protected oligosaccharide was then activated with CCl_3CN at the site of anomeric carbon to be coupled to growing peptide chain in solid phase synthesis. The glycopeptides were cleaved from the resin and were deprotected. The crude product was purified on reverse phase column chromatography and tested for biological activity.

INTRODUCTION

A protein performs its function only in specific areas of the cell, where biological selection processes occur predominantly on the intracellular and outer-cell membranes. This protein guiding to activity sites is based on a receptor-effectors interaction in which, in eucaryotic cells, the glycoproteins play a decisive role [1]. In common with most membrane proteins, G-protein-coupled receptors (GPCR) are usually glycosylated. GPCR possess at least one glycosylation site in their *N*-terminal domain or in one of extracellular loops [2]. Although, exceptions do occur as evidenced for the adrenergic receptor α_{2b} this does not contain any putative glycosylation site [3]. The *N*-glycosyl attachment site to protein receptor via galactosyl, fucosyl, sialyl and often *N*-acetylglucosaminyl (GlcNAc) residues is the Asn residue in the consensus sequence Asn-Xaa-Thr/Ser in correctly for glycosylation-oriented fashion. A separate class of *O*-glycosylation involves the glycan linking to the hydroxyl of Ser or Thr via an *N*-acetylgalactosamine, galactosyl, fucosyl, sialyl and *N*-acetylglucosamine residues. The transmembrane helical bundle provides the binding site for small ligands while the binding site for larger ligands such as peptides very often involve additional interactions with the extracellular domains [4] including carbohydrate part. Some naturally occurring ligand hormones are glycopeptides. In our approach, we aimed to verify biological impact of oligosaccharide in oligosaccharide-hormonal peptide (ligand to GPCR) conjugate. We hypothesize that a *C*-terminal hormonal peptide binding

to receptor protein is reinforced by additional interaction with oligosaccharide at the peptide *N*-terminus.

To demonstrate the proof of concept of this hypothesis, we used the bradykinin (BK) B_1 and B_2 receptor as a receptor model system and we synthesized glycoconjugates of bradykinin and its antagonists. The peptides were assembled on solid phase with continuous flow system using Fmoc-strategy. Highly deacetylated hydrolyzed chitosan oligosaccharide (tetra to heptamer) was dissolved in DMF with 5% LiCl and in presence of diisopropylethylamine (DIPEA) protected at amino group sites with Boc_2O. The Boc protected oligochitosan as shown in Fig.1, dissolved in DMF with 5% LiCl and presence of DBU, trichloroacetonitrile was added. This reacting mixture was shaking overnight with *N*-terminal amine free peptide on the resin. The peptide glycoconjugate on the resin was cleaved from the resin and deprotected with trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O (95:2.5:2.5). The peptide glycoconjugate was precipitated in ethyl ether and lyophilized. The crude product was then purified on C_{18} reversed phase column chromatography. The peptide glycoconjugates identities were verified by MALDI mass spectrometry.

The kinin surrogates were tested in vasoconstriction bioassays as agonist or antagonist using the human umbilical vein. Results demonstrated that peptide glycoconjugates are less potent by one log scale unit than their unmodified parent molecules in B_2 receptor model system while the B_1 receptor model system retained its potency level for unmodified parent molecule.

MATERIALS AND METHODS

Chitosan (98% deacetylated) used for enzymatic hydrolysis was purchased from Marinard (Canada). Endochitosanase used for enzymatic hydrolysis was prepared at Sherbrooke University (Canada). TentaGel S resins with the first amino acid attached to were purchased at Rapp Polymere (Germany). Fmoc-protected amino acids and TBTU coupling agent were purchased from Chem Impex (USA). All other chemicals used were purchased at Sigma-Aldrich Canada Ltd. DMF used in synthesis was purchased from Fisher (Canada). The continuous flow system synthesis was performed on Pioneer Perseptive Peptide Synthesizer. The NMR ^1H data were taken with Bruker 300 MHz. The final products identities were confirmed by MALDI mass spectrometry with Micromass Tof Spec 2F. Analytical HPLC identification was performed on Agilent system with ZORBAX SB-C18 column.

RESULTS AND DISCUSSION

Bradykinin and its B_1 and B_2 antagonists were chosen to demonstrate hypothesized glycosyl-oligosaccharide interaction. Peptides were assembled on solid phase in Fmoc strategy using TBTU as amino acids coupling agent. Synthesis was performed in continuous flow system.

The peptides were not isolated when assembled but activated oligosaccharide was coupled onto while peptide was still on solid phase resin.

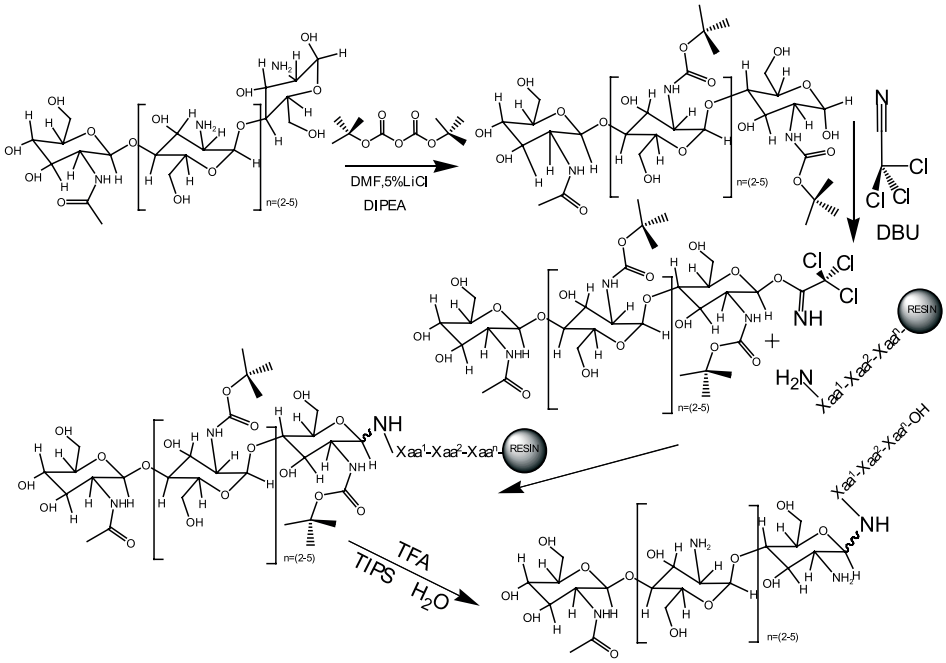

Figure 1. Scheme of oligoglycopeptide synthesis

Chitosan enzymatic hydrolysis was performed to final oligosaccharide with estimated mass of ~1000 Dalton as deduced from reducing sugar assay. This oligochitosan has shown in NMR spectra significant amount of acetyl methyl from residual either *N*-acetyl or acetate salts at δ 1.88 made in DMSO. NMR spectra of *N*-Boc protected oligochitosan have shown at δ 1.05 presence of *t*-butyl group in ratio of 2.8 times more than acetyl groups at δ 1.88.

N-Boc protected oligochitosan is soluble in DMF and this solvent is used to transform it into active derivative. Electron-deficient nitrile like trichloroacetonitrile is used to form base-catalyzed trichloroacetimidate at the anomeric site [5]. *N*-Boc protected oligochitosan DMF solution at room temperature is activated with trichloroacetonitrile in presence of DBU or DIPEA for 15 min. Then, reacting mixture is shaking overnight with free amine peptide on the resin. Glycopeptides are cleaved from the resin and deprotected in the same time with TFA/TIPS/H_2O (95:2.5:2.5). The trifluoroacetate salts of glycopeptides was precipitated in ethyl ether, centrifuged, dissolved in water and lyophilized. The crude glycopeptides were purified on reverse phase C_{18} column chromatography and separated glycopeptides were isolated. The chemical identities of the final products were given as mass spectrometry and analytical HPLC. The identities data and biological activity of synthesized compounds are shown in the Table 1.

In conclusion, we could not support our hypothesis on glycosyl-glycosyl interaction while the hormonal ligand binds to its receptor. We lost vasoconstriction potency for glycopeptides for B_2 receptor by 2 units in logarithmic scale and 1 unit for B_1 receptor. This does not mean that our hypothesis is wrong in general, it is wrong in the chosen hormonal system. There is also a good chance that in

the chosen system, the *N*-terminal site of the ligand is not free to interact with receptor glycosyl.

Table 1. Chemical and pharmacological characterization of synthesized glycopeptides and peptides

Glycopeptides	MALDI-MS	HPLC t_R (min)	Human umbilical vein
GlycoBK	2117	18.15	inactive
GlycoB$_2$ antagonist	2323	20.69	pA$_2$=6.20
GlycoB$_1$ antagonist	2224	23.36	pA$_2$=7.48
Peptides	MALDI-MS	HPLC t_R	Human umbilical vein
BK (B$_2$)	1060	13.80	pD$_2$=8.69 (mouse)
B$_2$ antagonist	1304	15.48	pA$_2$=8.28
B$_1$ antagonist	1140	20.61	pA$_2$=8.49

We solved the problem of hydrolyzed chitosan solubility in organic solvent using DMF-5% LiCl as solvent. This allowed us to find reaction conditions to protect amino groups of oligoglucosamine as *N*-*t*-butyloxycarbonyl derivative.

The most significant impact of our study is the development of glycoside synthesis at anomeric hydroxyl group on the solid phase. The well-known Keonigs-Knorr [6] method requires an exchange of anomeric hydroxyl group by bromine or chlorine and these active compounds could generate glycosyl group donor. This method has some limiting factors in several applications; therefore alternative methods were developed. The most promising approach for glycosylation on solid phase we found in use of trichloroacetonitrile [5]. We change originally used solvent and reaction conditions into required in solid phase synthesis, which is DMF and room temperature for coupling. Activation via trichloroacetimide additionally allows for stereochemistry control conditions. From our results, we conclude that peptide cleavage conditions we use were not destructive for glycoside bond.

In the future we intent to verify our principal hypothesis with similar biological systems.

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IDENTIFICATION OF NUCLEOTIDE BINDING RESIDUES FOR *N*-ACETYL-D-GLUCOSAMINE 2-EPIMERASE

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INTRODUCTION

Renin binding protein (RnBP) is an endogenous renin inhibitor originally isolated from porcine kidney. This protein inhibited renin activity strongly, with the apparent dissociation constant of 0.2 nM [1]. Recently, the human recombinant RnBP was identified as the enzyme *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [2]. The enzyme catalyses conversion between GlcNAc and *N*-acetyl-D-mannosamine (ManNAc). The human GlcNAc 2-epimerase activity specifically inhibited by sulfhydryl-alkylating and oxidizing reagents [3], and the essential cysteine residue of human GlcNAc 2-epimerase has been identified by site-directed mutagenesis [3, 4]. Our recent studies demonstrated that nucleotides such as ATP, dATP, ddATP, ADP, and GTP enhance human, rat, and porcine GlcNAc 2-epimerase activities [5]. Moreover, the middle domain of the GlcNAc 2-epimerase molecule participated in the specificity and binding of nucleotides [6]. In the present study, we constructed several mutants of middle domain of human and rat GlcNAc 2-epimerases to identify the amino acid residue conferring nucleotide binding.

EXPERIMENTAL

Materials

Restriction enzymes, DNA ligation kit Ver. 2.0, site-directed mutagenesis system (Mutan-Super Expression Km), and *Escherichia coli* MV1184 and JM109 competent cells were obtained from Takara-Bio (Tokyo, Japan). GlcNAc, ManNAc, 3-hydroxy 2, 4, 6-tri iodobenzoicacid (HTIB), and *N*-acylhexosamine oxidase (AHOX) (Lot 71K20041) were obtained from Sigma (St. Louis, MO, USA). Horseradish peroxidase was from Wako (Osaka, Japan). 4-Aminoanitipyrine was from Nacalai Tesque (Kyoto, Japan).

Construction of Mutant Plasmids

The oligonucleotide-directed dual amber method [7] using Mutan Super Expression System was used for the construction of mutants of human and rat GlcNAc 2-epimerases. Mutations were confirmed by nucleotide sequencing analysis. The mutant cDNAs were subcloned into *E. coli* expression vector pUK223-3 [8].

Western Blotting

Samples were loaded onto a 5–20% gradient polyacrylamide gel (PAGEL 520, ATTO) and then electrophoresed according to Laemmli [9] at a constant current of 20 mA. After the electrophoresis, the protein in the gel was transferred onto a nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20 (TBST) containing 5% skim milk, then incubated for 1 h at room temperature with rabbit anti-human or -rat GlcNAc 2-epimerase antiserum (1:1,000 dilution with TBST). After the incubation, the membrane was washed three times with TBST and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (1:5,000 dilution with TBST) (Promega, Madison, WI, USA). The membrane was washed three times with TBST and allowed to react with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for color development.

GlcNAc 2-Epimerase Activity

GlcNAc 2-epimerase was measured in an AHOX and peroxidase coupled system [10]. A reaction mixture contained 80 μl of 0.1 M Tris-HCl, pH 7.5, containing 10 mM MgCl2, 50 mM ManNAc, 5 mM ATP, and 20 μl of enzyme solution. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by the addition 0.9 ml of ice-cold distilled water. The diluted sample (20 μl) was mixed with 0.25 ml of 1 mM 4-aminoantipyrine, 0.5 unit/ml of AHOX, 5 units/ml

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     96                                                                155
Human: RVAPP GKKCAFVLTR DGRPVKVQRT IFSECFYTMA MNELWRATGE VRYQTEAVEM MDQIV
       ***** *********: ********** ********** *****::*** ::**@***** ****:
Rat  : RVAPP GKKCAFVLTQ DGRPVKVQRT IFSECFYTMA MNELWKVTGE MHYQREAVEM MDQII

    156                                                                215
Human: HMVQE DASGLGRPQL QGAPAAEPMA VPMMLLNLVE QLGEADEELA GKYAELGDWC ARRIL
       ***:* *::******* @*:@*:**** ********** ****:***:: :********* *:***
Rat  : HMVRE DPAGLGRPQL SGTLATEPMA VPMMLLNLVE QLGEEDEEMT DKYAELGDWC AHRIL

    216                                                                275
Human: QHVQR DGQAVLENVS EGGKELPGCL GRQQNPGHTL EAGWFLLRHC IRKGDPELRA HVIDK
       ***** ***:****** *:******** **:******* *******::@ :*****@*:@ *:***
Rat  : QHVQR DGQVVLENVS EDGKELPGCL GRHQNPGHTL EAGWFLLQYA LRKGDPKLQR HIIDK

    276                                 305
Human: FLLLP FHSGWDPDHG GLFYFQDADN FCPTQ
       ***** *******:** *********: :****
Rat  : FLLLP FHSGWDPEHG GLFYFQDADD LCPTQ
```

Figure 1. Comparison of amino acid sequence of middle domain of human and rat GlNAc 2-epimerases. Identical amino acids and similar amino acids are shown by asterisks and colons. @, different amino acids

of horseradish peroxidase in 0.1 M sodium phosphate buffer, pH 7.25 containing 0.1% NaN_3, and 0.25 ml of 2 mM HTIB in 0.1 M sodium phosphate buffer, pH 7.25 containing 0.1% NaN_3. After incubation at 37°C for 30 min, absorbance at 515 nm was measured.

RESULTS AND DISCUSSION

Construction of Mutant Plasmids

Recently, we have identified nucleotide binding region of GlcNAc 2-epimerase by constructing several chimeric enzymes of human and rat GlcNAc 2-peimerases. The middle domain of the GlcNAc 2-epimerase was important for the specificity and affinity for nucleotides [6]. Figure 1 shows the comparison of amino acid sequences of middle domain of human and rat GlcNac 2-epimerases. The middle domain of human and rat sequences are highly homologous. Only six amino acid residues are not identical. Thus, we constructed human (T145R, Q171S, P174L, C260A, E267K, and A270R mutants) and rat GlcNAc 2-epimerase mutants (R145T, S171Q, L174P, A260C, K267E, and R279A mutants) to identify the amino acid residue(s) conffering nucleotide binding.

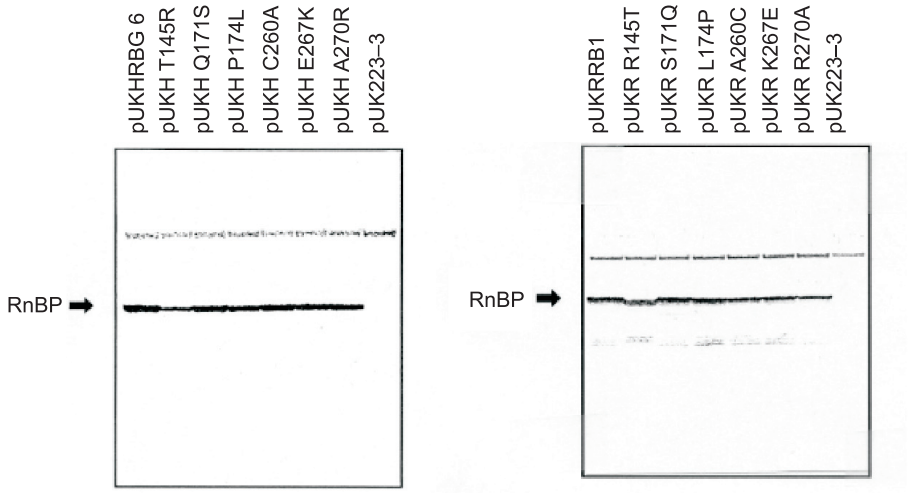

Figure 2. Western blotting of mutant human and rat GlcNac 2-epimerases *E. coli* extract harboring each plasmid was applied on the gel. pUK223-3, expression vector without insert DNA

Expression of Mutant GlcNAc 2-epimerases

An overnight culture (1 ml) of *E. coli* JM109 cells harboring an expression plasmid was used to inoculate 40 ml of 2 x YT (1.6% polypeptone, 1% Yeast extract, 0.5% NaCl, pH 7.4) containing 0.1 mg/ml ampicillin. The culture was incubated at 37°C for 5 h, and then expression of mutant protein was induced with 1 mM isopropyl-β-thiogalactopyranoside. Cells were harvested 3 h after induction. The cells were sonicated with 4 ml of 20 mM sodium phosphate buffer pH 7.0, containing 1 mM EDTA, 10 μM leupeptin, and 0.05% 2-mercaptoethanol, then cen-

trifuged at 20,000 x g for 30 min. The supernatant was used for Western blotting or assaying of GlcNAc 2-epimerase. When the extract of *E. coli* cells harboring mutant plasminds were used for the Western blotting, only single protein bands corresponding human and rat GlcNAc 2-epimerases were visible on the immunoblots (Fig. 2).

Effects of ATP on Mutants

To understand affinity of nucleotides on the mutant enzymes, the dose dependency of ATP on the mutant enzymes was investigated. Human and rat mutants were activated by ATP in a dose-dependent manner. Human T145R, P174L, C260A, E267K, and A270R mutants had nearly the same dose dependency curves to that of wild-type human enzyme. Human Q171R mutant had higher affinity for ATP than did the wild-type human enzyme. Rat R145T, L174P, A260C, K267E, and R270A mutants had nearly the same dose dependency curves to that of wild-type rat enzyme. But rat R145T and S171Q mutants showed lower affinity for ATP than did the wild-type rat enzyme (data not shown). These results indicate that residue 171 is critical for the nucleotide binding of GlcNAc 2-epimerases.

ACKNOWLEDGMENTS

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IN VITRO DEGRADATION OF CHITIN BEADS AND CHITOSAN FILMS

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INTRODUCTION

The degradation behavior of chitin and chitosan substrates is very important for biomedical applications such as controlled drug release, tissue engineering, and wound healing [1]. Enzymatic degradation of chitin is also interesting as an alternative to acid hydrolysis, used commercially to obtain amino sugars such as *N*-acetylglucosamine (NAG) and glucosamine, which are believed to possess therapeutic potential [2, 3]. Nevertheless, the number of studies on the degradation of potentially useful forms of chitin and chitosan, including beads and films, is very limited. The present study aims to investigate the degradation response of chitosan films and chitin beads to enzymatic activity and compare it to enzyme-free reference solutions. Enzymes found in human and other mammalian systems were used, namely lipase, lysozyme, and Flaviastase (an α-amylase-protease preparation simulating pancreatic enzymes). Other variables systematically tested were chitin concentration in the beads, enzyme concentration, pH, incubation period, and type of chitosan film. In addition, the effect of treatment with a crosslinking agent on degradation was studied.

EXPERIMENTAL

Preparation of Beads and Films

Chitin beads were formed by dropping 0.5, 1.0, and 1.5% (w/v) chitin-DMAc/LiCl droplets into ethanol, allowing for solvent exchange, rinsing several times with ethanol, filtering, and drying at 37°C [4]. Chitosan films were prepared by solvent evaporation at 37°C from chitosan solutions cast into Petri dishes. The degree of deacetylation of chitosan starting powders was 90%. Optimized chitosan solutions used for membrane formation were 1% (w/v) chitosan in 2% (w/v) acetic acid, 3% (w/v) chitosan in 3% (w/v) oxalic acid, and 3% (w/v) chitosan in 3% (w/v) ascorbic acid. Additionally, the former solution was crosslinked using

25 ppm and 125 ppm glutaraldehyde (GA). Similarly, some chitin beads were treated with 1% GA to see its possible effects on degradation.

Degradation Studies and Characterization

Duplicate samples ranging in weight from 2 to 10 mg were placed into glass tubes and immersed in 5 mL solution. All tests were performed using triple distilled water or solutions prepared from it. Phosphate buffer solution was used to keep pH at 7.4. This was not changed except for additional tests with Flaviastase at pH 1.2 to simulate the acidic conditions found in the gastric fluid [5]. Enzyme concentrations were 100 mg/L and 1000 mg/L. Samples were initially weighed with the aid of an Ohaus balance with a precision of 1×10^{-5} g and then incubated at 37°C for durations ranging from one day to 30 days. After the incubation period, samples were washed, dried at 70°C and weighed again to calculate the weight change. Solvent evaporation was carried out on some solutions at 90°C after the degradation tests in order to recover the degradation products. In some cases, cream, yellow, or dark brown colored sticky residues were recovered after drying. These were analyzed by FTIR spectroscopy, using KBr-pelletized powder samples and a Mattson Satellite 5000 FTIR instrument. Some of the corresponding films or beads cleaned in water and dried after the degradation periods were also studied by FTIR.

RESULTS AND DISCUSSION

Table 1 lists the degradation results for an incubation period of 1 day for chitosan films in different solutions with or without enzymes. It can be deduced from these results that in the case of chitosan films, the presence of enzymes in the solution makes only a small difference to weight reduction in one day. When the incubation period is longer, a significant difference can be noted, indicating enzymatic degradation. The effect of incubation period is more evident in the case of lysozyme solutions. In the case of chitosan film from acetic acid solutions, the amount of weight loss increased from 14.2% in one day to 28.3% in 20 days. The difference was not significant in the case of solutions containing lipase or Flaviastase. A similar trend was observed in the case of chitin beads. It can also be seen from Table 1 that crosslinking of chitosan films with glutaraldehyde (GA) decreases the degree of susceptibility to degradation. As can be seen from the table, as the amount of GA is increased, the weight reduction decreased in general.

In the case of chitin beads, weight reduction is expressed in units of mg/mm^2 (Table 2) instead of wt% to normalize the data since as the chitin concentration increased, so did the bead size. The amount of weight reduction did not change significantly with increasing chitin concentration either in water, acid solution or phosphate buffer. In enzyme solutions, on the other hand, the weight reduction was considerably higher for beads obtained from 1.5% solution than 0.5% and 1% chitin solutions. In addition to the susceptibility of the substrate to the enzyme, other factors that affect enzymatic degradation of chitin beads may be active such as bead morphology, network strength, and crystallinity. These factors in turn affect the swelling ability and the ease of diffusion of the solution into beads. As

the amount of polymer increases, stronger gels are expected to form that would be more resistant to degradative effects. While the crystallinity of beads obtained from 0.5% solution and 1% solution were the same (~70%), it was observed from their SEM photographs that beads obtained from 1% solution had cracks formed on them [4]. These cracks would allow easier diffusion of the enzyme solution into the beads making them more susceptible to enzymatic degradation. As a result of these two competing factors, increasing chitin concentration from 0.5% to 1% does not seem to affect the enzymatic degradability of the beads. However, when the concentration is 1.5%, the nonuniformity of the bead morphology predominates and the enzymatic degradability increases considerably.

Increasing the enzyme concentration from 100 mg/L to 1000 mg/L increased the amount of weight reduction in general. Unlike chitosan films, GA treated chitin beads lost more weight compared to untreated samples. This opposite behavior occurs presumably due to the decrease in crystallinity upon treatment with GA and easier degradation of the amorphous material. IR analysis supports this view since no crosslinking could be observed in chitin beads after treatment with GA and the beads of both types show no distinction after degradation.

Weight loss increased significantly in chitosan films incubated in acidic Flaviastase solutions as compared to Flaviastase solutions in phosphate buffer (Table 1). This implies that fast degradation of chitosan films can be obtained in gastric fluid.

Table 1. Average Weight Change (%) of Chitosan Films in Different Solutions*

Sample description**	W	A	P	LI100	LY100	F100	LI1000	LY1000	F1000	FA1000
1% Chitosan in 2% Acetic Acid Soln.	-14,1	-61,1	-8,8	-18,8	-15,0	-13,5	-9,1	-14,2	-10,4	-35,7
Same as above with 25 ppm GA			-1,4	-5,2	-2,2	6,9	-3,4	-5,0	-2,7	-16,2
Same as above with 125 ppm GA			-4,2		-3,4		-2,3	-4,6	-4,0	-3,2
3% Chitosan in 3% Oxalic Acid Soln.	-96,0	-100,0	-67,6				-45,3	-51,9	-53,3	-100,0
3% Chitosan in 3% Ascorbic Acid Soln.	-46,6	-52,9	-46,3				-45,7	-43,6	-46,7	-55,2

** W-Triple distilled water; P-phosphate buffer; A-pH 1.2 HCl in dist.water; LI100-lipase 100 mg/L in phosphate buffer; LI1000-lipase 1000 mg/L in phosphate buffer; same nomenclature for LY (lysozyme), F (Flaviastase), and FA (Flaviastase solution at pH = 1.2); GA - glutaraldehyde; weight change shown in percent, negative sign indicates a decrease while no sign indicates weight gain*
*** Films obtained from the solutions described*

Table 2. Average Weight Change (mg/mm^2) of Chitin Beads in Different Solutions

Sample description	W	A	P	LI100	LY100	F100	LI1000	LY1000	F1000	FA1000
0.5% chitin in DMAC-LiCl	-0,015	-0,033	-0,013	-0,002	-0,003	-0,013	-0,007	-0,011	-0,006	
1% chitin in DMAC-LiCl	-0,019		-0,012	0,001	-0,012	-0,005	-0,003	-0,010	-0,004	-0,001
1.5% chitin in DMAC-LiCl	-0,019	-0,028	-0,014	-0,009	-0,010	-0,011	-0,024	-0,047	-0,024	
1,5% chitin in DMAC-LiCl, treated with GA		-0,056	-0,051				-0,053	-0,069	-0,056	

** see Table 1 for abbreviations*

The spectrum of the chitosan film treated with the enzyme gives evidences for the degradative effects of the enzymes on the film. The amide I band at 1650 cm^{-1} shifts to 1633 cm^{-1} (Fig. 1). The bands at 1580 cm^{-1} and 1494 cm^{-1} which may be attributed to the C=C and C=N unsaturation due to the crosslinking reaction disappear and a new band appears at 1521 cm^{-1} (N-H bending vibrations) indicating chemical changes on the film. These changes could be taken as evidence for the increase in the fraction of the deacetylated units on the chitosan backbone. So, it can be proposed that enzymatic degradation of chitosan in Flaviastase solution involves mainly chain scission at the NAG bearing units. The weakening of $-CH_3$ absorptions in the region 1300–1400 cm^{-1} and at 2900 cm^{-1} support this view. The loss of peaks at 1580, 1494 and 863 cm^{-1} shows that the crosslinkages are also susceptible to degradation. The loss of the etheric stretching at 1039 cm^{-1} is a strong evidence of the cleavage of the glycosidic linkages of chitosan. The spectrum of the degradation product recovered from solution (Figure 1 (c)) exhibits a carbonyl stretching band at 1711 cm^{-1}, showing the formation of reducing sugars bearing an aldehyde group [6]. The amide I band at 1630 cm^{-1} and the C-O stretchings in the 1150–1030 cm^{-1} region confirm the formation of chitooligomers as degradation products.

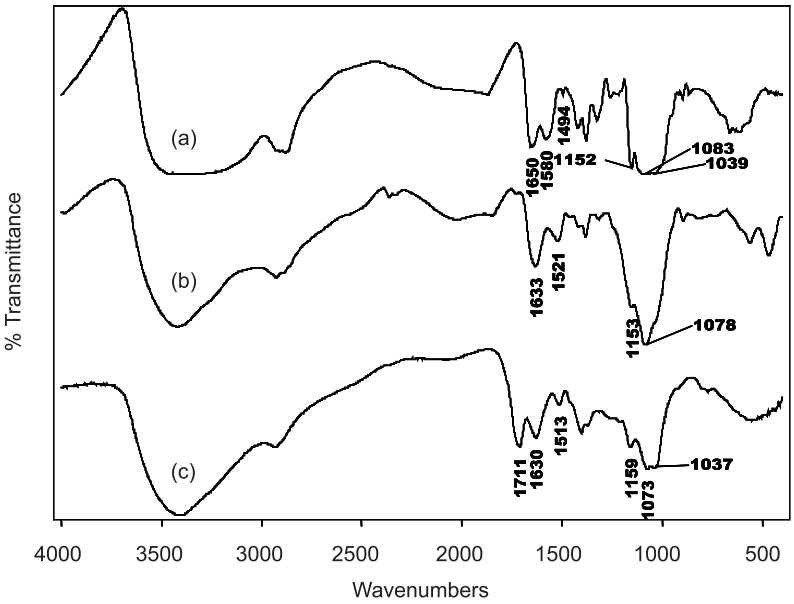

Figure 1. FTIR spectra of (a) chitosan film formed from 1%w/v chitosan/acetic acid solution crosslinked with GA (b) same film after incubation in 1000 mg/L Flaviastase solution under acidic conditions for 1 day, and (c) degradation product recovered by solvent evaporation

CONCLUSIONS

Chitosan films and chitin beads lost up to 28% and 15% of their weights, respectively, in lysozyme solutions in 20 days compared to 14% and 3%, respectively, in 1 day. Crosslinking of chitosan films with GA decreased enzymatic degra-

dation as a likely result of a stronger polymer network. Treatment of chitin beads with GA, on the other hand, increased degradation, which can be attributed to reduction in crystallinity. Degradation products from chitosan films (obtained from chitosan-acetic acid solutions) and chitin beads were identified as chitooligomers in most cases. Chitosan salt films (from oxalic and ascorbic acid solutions) dissolved much faster than other substrates and degradation proceeded in solution.

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A MATHEMATIC MODEL FOR CHEMICAL DEACETYLATION OF CHITIN AT RELATIVELY LOWER TEMPERATURES

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Key words: Mathematic model, Chemical deacetylation, Chitosan, Lower temperatures

INTRODUCTION

Chemical deacetylation of chitin is currently carried out at elevated temperatures often around 100°C [1–3]. Although enzymatic deacetylation has been proposed, it still needs to be more economic and practical. We have chosen three lower temperatures (288, 298, 308K) for deacetylation reaction instead of high temperatures, which cause several process problems. We proposed a quantitative empiric equation for the degree of deacetylation with respect to reaction time and temperature for each particle size. The mathematic formula was proved to be in good agreement with the experimental values.

EXPERIMENTALS

Deacetylation

Chitin of red crabs was obtained from local agent whose degree of deacetylation (DDA) was 42%. The chitin was grinded into 3 particle sizes, namely, 300–850 μm, 150–300 μm and 150 μm. The resulting groups were treated with 50% NaOH at 288K, 298K and 308K for different reaction times. Deacetylation was measured with IR method. To quantify the relationship between DDA and reaction parameters, we assumed the following model:

$$DDA = A \times \exp[-(1+t)^{(\alpha+\beta T)}] \quad (1)$$

t: reaction time, T: absolute temperature, A, α and β: constant

Molecular weight determination

Molecular weight was determined by size exclusion chromatography using TSK gel G4000PW column, whose temperature was 308K. Flow rate was 0.4 ml/min and 50 μl of the samples were injected for analysis.

RESULTS AND DISCUSSION

As seen Fig. 1, there was almost no difference in DDA between the particle size of 150 μm and 300 μm. However for the size of 850 μm, DDA was lower than those of smaller particles. It is reported that 68% of DDA were obtained after a 40 hour treatment at 30°C with cuttlefish chitin [4]. This value of DDA in cuttlefish at 30°C is very close to those obtained in this experiment.

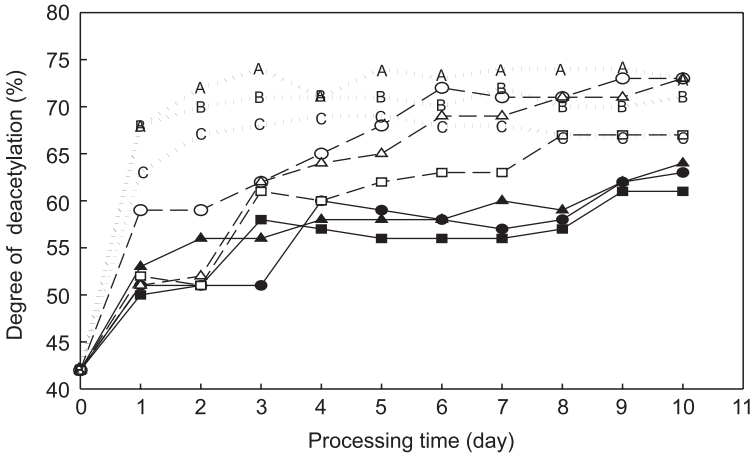

Figure 1. Effect of processing time on DDA of chitosan-150, chitosan-300 and chitosan-850. 150, 300 and 850: Particle size in μm; 288, 298, and 308: Temperature in K

— ● —: 150–288. — ▲ —: 300–288. — ■ —: 850–288.
— ○ —: 150-298. — △ —: 300–298. — □ —: 850–298.
— A —: 150–308. — B —: 300–308. — C —: 850–308.

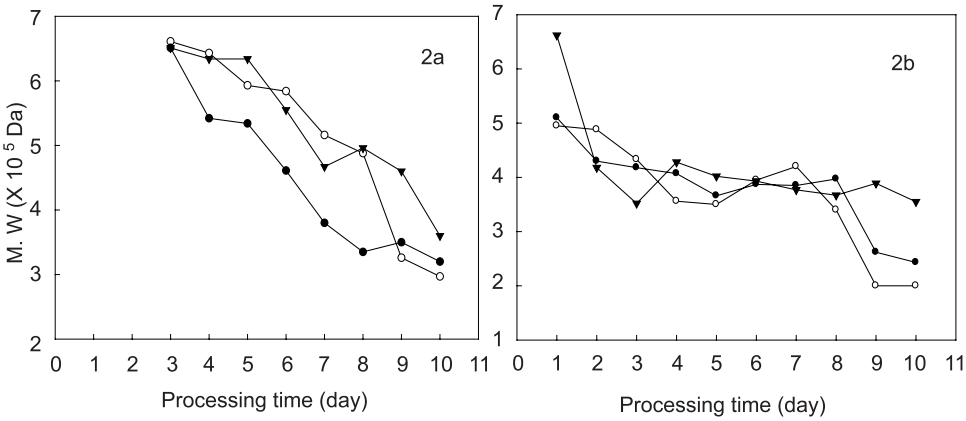

Figure 2. Effect of processing time on molecular weight of chitosan produced at different temperatures

2a: 298K. — ● —: chitosan–150, — ○ —: chitosan–300, — ▼ —: chitosan–850
2b: 308K — ● —: chitosan–150, — ○ —: chitosan–300, — ▼ —: chitosan–850

The solubility of chitosans produced at 288K was not improved with the reaction time. At 298K, the chitosans started to be soluble after 2-day reaction, while at 308K only one day was enough to produce soluble chitosan, regardless of the particle size (Data not shown).

As seen in Fig. 2a, at 298K the molecular weight was almost linearly diminished with reaction time. However, at 308K no change in MW was observed between day 3 and day 8 and thereafter a sharp decrease was seen for the particle size of 150 and 300 μm (Fig. 2b).

Mathematic model for DDA

As seen above, DDA increased at the early stage of the reaction and reached at the plateaus. It is also noticed that higher temperature increased DDA and shortened the reaction time.

We determined the constants (A, α and β) of the equation (1) from Fig. 1 and the values were given in Table 1.

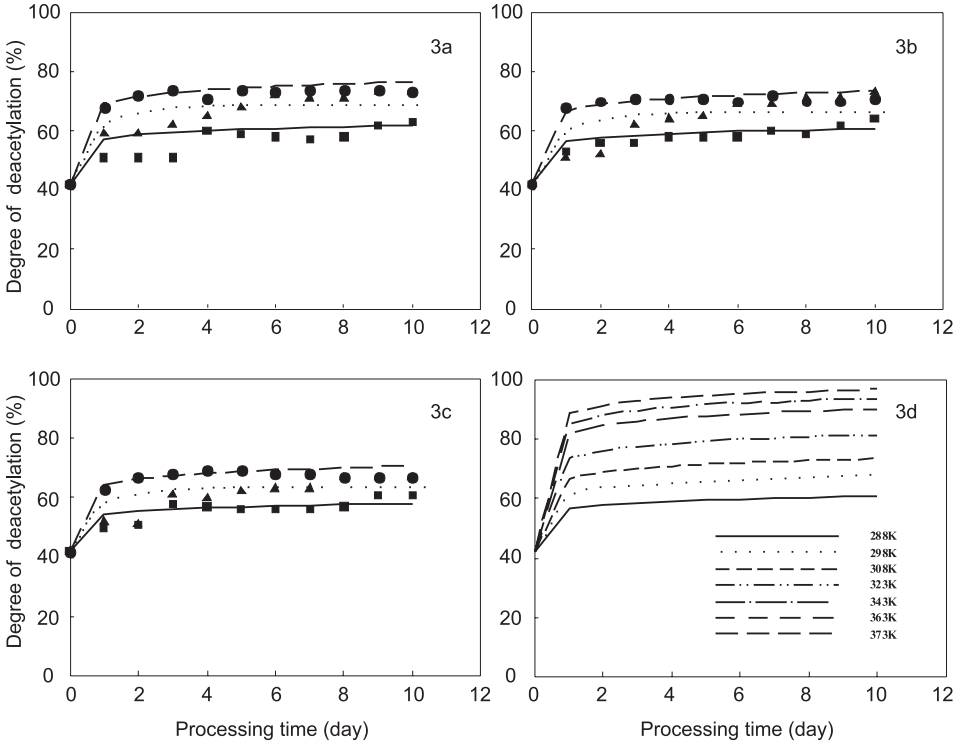

Figure 3. Comparison of experimental DDA with estimated DDA and estimation of optimal parameters for the size of 300 μm 3a: 850 μm particles, 3b: 300 μm particles 3c: 150 μm particles 3d: Estimation of optimal parameters for DDA of 300 μm particles. —■—: 288K. —▲—: 298K. —●—: 308K

Comparisons of experimental DDA with estimated ones were made in Fig. 3a, 3b and 3c. The best concordance between experimental and estimated values was observed at 308K, regardless of the particle size. For the given particle size, higher DDA was always obtained at 308K than at 288K and 298K.

As seen in Fig. 3d, an estimation of optimal temperature and reaction time was made for DDA of the particle size 300μm. DDA increased as the reaction temperature became higher. However for a given temperature, DDA remained

constant after one day of reaction (Fig. 3d). To obtain DDA above 80%, the reaction temperature must be above 343K (Fig. 3d).

Table 1. Values of the constants in Equation (1), DDA = A x exp[–(1+t) $^{(\alpha+\beta T)}$]

Constant	Particle size (μm)		
	150	300	850
A	42	42	42
α	0.59087	0.49325	0.48048
β	−0.00223	–0.00188	–0.00181

CONCLUSIONS

Since there were no observable differences in DDA between 150 μm and 150–300 μm, the particle size 150–300 μm was considered as optimal for chitin deacetylation at lower temperatures. When the chitin of 300 μm was deacetylated at 308K for one day, the DDA was about 70%. According to the estimated results by the proposed equation, optimal reaction time for 300 μm particles was also one day and reaction temperatures can be adjusted along with the final DDA.

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COMPARISON OF THE INHIBITORY ACTIVITY OF CHITOSAN ON LACTIC ACID BACTERIA AND YEASTS

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Abstract: Yeasts and lactic acid bacteria are major microbial groups that are found in most fermented products. A variety of indigenous fermented foods and beverages are made using the activities of both yeast and lactic acid bacteria, either simultaneously or successively. It is extremely difficult to control microbial species during fermentation, especially spontaneous fermentation due to the complexity of the microorganisms involved [1]. Therefore, we compared the antimicrobial activity of chitosan against two lactic strains, *Lactobacillus plantarum* and *Leuconostoc mesenteroides,* and two yeast strains, *Hanseniaspora guillermondii* and *Candida zelanoides* from korean fermented foods, to investigate the possible use of the non-toxic biopolymer chitosan for selective control [2]. In addition, the effect of food additives on the antimicrobial activity of chitosan was determined.

Key words: lactic acid bacteria, yeast, antimicrobial activity, chitosan

INTRODUCTION

In many countries, a variety of indigenous fermented foods and beverages are made using the activities of both yeast and lactic acid bacteria, either simultaneously or successively. In this study, we have investigated the antimicrobial effect of lomolecular-weight chitosans against lactic acid bacteria and yeast isolated from typical korean traditional fermented foods for the possible use of the non-toxic biopolymer chitosan for control of microflora of food fermentation. Lactic strains were isolated from kimchi, traditional fermented vegetables, and yeast strains from soybean paste “doenjang”.

MATERIAL AND METHODS

Chitosan Preparation

Low molecular weight chitosans A (MW 3,000), B (MW 10,000) and C (MW 50,000) were obtained from Amicogen Inc.(Jinju, Korea). These chitosans had minimum degree of deacetylation 95%. Stock solution containing 1,000ppm of each chitosans in 0.5% acetic acid was sterilized by membrane filtration.

Strains

Lactobacillus plantarum and *Leuconostoc mesenteroides* from the Lab of applied microbiology, Ajou University were subcultured twice in TS broth for 16 h at 30°C for all experiments. Yeast strains were isolated from korean home-made doenjang using YM agar at 30°C.

The isolates were identified with the Biolog automated identification system (Biolog Inc., Hayward, CA,USA). The strains were stores at YM agar at 4°C. Stock cultures were propagated twice in TS broth (pH 6) for 16 to 18 h before each experiment.

Inhibition test

Chitosan A, B, C from stock solution were added to TS Broth at different concentrations from 25 ppm up to 2,000 ppm, and an inoculum of 16–18h culture was added to the broth to give a final concentration of 106 cells per ml. The inoculated broth was incubated at 30°C and cell growth was determined by measuring optical density at 600 nm.

RESULTS

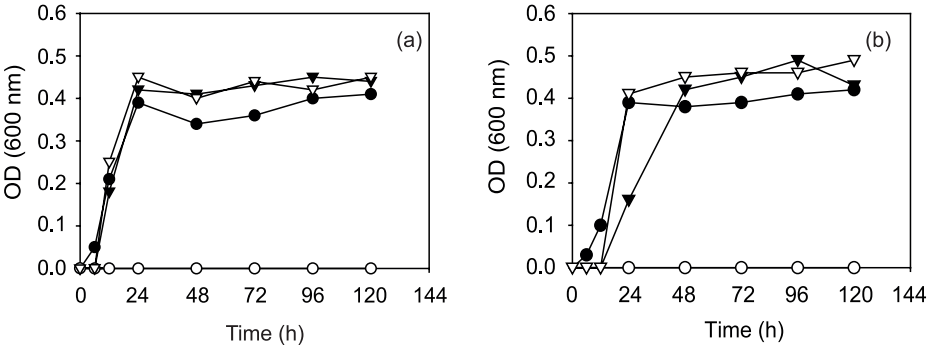

Figure 1. Inhibition of Lactic acid bacteria, *Lactobacillus plantarum* (a) and *Leuconostoc mesenteroides* (b) in by 50ppm of chitosan (●: control ○: 3 kDa chitosan ▼: 10 kDa chitosan ▽: 50 kDa chitosan)

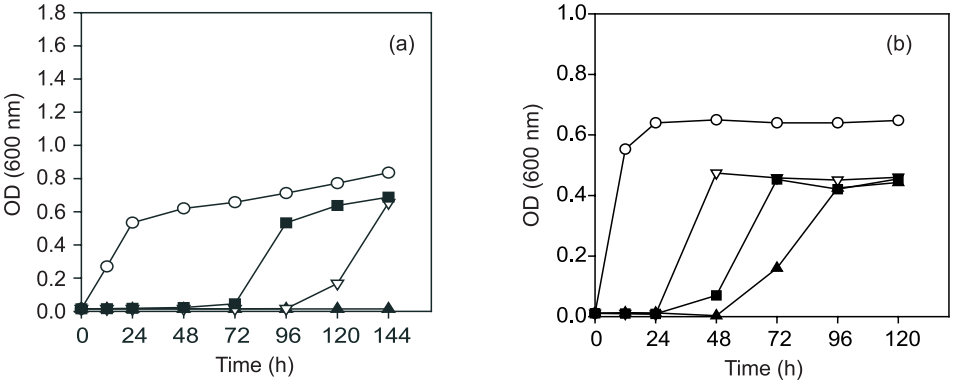

Figure 2. Inhibition of yeast *Candida zeylanoides* SW 1j (a) and *Hanseniaspora guillermondii* S 9–3 (b) by 50 ppm and 300 ppm of chitosan respectively (○: control ▲: 3 kDa chitosan ▽: 10 kDa chitosan ■: 50 kDa chitosan)

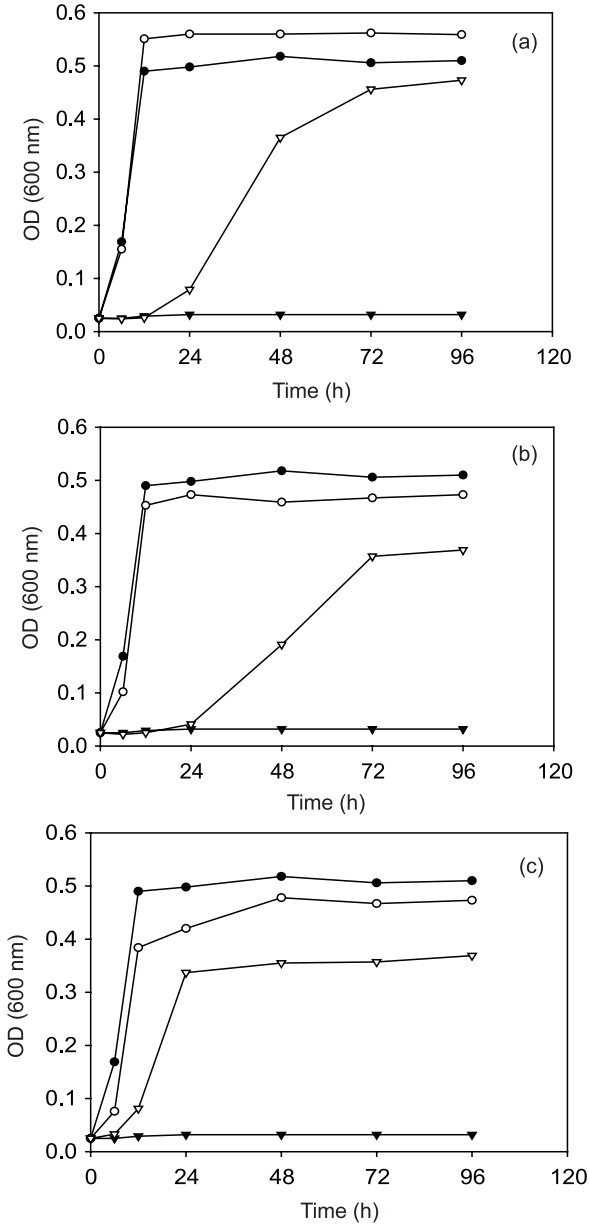

Figure 3. Influence of 2% sucrose (a), ethanol (b) and NaCl (c) on the inhibitory activity of 100 ppm chitosan against *Hanseniaspora guillermondii* S 9–3(●: control ○: sucrose, ethanol and NaCl added ▼: chitosan added ▽: sucrose, ethanol, NaCl and chitosan added)

CONCLUSIONS

The chitosan sensitivities of the lactic acid bacteria and yeast were variable. The growth of both organisms were more effectively inhibited by chitosan of MW 3,000 than did chitosan of MW 10.000 or MW 50,000. However, the lactic

acid bacteria were more sensitive to the inhibitory activity of chitosan. The results suggest the possible use of low molecular-weight-chitosan for the control of food fermentation in which both groups of organisms frequently occur together.

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CHAPTER VII

CHEMISTRY OF CHITIN AND CHITOSAN

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PREPARATION OF CHITOSAN-HYBRIDIZED ACRYLIC RESINS IN EMULSION POLYMERIZATION AND THEIR ADSORPTION CHARACTERISTICS FOR FORMALDEHYDE

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Key words: Chitosan, Emulsion polymerization, Formaldehyde, Adsorption, Emission rate

INTRODUCTION

Recently, in living environmental, there has been an increased as sick-building syndrome which occurs by indoor air quality containing harmful substances [1]. This cause is the deficiency of ventilation occurred by high airtight and high heat insulation, and the solvents and antiseptics which are contained in furniture and building materials. The reduction of various organic solvents and toxic chemicals contents in building materials is expected. Especially, the elimination of volatile organic compounds (e.g. formaldehyde and so on.) in adhesives and interior finishing coatings is strongly required for our health [2]. The interior finishing coatings are required water-based system without contained volatile organic compounds. Therefore, it is very important to develop the high quality interior coating of safety. Chitosan is an effective natural cationic polymer for the adsorption ability of formaldehyde and an environmental friendly material. However, in general, when cationic polymers are applied to waterborne coatings using acrylic emulsion, the precipitates are formed. To resolve this problem, chitosan-hybridized acrylic resins were investigated in the emulsion polymerization between chitosan-acrylic acid (AA) ion complex and acrylic monomers. In this study, their formaldehyde adsorption ability and emission are discussed.

EXPERIMENTAL

Chitosan- hybridized acrylic emulsion and coating material were prepared by a previously described procedure [3].

The adsorption experiment for formaldehyde was performed in 10 L of the tedler-pack.

Chitosan-hybridized acrylic emulsion was coated on 15 cm × 15 cm of a glass plate and were dried at room temperature. Two pieces of the glass plates were put into the tedler-pack and formaldehyde gas was injected into it. After a while, the DNPH cartridge was connected to tedler-pack outlet, and the concentration

of formaldehyde in the tedler-pack was measured. After the DNPH derivative in a DNPH cartridge was desorbed using acetonitrile, it is dissolved.

The formaldehyde concentration were measured by HPLC (Shimadzu). Analysis conditions were shown in Table 1.

Table 1. Analysis condition of HPLC

Detector	UV-VIS DETECTOR SPD-10A VP
Column	STR-ODS II (4.6ø×150mm)
Mobile phase	1.0 mL/min, acetonitrile/water=60/40
Column temperature	40°C
Detector wave number	360 nm
Injection volume	20 μL

Table 2. Small chamber condition

Surface area	0.044 m^2
Air volume in a small chamber	0.02 m^3
Product loading factor	2.2 m^2/m^3
Ventilation rate	167 mL/min
air exchange rate	0.5/h
Sampling period	360 min

The determination of the emission of formaldehyde was performed using a small chamber method. This method is equivalent to ENV 13419-1:1999. The small chamber method conditions were shown in Table 2. The area specific emission factor at a given time, t, after placing the test specimens in the small chamber can be expressed as in equation (1).

Area specific emission rate

$$(EF) = (C_t - C_{tb,t}) \times n/L \quad (1)$$

where C_t is the concentration of formaldehyde at a given time, $C_{tb,t}$ is a travel blank concentration at a given time, n is ventilation rate, L is product loading factor.

RESULTS AND DISCUSSION

Adsorption ability for formaldehyde

The chitosan-hybridized acrylic emulsions with chitosan contents of 0 wt%, 3 wt%, 5 wt% and 10 wt% in solid were synthesized. After their films were formed, adsorption tests of their films for formaldehyde were carried out. Figure 1 shows the result of the adsorption performance of formaldehyde with each chitosan-hybridized acrylic resin film. In the adsorption test of the chitosan-hybridized acrylic resin films, the formaldehyde concentration in the atmosphere almost became 0 ppm in 30 min. On the other hand, in the adsorption test of the

film without chitosan, the formaldehyde concentration gradually decreased, but it took 180 min. to reach 0 ppm.

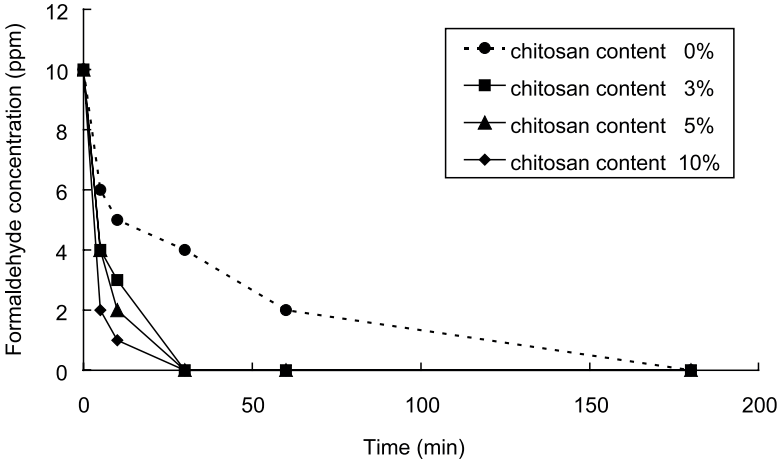

Figure 1. Adsorption performance of the chitosan-hybridized acrylic resin film for formaldehyde

Emission of formaldehyde

The chitosan-hybridized acrylic resin film with chitosan contents of 3wt% and coating material film using their absorbed by initial formaldehyde concentration of 19.7 ppm were examined. To remove formaldehyde, which was physically adsorbed into the film, atmosphere in the tedler-pack was exchanged by nitrogen gas three times. Table 3 shows these results.

Table 3. Adsorption performance of the chitosan-hybridized acrylic resin film and coating material film by initial formaldehyde concentration of 19.7 ppm

Sample	Formaldehyde concentration after 24 hours	Release of folmaldehyde after exchanged by nitrogen gas		
		1	2	3
Glass plate	8.67 ppm	2.09 ppm	1.06 ppm	0.35 ppm
Chitosan hybridized acrylic resin film	0.14 ppm	0.15 ppm	0.12 ppm	0.11 ppm
Coating material	0.11 ppm	0.08 ppm	0.07 ppm	0.07 ppm

And then, emissions of formaldehyde from these samples were carried out in the small chamber. In Figure 2, emission factor of formaldehyde from chitosan-hybridized resin film and coating material under various conditions after 1 day are showed.

Emission factor from most samples were about 10 $\mu g/m^2h$. Mass of adsorption in each sample was much, but low emission factor were showed. In this case, formaldehyde, which was physically adsorbed into the films, would be released. On the other hand, formaldehyde, which was chemically adsorbed, would not be released.

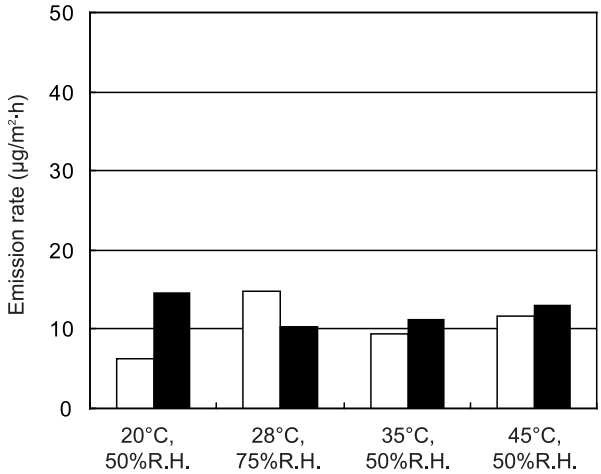

Figure 2. Emission of formaldehyde from the coating material and the chitosan-hybridized acrylic resin film. (□):coating material, (■): chitosan- hybridized acrylic resin film

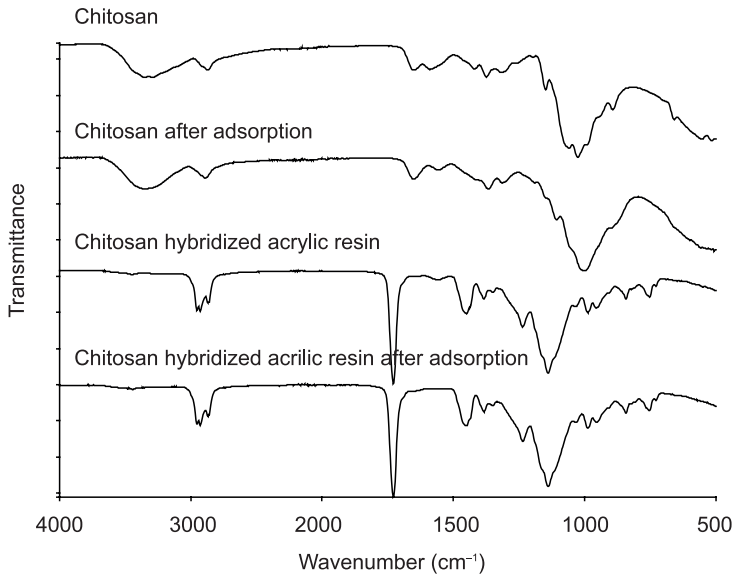

Figure 3. FT-IR spectra of chitosan, chitosan hybridized acrylic resin before and after adsorption for formaldehyde

In Figure 3, FT-IR spectra of chitosan-hybridized acrylic resin before and after adsorption for formaldehyde are showed. The chitosan-hybridized acrylic resin has strong and weak peaks based on acryl resin and chitosan, respectively. Moreover, the chitosan hybridized acrylic resin has weak peak of amino group band at 1,580 cm^{-1}. On the other hand, a chitosan -hybridized acrylic resin, which adsorbed formaldehyde, doesn't have peaks based on amino group, because of a combination between chitosan and formaldehyde. It was difficult to make a sure to combine between chitosan and formaldehyde in the chitosan- hybridized acrylic resin directly, because the chitosan-hybridized acrylic resin had IR adsorption for acryl resin. Therefore, FT-IR spectra of chitosans before and after adsorption

test for formaldehyde were measured. The both chitosans have peaks based on glucosamine structure and acetyl amino group. Chitosan shows a peak based on amino group band at 1,580 cm^{-1}. Chitosan adsorbed formaldehyde doesn't show the peak but a peak based on C=N band at 1,600 cm^{-1}. It was ensured that $-N=CH_2$ was made from an amino group of chitosan and a formaldehyde.

CONCLUSIONS

Adsorption experiment for formaldehyde and emission of formaldehyde using a small chamber were studied. Chitosan hybridized acrylic resin film showed high adsorption ability for formaldehyde. Emission factors of formaldehyde from chitosan hybridized resins and coating material were not depended on experimental conditions, for example temperature, humidity and so on. A chemical combination between chitosan and formaldehyde might be stable.

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CHARACTERIZATION OF BIODEGRADABLE PHOTO-CURED CHITOSAN FILMS WITH ACRYLIC MONOMERS

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Abstract: Natural polymer, chitosan was obtained from dried prawn shell waste through the preparation of chitin and was characterized. Thin film of chitosan was prepared by casting method from its 2% chitosan solution. Mechanical properties like Tensile strength (TS), elongation at break (Eb) of chitosan film were studied. Five formulations were developed with 2-ethyl 2-hydroxy methyl 1,3-propandiol trimethacrylate (EHMPTMA), a trifunctional monomer and 2-ethylhexyl acrylate (EHA), a monofunctional monomer in the presence of photoinitiator Darocur-1664 (2%). The film was soaked in those monomer formulations in dissimilar soaking times and irradiated under UV-radiation at different radiation intensities for the improvement of the properties of chitosan film. The cured films were then subjected to various characterization tests like TS, Eb, polymer loading (PL), water absorbency, gel content etc. The formulation, containing 25% EHMPTMA and 73% EHA showed the best performance at 10th UV passes of UV radiation for 4 minutes soaking time.The cured films were then characterized by X-ray diffraction, solid-state ^{13}C CP/MAS-NMR, FTIR, DSC and SEM. Various degradable tests were also performed.

INTRODUCTION

Chitosan, β-1,4-linked glucoseamine, is the second most abundant polysaccharide found on the earth next to cellulose. Chitosan has a number of unique properties which enable chitosan to attract scientific and industrial interest in different fields. There has been a growing interest in grafting of vinyl monomers onto chitosan for biomedical and industrial applications [1–2]. Prawn production and catching has grown as an important income for Bangladesh. Bangladesh, the largest delta country of the world having 724 km long costal border and Riverine County too. Large quantities of prawns are produced in Bangladesh. Approximately, 45% of total weights of prawn are west and majority of the wastes are deposited offshore or inland causing pollution. The aim of this research is to extract chitin and chitosan from waste prawn shell and to prepare biodegradable polymers plus to reduce the fishery waste and environmental pollution.

MATERIALS AND METHODS

Chitin was extracted from washed and dried prawn shell powder by deprotenization with 3% NaOH and demineralization with 3% HCl. Chitosan was prepared from the extracted chitin by deacetylation with NaOH. Both the extracted chitin and chitosan were characterized by FTIR. Chitosan films were developed by casting method from acidic aqueous solution of chitosan. Chitosan films were cured with different formulations prepared by 2-ethyl 2-hydroxy methyl 1,3-propandiol tri-methacrylate (EHMPTMA) and 2-ethylhexyl acrylate (EHA) acrylic monomers in the presence of photoinitiator Darocur-1664 (2%) under UV-radiation Table 1.

Table 1. Composition of Different Monomer Formulation (%w/w)

Formulations	EHMPTMA	EHA	Darocur-1664
M1		98	2
M2	25	73	2
M3	50	48	2
M4	75	23	2
M5	98	–	2

RESULTS AND DISCUSSION

Optimization of monomer formulation

Monomer formulation plays an important role because it affects the polymerization rate and the overall conversion as well as the properties of cross-linked polymerization. Chitosan films were soaked in different formulations for different soaking time (1, 2, 3, 4 and 5). The chitosan films were soaked in different formulations (M1 to M5) for 4 minutes soaking time to optimize a definite formulation for curing of the chitosan film. After soaking, the films were cured under UV radiation at different intensities (2, 4, 6, 8, 10, 12 and 15 passes). After 24 hours of radiation the samples were subjected to different characterization tests to find an optimum formulation.

Polymer Loading (PL)

The results of PL values of the cured film are presented in Fig. 1 against number of UV radiation passes as a function of monomer formulation for 4 minutes soaking time. It is observed from the Fig.1 that, the highest PL is found 49 with the treatment of formulation M2, at 10th UV passes for 4 minute soaking time. In all cases, the maximum PL values are obtained at 10th UV passes. Beyond 10th pass, the PL values decreases. This may be due to the radiation degradation of the film at higher UV passes [1]. Triacrylate possesses more cure speed than monoacrylate [2]. Percentage of PL increases with EHMPTMA, a denser trifunctional monomer, up to 25% but more than 25% EHMPTMA, reduces the curing of chitosan film. At low concentration, a multifunctional vinyl monomer like EHMPTMA promotes reaction with the help of photoinitiator leading to network poly-

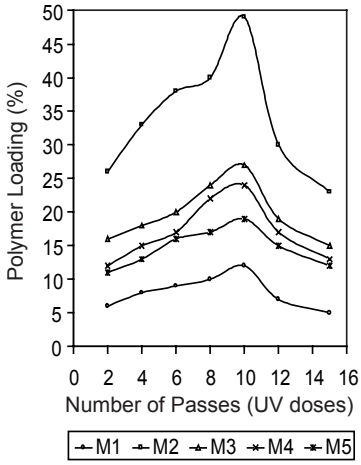

Figure 1. PL(%) vs. radiation regarding formulation for 4 min. soaking time

mer structure through curing via their double bonds [3]. As the increase of EHMPTMA concentration, the amount of residual unsaturation is also increased with the consequence of faster rate of formation if tree dimensional network causing restricted mobility. The decrease in PL values at higher EHMPTMA concentration may be caused that radical-radical recombination process may be dominating, thus creating homo-polymer rather than monomer-chitosan backbone reaction, where the hydroxyl and amino group of chitosan may be react with vinyl radicals in EHMPTMA.

Tensile Strength

The results of TS values are shown in the Fig.2, where TS values are plotted against number of UV passes as a function of monomer formulation for 4 minutes soaking time. The highest TS value was 29.1 MPa, achieved with the monomer formulation M2, at 10th UV. The presence of hydroxyl and amino group of chitosan film may be allow for photo-curing reaction with EHMPTMA, a more cross-linking denser trifunctional monomer in EHA. The hardness of the UV-cured polymer film depends on the functionality of the acrylic monomer. In this case, the chitosan film become brittle, twisted and shrinkage after 25% EHMPTMA concentration and the TS decreases. This may be due to the fact that, the homo-polymerization reaction between EHMPTMA and EHMPTMA radical is dominant and the reaction of chitosan film with EHMPTMA is less prominent. It is observed that the TS value of the chitosan film increases with the increase in radiation intensities and after attaining a maximum level (10th pass), the TS value decreased. This may be due to the degradation of polymer at higher radiation doses and the film become hard, twisted and brittle.

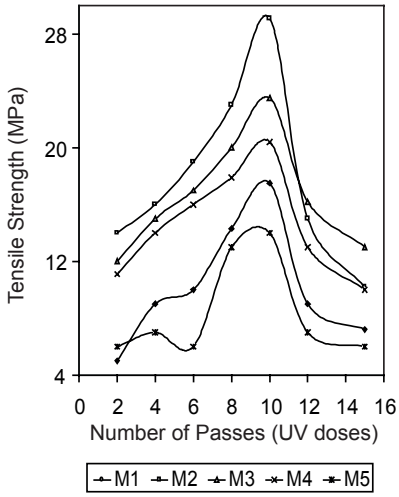

Figure 2. TS (MPa) of chitosan vs. UV regarding formulation 4 min. soaking time

Elongation at Break (Eb)

The results of Eb are plotted in Fig.3 against number of passes as a function of monomer composition for 4 minutes soaking time. The maximum Eb 16% of the chitosan film is observed with the monomer formulation M1, at the 10th passes of UV radiation. There is a rise in Eb at the initial stage of UV radiation like TS value. The Eb value increases with the number of UV passes and after this, Eb value decreases. M2, the treated film that gives higher TS; but does not give higher Eb. The lowest Eb value 4.2% is obtained at M5, containing 98% EHMPTMA. TS achieved the overall cross-linking network within the cured film. EHMPTMA gives the highest TS and this produces brittle film that crack easily during stretching. On the other hand EHA increase high elasticity and impart some flexibility. So, it is clear that TS and Eb are very much dependent on the nature of the formulation, where the monomer is monofunctional or multifunctional. The combination of EHMPTMA and EHA at the ratio of 25 and 73 (M2), yields the suitable condition for better cross-linking phenomenon at the equilibrium condition that create the chitosan film with highest TS and moderate Eb.

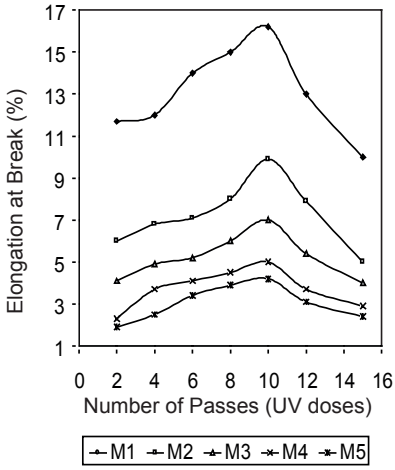

Figure 3. Eb(%) of the chitosan vs. UV regarding formulation 4 min. soaking time

Optimization of Soaking Time

On having the highest TS and PL at M2 formulation, the chitosan films were soaked in M2 for different soaking time and then irradiated under UV radiation with different number of passes.

Polymer Loading (PL)

The results are graphically represented in the Tabl21 where PL values are plotted against number of UV radiation pass as a function of soaking time for optimized monomer formulation, M2. It is observed that the PL values increase with soaking time until it reaches to 4 minute and give maximum value 49 at 10th UV pass and above 4 minute soaking time PL value decreases with increasing soaking time. Soaking increases the cross-section area of the film. As a result monomer can easily diffuse in the chitosan film and may be react with hydroxyl and amino group of chitosan in low swelling time. In higher soaking time, the film becomes twisted, shrinkage and pale to look [18]. The PL value increases with initial UV radiation doses, attain the maximum value at 10th passes of UV radiation and then decreases as the radiation doses increases. This may be caused the radiation degradation at higher UV doses.

Tensile Strength (TS)

The results of TS values are represented in Table 2, The highest TS value 29.1 MPa is achieved with 4 minutes soaking time at 10th pass followed by 23 MPa for

3 minute soaking time at the same UV radiation doses.TS value increases with the increase in soaking time. This may be due to the increase soaking, lead to increase diffusion of monomer into the sites of reaction and the amount of curing increases [18]. Hence, the TS value increased. After attaining the maxima, TS value decreases with increase in soaking time. TS value also increase with the increase of number of passes up to the 10th pass beyond which it decreases as the radiation passes are increased.

Elongation at Break (Eb)

The Eb values are plotted in Fig.6 against number of passes as a function of soaking time. For M2, Eb increases with the increase in soaking time and it reaches maxima 9.9% at 10th UV passes for 4 minute soaking time then decreases. It indicates that, in 4 minute soaking time, the inherent character of the film appears to be superior as compared to other soaking times. So optimization is established for 4 minute soaking time and monomer formulation M2, containing 25% EHMPTMA, 73% EHA and 2% Darocur-1664.

Table 2. Effect of soaking on PL, and mechanical properties of cured chitosan film

No. Pass	Soaking time in minute														
	1			2			3			4			5		
	PL (%)	TS (MPa)	Eb (%)	PL (%)	TS (MPa)	Eb (%)	PL (%)	TS (MPa)	Eb (%)	PL (%)	TS (MPa)	Eb (%)	PL (%)	TS (MPa)	Eb (%)
2	11	8.1	4.1	16	9	4.5	23	10.3	5.1	23	14	6	20	9	5.3
4	13	8.9	4.3	19	11	4.7	24	14	5.7	24	16	6.8	21	12	5.9
5	16	11	4.8	22	13	5	29	16	6.1	29	19	7.1	26	14	6.4
8	17	13	4.9	23	15	5.4	31	20	6.3	31	23	8	29	17	6.9
10	19	15	5.1	26	19	5.9	35	23	7	35	29.1	9.9	31	19	7.3
12	13	10	4.4	21	12	5	24	19	5.3	24	15	7.9	22	13	5.8
15	10	9	3.3	15	10	4.1	19	13	4.9	19	23	6	16	11	4.8

CONCLUSIONS

Mechanical properties like tensile strength (TS 7.5 MPa), elongation at break (Eb, 8%) of chitosan films were studied. The film cured with formulation, containing 25% EHMPTMA and 73% EHA showed the best performance at 10th UV passes of UV radiation for 4 min soaking time. The cured films showed the enhanced TS (29 MPa) and Eb (10%) along with 49% polymer loading and 98% gel content. The cured films were then characterized by X-ray diffraction, solid-state ^{13}C CP/MAS-NMR, FTIR, DSC and SEM. The X-ray diagrams of treated and untreated chitosan are comparatively same but additional lines with low crystal orientation are seen in cured film. Smooth surface without crack and particle deposition are observed in SEM photograph of cured chitosan films. The SEM picture of the cross-section of films demonstrate same differences. Diminutive

variations are perceived in decomposition temperature and enthalpy values of treated and untreated films. No noteworthy difference is scrutinized in NMR spectra. The degradable properties of both virgin and grafted films in various environmental conditions such as in soil, water as well as simulating weathering, water uptake as well as get content and swelling properties were studied.The grafted film shows the minimum loss of mechanical properties and weight. The TS of the cured film is 29 MPa, which, is about 300% higher that that of untreated one and it retains its properties in water but highly degradable in soil. The photo-cured films are biodegradable with superior mechanical properties, and could be used in versatile applications.

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PREPARATION AND CHARACTERIZATION OF PHOTO-CURED BIOBLEND OF CHITOSAN AND ETHYLENE GLYCOL

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Abstract: Chitosan was extracted from the dried prawn shell (head) waste through the intermediate product chitin. The prepared chitin and chitosan was investigated by FT-IR in order to ensure the deacetylation. Thin films of chitosan were prepared and its mechanical properties like tensile strength, elongation at break were studied. Chitosan films were cured with ethylene glycol (EG) at various ratios using photo-curing technique. Soaking time, concentration of EG at various UV radiation intensities were optimized with extent of mechanical properties of the cured films. The EG-cured chitosan films after treated with 5% ethylene glycol (EG) and 2% photoinitiator (Irgacure-651) showed the best performance at 15th UV passes for 20 min soaking time. Bioblend of chitosan and EG of different proportions were prepared and their mechanical properties were also studied at raw and after UV irradiation (15th passes).

INTRODUCTION

The field of photocuring has been developed to an important branch of photocrosslinking of polymers [1]. Photocrosslinkable polymers possess functional groups, which can undergo light-induced reactions to form directly a crosslinked polymer [2]. Chitosan possess the primary structure corresponds to a linear chain of β-1-4 linked 2-amino-2-deoxy-D-glucopyranose residue [3, 4]. Grafting polyethylene glycol (PEG) onto chitosan is a convenient approach to water-soluble chitosan derivatives [5]. The extent of grafting and homopolymer formation was found to depend on chitosan, amount of initiator, monomer concentration, reaction temperature and reaction time [6]. The present study describes the mechanical properties of EG cured and EG blended chiosan films with dissimilar ratios. Grafting is carried out at different soaking time with different UV radiation intensities.

EXPERIMENTAL

Materials

Chitosan was extracted from the shell (head) of fresh water Prawn. Ethylene glycol, Sodium hydroxide (NaOH), Hydrochloric acid (HCl), Ethanoic acid

(CH_3COOH); purity 99.9% was supplied by Merck,Germany. And photoinitiator (Irgacure-651) was from Ciba-Geigy, Switzerland.

Method

Chitosan was extracted from prawn shell waste. The chitosan films were prepared by a casting method .The soaking formulations for grafting were prepared with four different ratios of EG in methanol with 2% photoinitiator .The films were soaked into these formulations for dissimilar soaking times and irradiated under UV radiation of different passes, using UV machine (IST Technik,Germany). Blend of chitosan and EG was prepared with different EG ratios in 2% ethanoic acid solution. The films were prepared like raw chitosan films. The mechanical properties of both types of films were investigated after 24 hr of irradiation. Tensile strength, elongation at break were measured by Universal Testing Machine (INSTRON, model 1011,UK).

RESULTS AND DISCUSSION

Characterization of Ethylene glycol (EG) Grafted Chitosan films

Mechanical properties like tensile strength (TS), elongation at break (Eb) were investigated. The tensile strength of raw chitosan film was obtained 28.8 MPa and Eb was 15.5%.

Optimization of EG concentrations

At first the raw chitosan films were soaked in different formulations; F1, F2, F3,F4 of 3%, 5%, 10%, 20% (weight percent) ethylene glycol respectively with 2% photoinitiator in methanol for 20 min soaking time. The soaked fims were cured under UV radiation at different intensities (number of passes; 5, 10, 15 and 20 passes). Nearly 24 hr later of UV radiation, the mechanical properties TS and Eb percent were investigated.

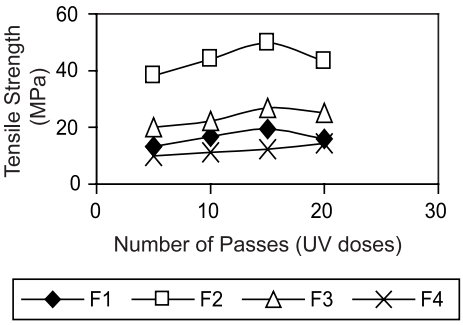

Figure 1. Tensile strength of the cured chitosan films against the number of UV passes with respect to soaking formulations (F1–F4) for 20 min soaking time

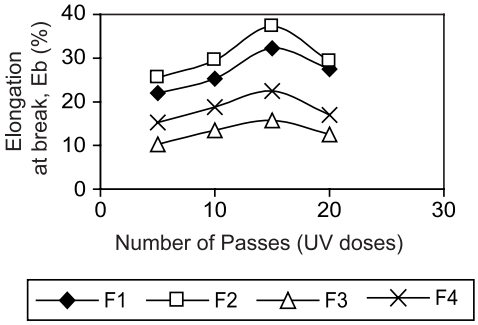

Figure 2. Elongation at break of the cured chitosan films against the number of UV passes with respect to soaking formulations (F1–F4) for 20 min soaking time

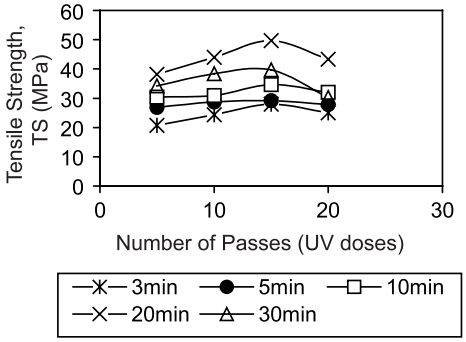

Figure 3. Tensile strength of the cured chitosan films against the number of UV passes with respect to different soaking times for F2 formulation

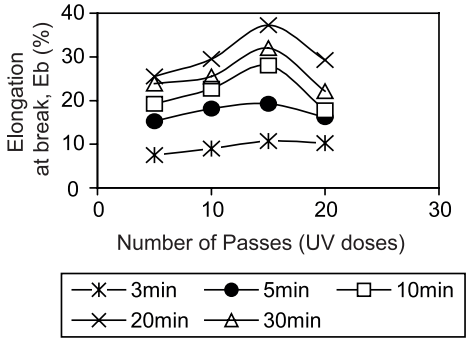

Figure 4. Elongation at break of the cured chitosan films against the number of UV passes with respect to different soaking times for F2 formulation

Optimization of Soaking Time

After having the optimized EG concentration (5%), the chitosan films were soaked in F2 formulation for various soaking times (3, 5, 10, 20, 30 min) and then irradiated under different number of UV passes (5, 10, 15, 20) nearly 24 hr later of UV radiation, the mechanical properties TS and Eb percent were investigated.

Tensile Strength and Elongation at Break

The tensile strength values and the elongation at break percentages of the cured films were plotted in figure 1. and in figure 2. respectively against UV radiation (number of passes) with respect to formulations; (F1, F2, F3, F4) the soaking time was 20 minutes . From the figure it is observed that TS and Eb percent both increase with UV radiation up to 15th passes and with EG concentrations up to 5%, then decreases. F2 formulation show highest TS value 49.69 MPa and Eb percent 37.25 at 15th UV passes.

The tensile strength values and the elongation at break percentages of the cured chitosan films soaked in F2 formulation for different soaking times (3, 5, 10, 20, 30 min) were plotted against the number of UV passes in figure 3. and in figure 4. respectively. It is observed both in figure 3. and in figure 4. that, with the increase of soaking time up to 20 minutes and UV passes up to 15th the tensile strength and Eb percent of the films increase then those decrease. In the figure 3. the best TS 49.69, is observed at 20 min soaking time and at 15th UV passes and in the figure 4. the best Eb percent 37.25, is observed at 20 min soaking time and at 15th UV passes .

The reason may be the higher UV doses and higher EG concentration which may impart compactness of molecular chains in the amorphous region of the polymer, which increases the brittleness of the polymer backbone. Higher UV doses may also cause the degradation of the polymer that may also brittleness.

Characterization of Ethylene glycol – Chitosan Blend films

Chitosan and Ethylene glycol bioblend was prepared in different composition; B1, B2, B3, B4 of 0.3%, 0.5%, 1.0%, 1.5% ethylene glycol respectively with 1% chitosan in every formulation with 2% acetic acid solution. The composition is

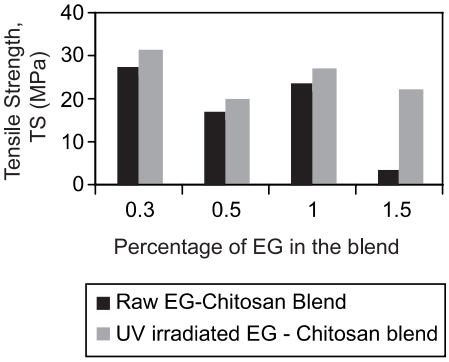

Figure 5. The Tensile strength of EG-Chitosan blend of raw and UV irradiated (15th passes) of different ratios

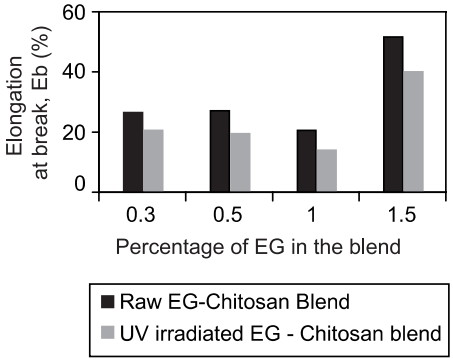

Figure 6. Elongation at break of EG-Chitosan blend of raw and UV irradiated (15th passes) of different ratios

taken here in weight percent .The mechanical properties were investigated with raw and UV (15th passes) irradiated bioblend films.

Tensile strength and Elongation at break

The tensile strength values and the elongation at break percent of raw and UV irradiated bioblend were plotted against the different ratios of EG in the blend with chitosan in the figure 5. and figure 6. respectively. When the raw blend films were irradiated, their TS was increased and respective Eb percent was decreased. It may be due to the photoinduced coplymerization between EG and chitosan. When very small amount EG is blended with chitosan, the film possesses the TS (27.13 MPa) close to the raw chitosan film (28.80 MPa) with slight increase in Eb percent. And with the increase of EG ratio in the blend decrease the TS with the increase of Eb percent. The reason may be when EG was blended with the chitosan it formed weak bond with each other may be weak hydrogen bond and when these were irradiated significant change in TS was obtained may be due to the crosslinking between EG and chitosan. The exception of 50/50 ratio may be due to its inherent character, which just got fit with this combination.

CONCLUSIONS

A significant variation in mechanical properties of chitosan is observed upon grafting and blending with ethylene glycol. In case of grafting the properties have changed with different EG concentrations, soaking times and number of UV passes. Optimum grafting condition increases the TS about 72.53% and Eb percent about 140.32% than the raw chitosan film. In case of blending although the TS has no significant change but the Eb percent has increased much significantly. The Eb percent has increased about 232.25% in the raw blend and 158.06% when the raw blend was UV irradiated with considerable improve in TS than the non-irradiated (raw) one.

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CONFORMATIONAL PROPERTIES OF NAPHTHOYL CHITOSAN IN SOLUTIONS AND FILM

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Abstract: An *O*-naphthoyl chitosan derivative (degree of substitution 0.56 per glucosidic unit) was prepared and its chiroptical properties in dilute solutions and film were characterized by circular dichroism (CD) spectroscopy. The conformational properties in dilute solutions were then deduced according to the CD results. It is suggested that the helical conformation of the polymer is dependent on concentration and solvent, but independent on temperature. A higher ordered right-handed helical conformation was also suggested in a cast film.

Key words: naphthoylchitosan, conformational properties, circular dichroism

INTRODUCTION

Chitosan, a fully or partially deacetylated product of its parent polysaccharide chitin, is capable of forming rigidly helical conformations in the crystalline state, as reported by Ogawa and his coworkers [1, 2]. However, little is known about the chiral conformations of chitosan and its derivatives, due to the difficulty in preparing specimens suitable for X-ray diffraction studies. On the other hand, there has been considerable work contributed to the conformation studies of chitosan in aqueous solutions by intrinsic viscosity measurements [3–5] and light scattering methods [6, 7]. These experiments provided very important structure parameters (i.e. the exponent of the Mark-Houwink equation a, and the persistent length q) required to evaluate the conformation of this promising biopolymer. Unfortunately, these results did not give us any information on the chirality of the polymer chains.

The present work was carried out in order to obtain information concerning the helical structure of naphthoyl appended chitosan derivative in dilute solutions and a cast film by circular dichroism (CD) spectroscopy. The influence of

concentration, solvents and temperature on the chain conformation was investigated in this study.

EXPERIMENTAL

Materials and Reagents

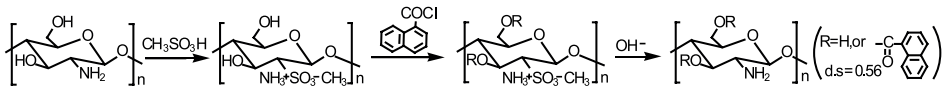
Chitosan powder was a product of Katakura Chikkarin Co. Ltd. (Japan), with an average molecular weight of about 1,090,000 g/mol (M_V) and a degree of deacetylation of 96%. 1-Naphthoyl chloride was purchased from Tokyo Kasei Kogyo Co. Ltd.

Spectroscopy

CD spectropolarimetry was done using a Jasco-500 A-type. A jacketed quartz cell with light-path length of 0.5 cm was used in our measurement. Absorbance spectra were recorded using a Hitachi U-3000 spectrometer. All the CD and UV spectra were recorded at room temperature (20°C). FTIR spectrum measurement was carried out using a Nicolet Magna 560. ^{13}C-NMR and ^{1}H-NMR spectrum was obtained by using a JNM-LA 500.

Preparation of Naphthoyl Chitosan

Five hundred milligrams of chitosan powder was added to 15ml of methane sulfonic acid and the mixture was stirred at room temperature (20°C) until the polymer was completely dissolved. Then, 3.0g of 1-naphthoyl chloride was gradually added to the above solution over 30 min under cooling with ice water. The mixture was then stirred for 3h at room temperature, and then the reactor was kept in ice water over-night. To precipitate the product, 200 mL ice water was poured into the reactor. The precipitate was separated and then washed with distilled-deionized water and neutralized with 200 mL of 1.4% ammonium hydroxide. The product was further washed with water and ether several times



and dried in a desiccator for 3 days until the weight became constant. The product was purified by re-precipitation from DMAc solution in methanol and then by washing with water and ether several times. The structure of the product was confirmed by IR, ^{1}H-NMR and ^{13}C-NMR spectra. The degree of substitution was about 0.56 per glucose unit.

RESULTS AND DISCUSSION

CD Spectra of the dilute solutions

Figure 1 shows that the CD spectrum of a 25 mg/L naphthoyl chitosan/DMSO solution exhibits a negative band at about 295 nm which matches well with the absorption band. Obviously, this peak is assigned to the $^{1}L_a$ electronic dipole transition moment of the naphthalene group (along the short axis) as a consequence of the interaction between the chromophore and the chiral helix of the polymer.

With increasing polymer concentration, the magnitude of this CD signal rose and underwent a sign reversion from negative to positive at a concentration of 1mg/mL (Figure 2). The inversion of the CD pattern suggests that there is a change of chiral structure of the polymer system. Such a phenomenon of CD sign inversion was also found to occur in liquid crystalline solutions of some polysaccharide derivatives. [8, 9] These were interpreted as the helical arrangement of the chromophores that are enforced by the macroscopic three-dimensional structure (chirality in supermolecular level). In our case, as a highly ordered three-dimensional structure is absent at low concentrations, the CD inversion is

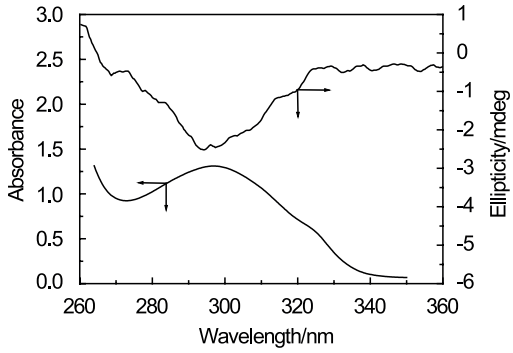


Figure 1. CD and UV spectra of naphthoyl chitosan in DMSO solution. The polymer concentration is 25 mg/L

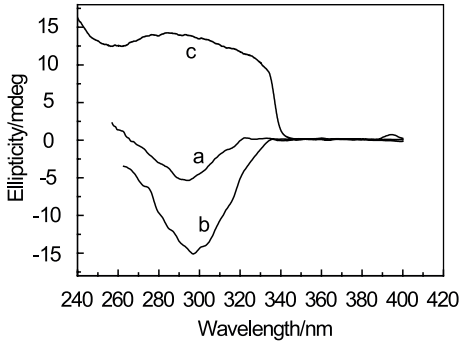


Figure 2. CD spectra of naphthoyl chitosan in DMSO solutions with various concentrations: (a) 50 mg/L; (b) 100 mg/L; and (c) 1000 mg/L,×1/10

independent of supermolecular structure. Therefore, the observed CD reversion can only be assigned to the helix-sense reversion of the polymer conformation (chirality in molecular level). We suppose that the reversion is relevant to the enhanced interaction of the hydrophobic groups appended to the chains accompanied by an increase in concentration. Indeed, the peak position of the absorption spectrum shows a small red shift with increasing concentration (See Figure 3), which may be attributable to the gradual enhancement of chromophore interaction.

Adding a small amount of water (10 v/vol%) to the DMSO-naphthoyl chitosan solution gives a drastic decrease in CD intensity (Figure 4). With further increas-

ing the water content, the intensity gradually decreases. Obviously, the large change in CD intensity was not due to the change in the extinction index of solvent, but most likely to the change in conformation. We suppose that the intra-molecular hydrogen bonds support the helical chain of naphthoyl chitosan to sustain a relatively extended helix in solution. As a consequence of the collapsing of the intra-molecular hydrogen bonds by adding water to DMSO solution, naphthoyl chitosan suffers a conformational change from a helical structure to a looser one. As a result of the decrease in helical content in the solution, the CD intensity is reduced.

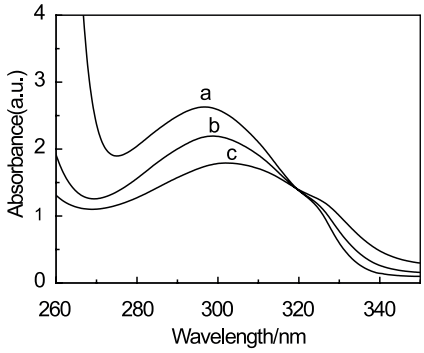


Figure 3. UV spectra of naphthoyl chitosan in DMSO solutions. (a) 50mg/L; (b) 100mg/L; and (c) 1000 mg/L. The light-path length of the cell used was 1.0 cm, 0.5 cm and 0.1 cm, respectively.

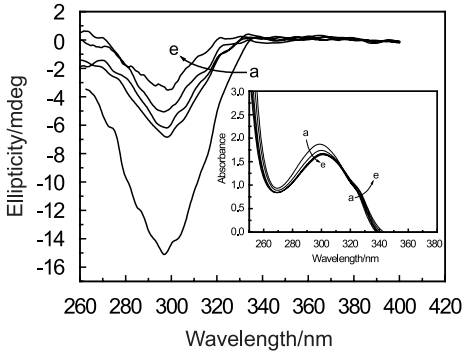


Figure 4. CD and UV spectra of naphthoyl chitosan in DMSO/water mixed solvents with various mixing ratios (v/vol%): (a) 100/0; (b) 90/10; (c) 80/20; (d) 70/30 and (e) 60/40. The polymer concentration is 100mg/l.

Figure 5 shows the temperature dependent on the CD spectra of a naphthoyl chitosan/DMSO solution. The increase in temperature from 20°C to 60°C does not change the sign or intensity of CD signal. This indicates that the conformation of the polymer in DMSO may be insensitive to a thermal stimulus.

The induced CD spectra of a film cast from a solution of naphthoyl chitosan are shown in Figure 6. The signal in the higher energy region was strongly biphasic, with a positive peak centered at about 232 nm and a negative one at about 203 nm (positive Cotton effect). The biphasic bands has a crossover at 216 nm, which consists with the UV absorption maximum of the ^1B_b transition moments.

The positive sign of the Cotton effect is in agreement with a right-handed helical conformation of naphthoyl chitosan chain in film, according to the "exciton chirality method" [10].

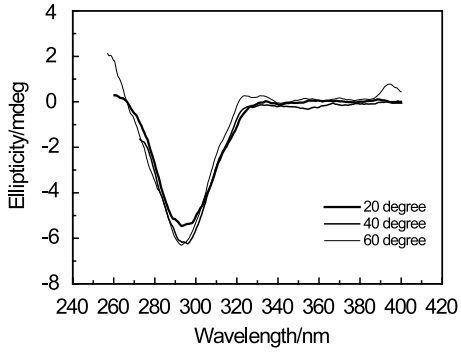


Figure 5. CD spectra of a naphthoyl chitosan/DMSO solution with concentration of 50 mg/l.

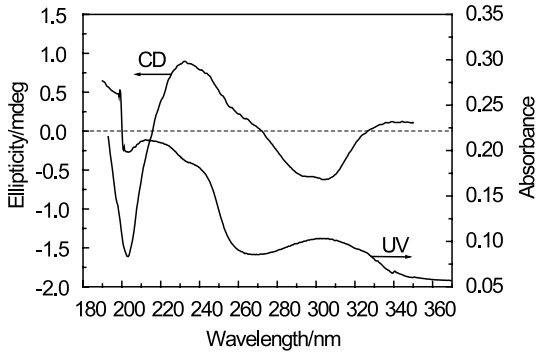


Figure 6. CD and UV spectra of a film cast from a naphthoyl chitosan/DMSO solutions

CONCLUSIONS

The present study revealed that naphthoyl chitosan adopted a helical conformation in DMSO as evidenced by circular dichroism. The helical conformation of the polymer is dependent on concentration and solvent, but independent on temperature. A higher ordered conformation was suggested to be a right-handed helical one in the cast film.

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STUDY ON THE EFFECT OF REACTION VARIABLES ON GRAFT COPOLYMERIZATION OF POLY (VINYLKETONE DERIVATIVES) AND ONTO *N*-SUCCINYL-*β*-CHITOSAN

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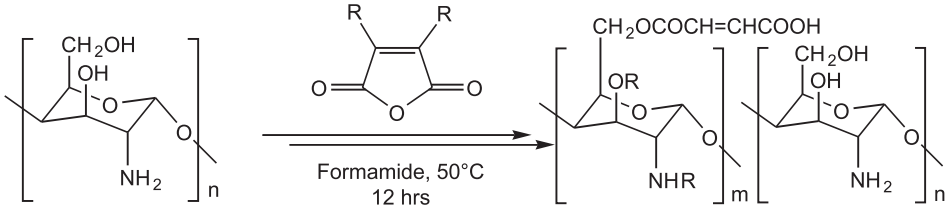
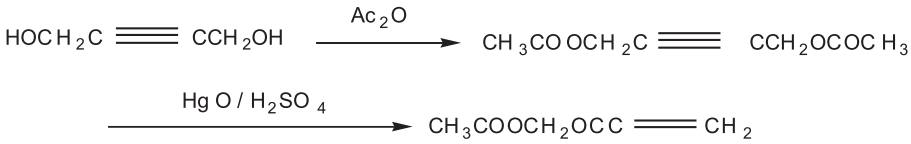
Key words: graft of *β*-chitosan derivatives, maleic acid *β*-chitosan

INTRODUCTION

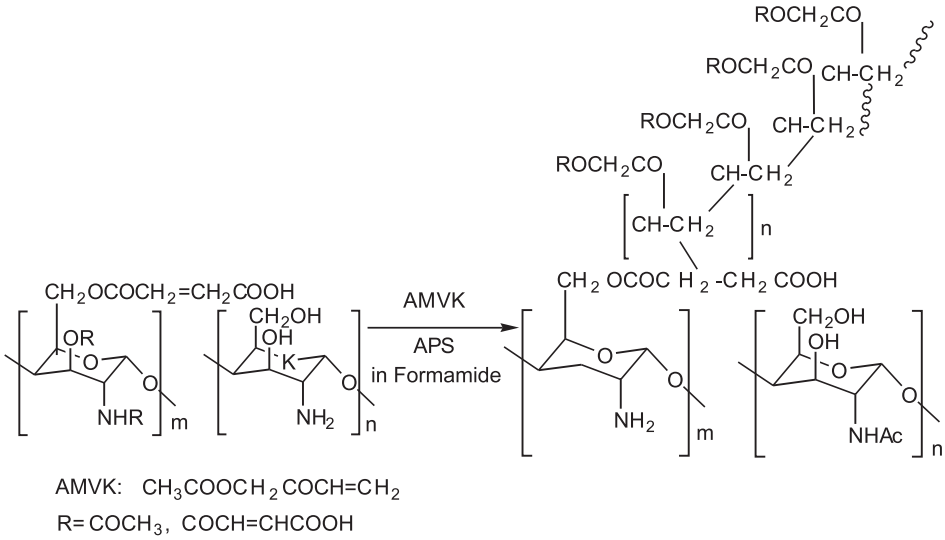
Chitin is a natural polymer consisting of *N*-acetyl-D-glucosamine monomers linked together with *β*-(1→4) linkages. And A chemical structure of Chitosan, (CTS), (poly-*β*-(1→4)-2-amino-2-deoxy-D-glucose) is obtained through partial deactylation of chitin, Recently CTS has attracted great attention since the range of its application has many expanded to medical, biotechnological, drug delibery systems, wound dressing, artificial membranes among other uses [1, 2, 3] Thereafter, the modification of its structure appears to be interesting in other to expand the potential uses of this biopolymer. A useful approach in other to graft vinyl monomers onto polysaccharide macromolecules consists in creating radical site by using appropriate initiators. In this paper we report the synthesis and the characterization of partially maleic anhydride coupled-*β*-CTS graft with acetomethoxyvinylketone (AMVK). *β*-Chitosan hydro gels which can exhibit dramatic intermolecular in their swelling behavior, network structure, or mechanical strength in response to change in pH [4], temperature [5, 6]. Cross linked poly (maleic acid-*co*-AMVK *β*-chitosan) has temperature-sensitive hydro gels and shows a behavior above than at 30°C in aqueous solution. Poly (MA-*β*-chitosan and AMVK) compound can also be modified by random co polymerization with hydrophilic or hydrophobic monomer in the gel composition. In particular, hydro gels prepared from random co polymerization of DCMA with ionic monomer such as acetomethoxyvinylketone(AMVK) show a different critical solution temperature depending on the pH and temperature.



Scheme 1. Reaction scheme for preparation of maleilated chitosan and chitin



Scheme 2. Reaction scheme for preparation of AMVK



Scheme 3. Reaction scheme for preparation of cross-linked maleilated chitosan polymer

EXPERIMENTAL

Preparation of maleilated *β*-chitosan (MC)

MA-*β*-CTS was synthesized according to the procedure reported by Berkovich, *et. al* [19]. As shown in Figure 1, maleic anhydride (two V%) and *β*-chitosan were added to dimethylformamide. The quantity of chitosan in the reaction mixture amounted to 2%. The reaction mixture was stirred at 40°C. After stirring and washed with methanol and acetone. The product was filtering and the filtrate solution was dialyzed against deionized water for 24 h.

RESULTS AND DISCUSSION

Graft co polymerization as hydro gels [MACTS-*co*-AMVK]

Hydro gels composed of MA-β-chitosan and AMVK were prepared by free radical polymerization in the presence of ammoniumpersulfate as an initiator and *N*,*N*,*N*,*N*-tetramethylenediamine.

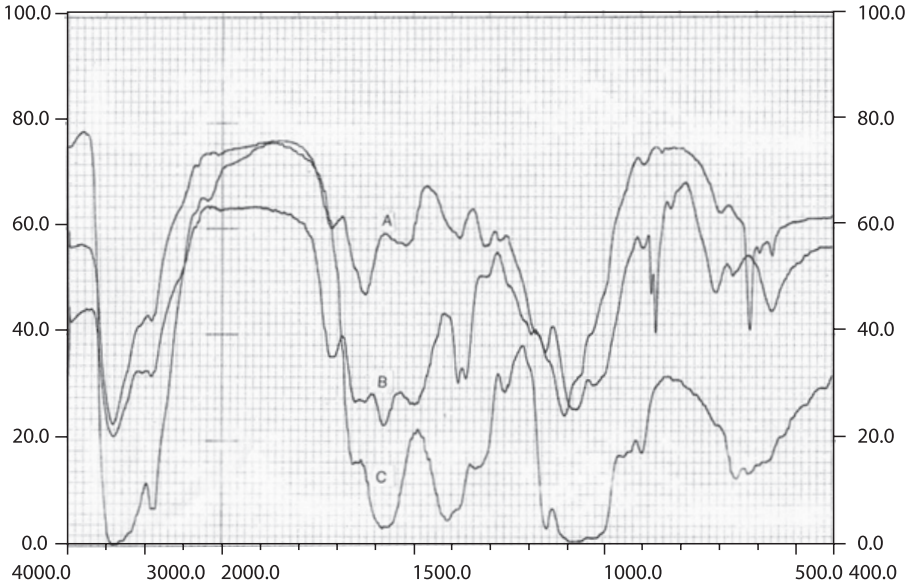


Figure 1. IR spectrum of β-chitosan, MACT

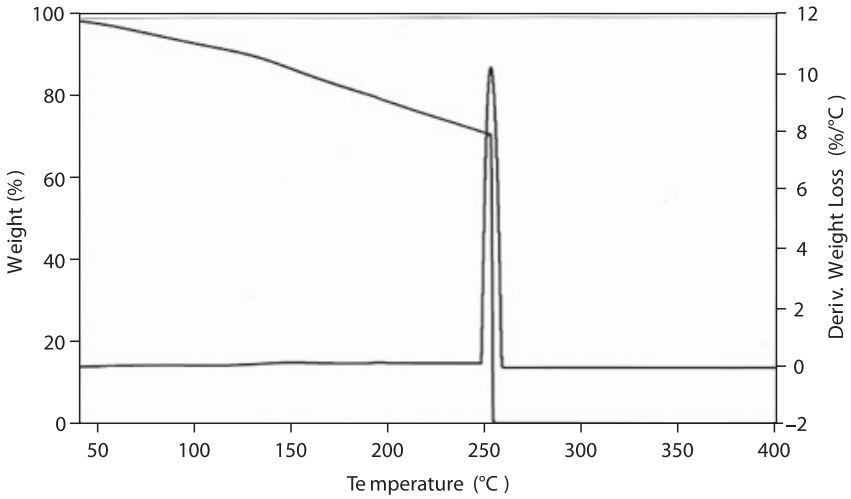


Figure 2. DSC and TGA of *N*-MA β-chitosan MA.CTS-*co*-AMVK

(TEMED) as an catalyst. The cross-linked hydro gels were prepared at various weight ratios of MA-β-chitosan. Polymerization mixture was bubbled through

with nitrogen for 10 min and the mixture was immediately injected into the space between the MA-β-chitosan and AMVK (MA-β-chitosan and AMVK) was carried out at room temperature for 15 h. After the gelation was completed, the gel membrane was cut into plate dish, 6 mm in diameter, and then immersed in an excess amount of deionized water for 2 days and filtering to remove the unreacted MA-β-chitosan. Swollen polymeric chitosan gels were dried at room temperature for 3 day, and these samples were further dried in a vacuum oven for 1 day at 50°C.

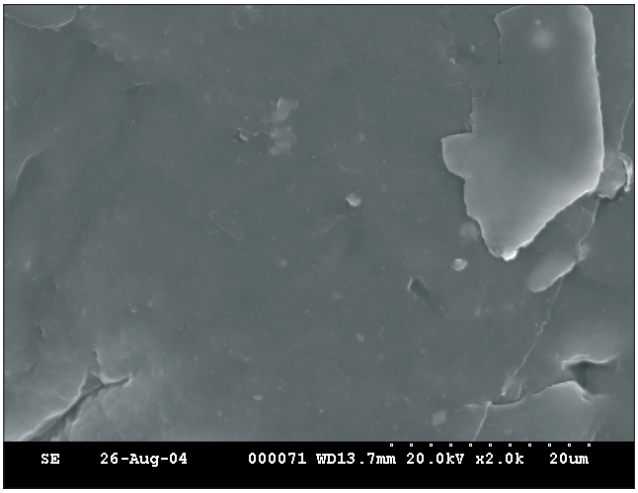


Figure 3. SEM photograph of *N*-β-chitosan *co*-MA.CTS-*co*-AMVK

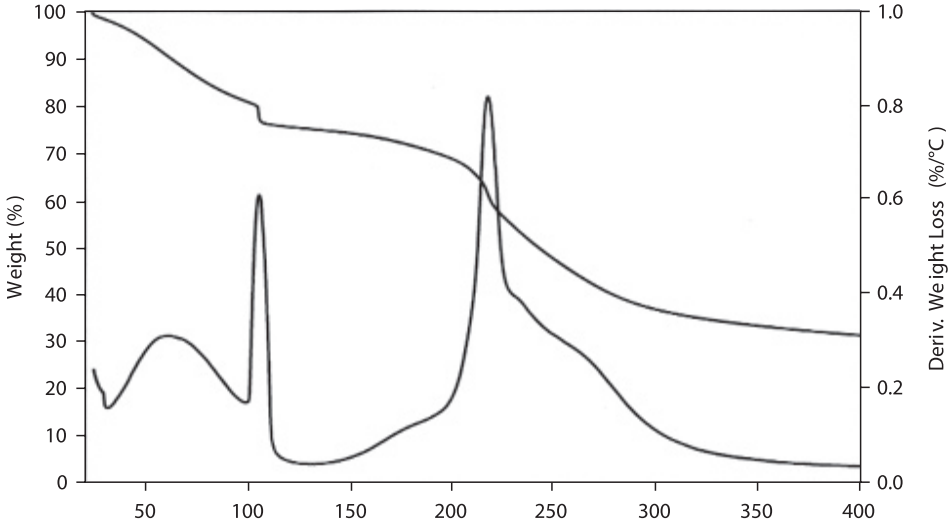


Figure 4. DSC and TGA of *N*-MAAMBK

Swelling measurement and thickness of film

1. For the pH dependent swelling studies, gels in triplicate were incubated in buffer solutions (ionic strength = 0.1M) ranging from pH 2 to 10 at a particular temperature

The Equilibrium Water Content(EWC)(%)= was calculated by the following equation, (EWC)(%)=[(Ws-Wd)/Ws]. 2). The thickness of each film was measured by micrometer (B.C. Ames, Co, Waltam, MA), having precision of 2.58 μm (0.1 mil).

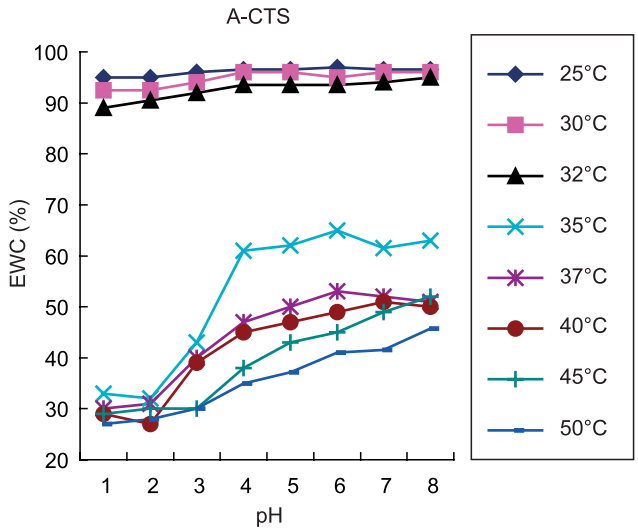


Figure 5. A-CTS of hydrogels as a function of pH

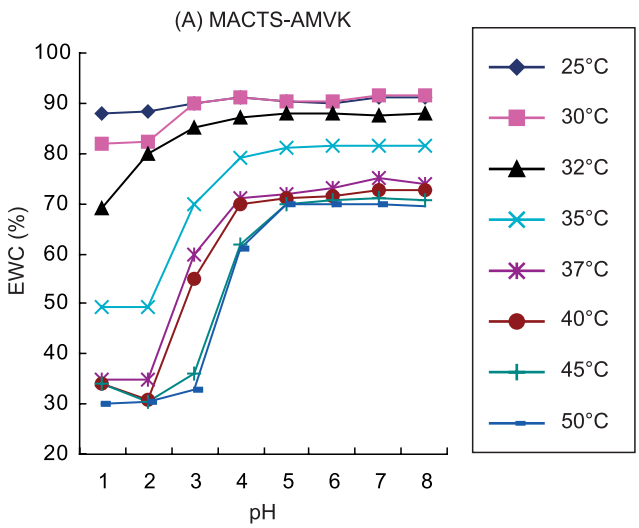


Figure 6. (A) MACTS-AMVK of hydrogels as a function of pH

Table 1. Feed composition of formation of hydrogelic graft polymerization

Sample	MA	AMVK	[COONa]/ [AMVK + COONa]	APS
(mg)	(g)	X 100 (mol%)	mg	
[MA- AMVK]	100	1.5	5	10
„	200	1.5	15	10
„	400	1.5	20	10

CONCLUSIONS

1. It has been established that maleic anhydride is capable to polymerize in presence of small quantities of β-chitosan, when amino groups have been protonated with acetic acid. Macromolecular soluble species have been obtained employing a critical concentration of β-chitosan (approximately 1% equivalent of group NH_2 in relation to initials equivalent of COOH groups). We have found that molar mass, yield and polydispersity decrease when the initial concentration of β-chitosan is diminished.
2. Maleilated chitosan was synthesized by β-chitosan, maleic anhydride and their conductor. Hydrogel sensitive to temperature and pH was produced from Maleilated β-chitosan, AMVK. In hydrogel, pH of buffing solution was increased due to the increased content of water in equilibrium caused by the increment of ionic reactive power.
3. The equilibrium constant for water content of hydrogel for temperature and pH increased with high temperature. And polar solubility of MACTS was increase. By contrast, cross-linkable β-chitosan material was resistant to aqueous solution.
4. This result could be interpreted as the physical and cross-linkable phenomenon caused by the reactive power of ion with free volume. Furthermore, heat-treating high polymer sharing the property of crystallization and amorphous state in this experiment, we could ascertain that thermal and mechanical property could be improved.

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PREPARATION AND CHARACTERIZATION OF CHLOROSTARCH COUPLING OF BASED SEAWEED PULP AND *β*-POLY(GLUCOSEAMINE)-(*N*-2,3-DIHYDROXYPROPYL) DERIVATIVES COMPOSITE FOAMS

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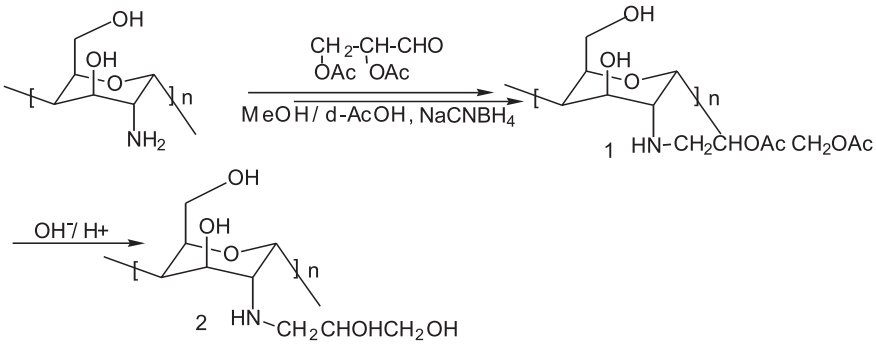
Key words: Chlorostarch polymer, Seaweed pulp, *β*-Poly(*N*-2,3-dihydroxypropyl- glucoseamine, Thermal properties, decomposition

INTRODUCTION

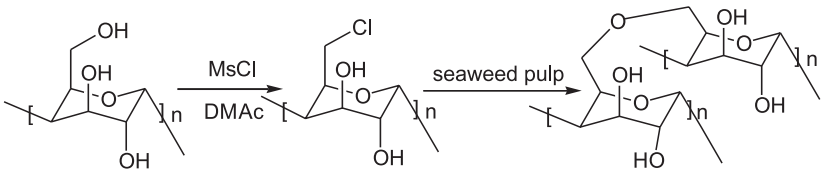
β-Chitin is characterized by weak intermolecular forces [1] and has been confirmed to exhibit higher reactivity in various modification reactions as well as higher affinity for solvent than *α*-chitin [2]. It is noteworthy that *β*-chitosan prepared from *β*-chitin also exhibited high reactivity compared to that from *α*-chitin and significant bactericidal activity [3, 4]. These results suggest the high potential of chitosan derived from *β*-chitin as a novel functional biopolymer. They have attracted much interest in biomedical application such as wound healing, anti microbial agents, cholesterol reducing agent, and drug delivery carrier, burn healing bio-dressing, drug delay-releasing material and blood vessel disease medicine, and food for lower blood-fat. etc. By contrast, starch, on of the natural biodegradable polymers obtained from agricultural crops, is produced at a relatively low price. This natural polymer is currently used as a raw material or an ingredient in degradable or decomposable various products. Here we report the formation of Cl-starch coupling to *N*-2,3-dihydroxypropyl *β*-chitosan and seaweed pulp from our laboratory were compared with their physical property, chemical spectroscopy, ets. In this study reports on the thermal decomposition (cleavage of bonds) of insoluble powdered chitosan coupled Cl-starch, with the objective of identifying key parameters that govern their thermal decomposition. And corn starch was chemically substituted with PCl via chlorination under several reaction conditions. The changes in structural and physical properties such as intrinsic viscosity and solvent resistance of the products were investigated

EXPERIMENTAL

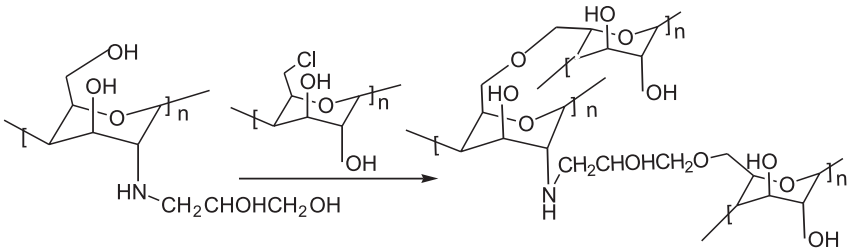
β-Chitosan was isolate from squid (*Dasidicus gigas*)pens was prepared in our laboratory. Briefly, *β*-chitin were treated with 40% NaOH at 100°C for 2 h. filtered, and washed with water. The deacetylation procedure was repeated three more times to give *β*-chitosan. Other chemicals: acrolein, potassium permanganate were purchased from Wako Chemical Co., Japan. Characterization of structural changes in *β*-chitosan and its derivatives were determined by the Nicole 5DX FT-IR spectrophotometer and ^{1}H NMR spectra were recorded on a Varian T60 and HA-100 spectrophotometer. Thermo gravimetric analysis (TGA) was performed. Using a Mettler TG50, Scanning the temperature linearly at 10°C/min from 30°C to 450°C, with N_2 as purge gas at 100 ml/min.



Scheme 1. Reaction scheme for preparation of maleilated β-Chitosan



Scheme 2. Reaction scheme for preparation of chloro starch and coupled seaweed pulp



Scheme 3. Reaction pathway for synthesis of Cl-Starch coupled compound 2

Coupling of *N*-2,3-dihydroxypropyl-*β*-chitosan (NDHPCTS) with Cl-starch

NDHPCTS(2) 1.5 g was dissolved in 50 ml of DMAc: 2 ml of acetic acid solution to prepare NDHPCTS solution and Cl-starch resolved in 5% LiCl/DMAc was added drop wise to the above viscous *β*-chitosan derivatives at 50°C under N_2,

and stirred for 30 mines. The mixed solution was then stirring until to 25°C and the more stirred for 6 hr and neutralized by adding 20% NaOH(aq). After neutralization, the solution was precipitated by adding 300 ml MeOH, followed by centrifugation and was dialyzed with RO water. The dried product was obtained by $CaCl_2$ contained vacuum oven.

RESULTS AND DISCUSSION

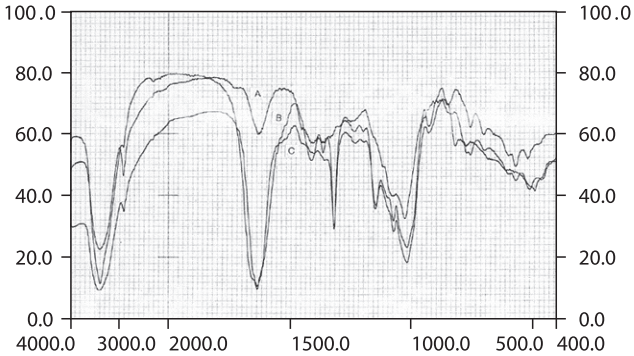


Figure 1. IR spectrum of Cl-starch coupling on Seaweed Pulp

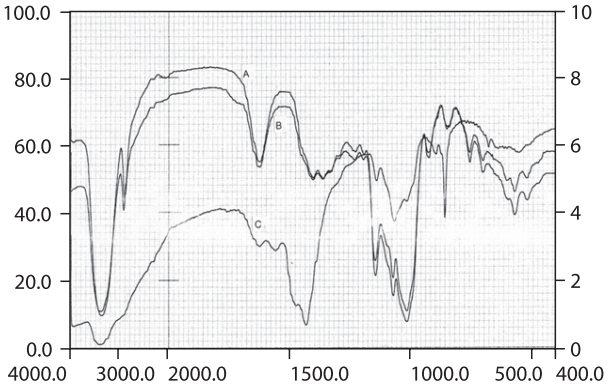


Figure 2. IR spectrum of β-chitosan,NPDHCTS coupled β-CTS-NPDHCTS

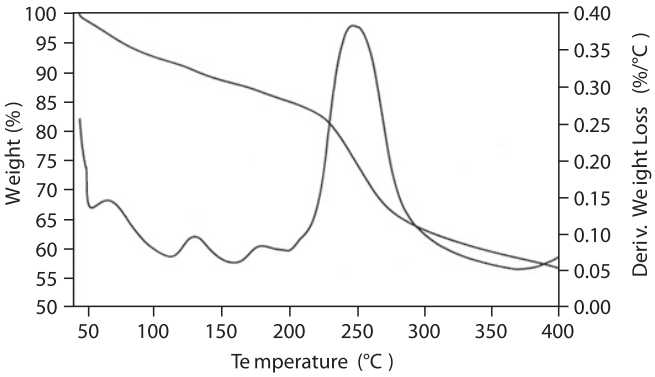


Figure 3. DSC and TGA of seaweed pulp

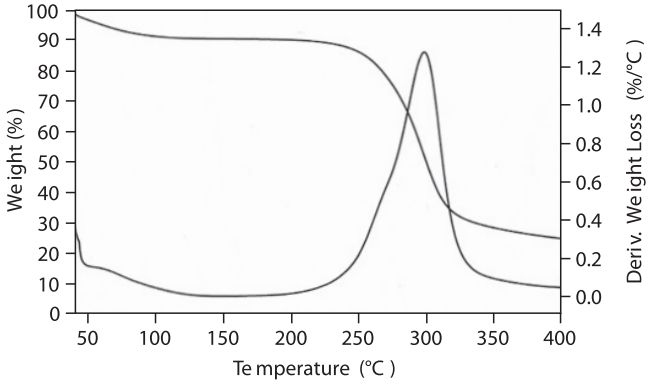


Figure 4. DSC and TGA of Cl-starch

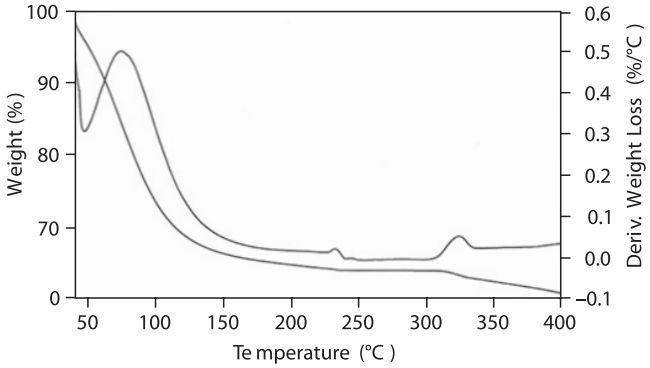


Figure 5. DSC and TGA of NDHPCTS

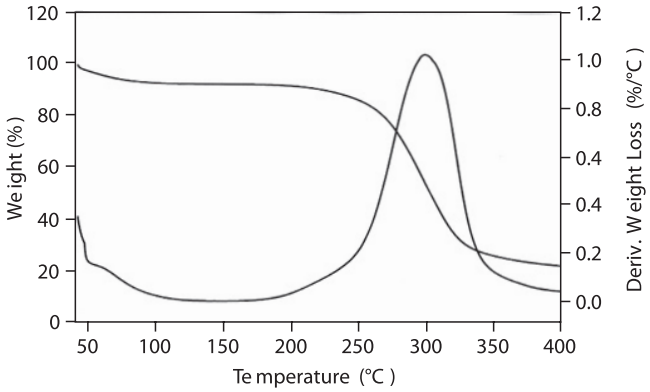


Figure 6. DSC and TGA of Cl-starch coupling to NDHPCTS

The characteristics of the different studied β-chitosans are shown in Tables. As can be seen they have different molecular weight and different coupling. Very different composite foams obtained from seaweed pulp and β-chitosan derivatives showed good mechanical resistance but skin grafting composite forms from NDHPCTS were soft and insoluble in water. The thermal properties observed for β-chitosan, are strikingly different from those of β-chitosan on basic form. The

latter display exothermic decomposition at temperatures around 300°C and The exothermic peaks temperature increased with seaweed pulp and β-chitosans

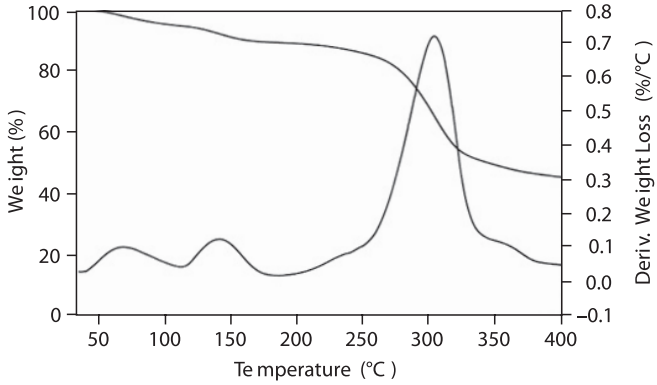


Figure 7. DSC and TGA of Cl-starch coupling to Pulp

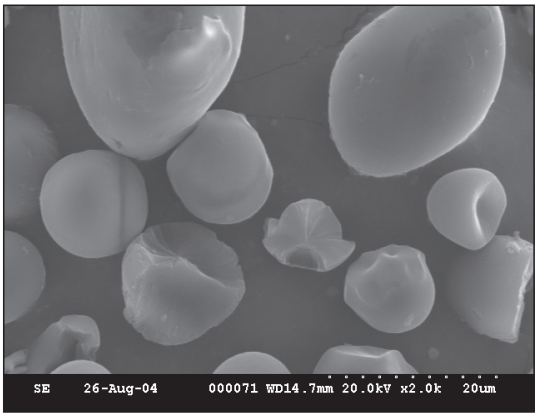


Figure 8. SEM photographs of Cl-Starch to seaweed pulp; surface(X1,000)

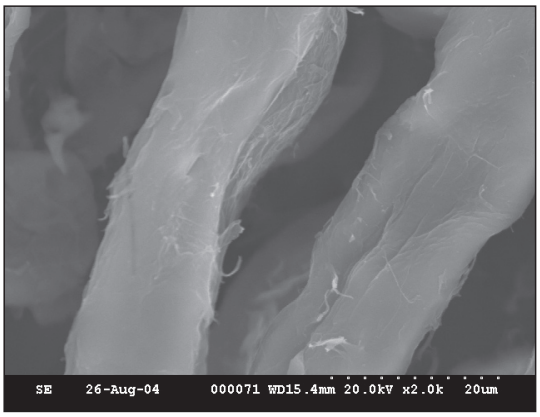


Figure 9. SEM photograph of Cl- Starch to β-chitosan derivative

CONCLUSIONS

In order to check the appropriateness for skin and pharmacological efficacy, chitosan was converted to cross-linked. To be used as biological adhesive, α, β-dihydroxypropionicaldehyde was coupling to β-chitosan by synthesizing 2 with acrorein and the following result came out. The data presented herein strongly suggests that the Thermal decomposition of β-chitosan derivatives and sea weed pulp are low hydrophilic solubility by aqueous solution.

1. For Cl-starch: pulp = 5: 3(mole ratio), Cl-starch: *N*-2,3-dihydroxy chitosan=1:1(mole ratio). The temperature under 65°C was desirable.
2. The TGA and DSC curves were identical in N_2 and air below 400°C. Modification of chitosan is one of the mast important ways to improve its physical properties, including solubility and biodegradability. Utilization of physical-chemical methods for chitosan modification makes it possible to prepare the chitosan forms with controlled structure. At the same time it could support the unification of the properties of the biopolymer produced mainly from naturally occurring residues of crustaceans.

ACKNOWLEDGMENTS

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REMOVAL OF SOLUBLE DYES FROM SOLUTION USING CHITINCONTAINING COMPLEXES

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Key words: chitin, chitosan, chitincontaining complexes, *Aspergillus niger*, dyes, sorption

INTRODUCTION

The synthesis of organic dyes, and also process of their use is accompanied by formation of a plenty of the polluted waste water. The high degree of pollution of waste water by dyes is characteristic for factories of textile and chemical industry. The waste water polluted with soluble dyes (acid, direct, active), very difficultly gives in to technological clearing. The dyes in reservoirs practically are not decomposed and negatively influence growth and development of microorganisms. For example, staying in reservoirs about 20 days the contents of direct dyes decreases only on 5–10%, and acid dyes on 60–80%. For clearing waste water of soluble dyes various methods including sorption are used. As a sorbent in this methods carbon is generally used, by the way there is a search of new cheaper and more effective sorbents.

Chitin, chitosan and various derivatives of chitin are effective sorbents of organic and inorganic substances from water solutions [1, 2]. McKay et al. showed that chitin could be used as an adsorbent of dyes from solution [3].

The aim of our work is to study the sorption of dyes on chitincontaining complexes, obtained from fungus mycelium of *Aspergillus niger* – the waste of production of citric acid.

EXPERIMENT

Insoluble chitincontaining complexes (ChCC) were obtained from waste received during biotechnological production of a citric acid. The waste is bioweights of Aspergillus niger. The bioweights was consecutively treated by hot (60°C) 1% water solution of NaOH for 90 minutes, then it was washed out by distilled water, by 1M solution HCl and finally by organic solvent. The finished product was dried at 40°C for one hour. The degree of deacetylation (DD) of ChCC is 0.8%. ChCC, grinded by the ball mill to the size of particles 100–200 meshes, were used as a raw material for obtaining of chitosancontaining complexes by a known method [4]. This method includes the treatment of an initial raw material by 40%

water solution NaOH for 4 hours at temperature 118°C. Such conditions of treatment were chosen as the most favorable for obtaining of chitosancontaining complexes with good sorption characteristics and with degree of deacetylation 96%. The degree of deacetylation was determined by conductimetric analysis.

In our work was researched the sorption of soluble dyes: direct light-blue(DB-K), acid red (AR-2C), active bright-light-blue (ABB-2KT). The experiments carried out in static conditions:

The solutions of dyes with concentration 20–200 mg/l were used. To each solution 0.02 g sorbent was added and mixed for 6 hours at 20°C and pH 6.8. Equilibrum concentration of dyes in solutions determined by photocolorimetry method. For the description of process sorbtion was used the Langmuir equation. The constants were calculated by a graphic method using the Langmuir equation in the linear form:

$$\frac{C_P}{\alpha} = \frac{1}{\alpha_\infty \cdot K} + \frac{C_P}{\alpha_\infty} \tag{1}$$

where C_P – equilibrium concentration, mg/dm^3; α – sorption capacities of dyes, mg/g; α_∞ – maximum sorption capacities, mg/g; K – constant of sorption equilibrium.

RESULTS AND DISCUSSION

The equilibrium isotherms represented on Figure 1 show that sorption capacities of dyes essentially depends on a type of dye: i.e. from its spatial structure, ability to association, presence of functional groups in a molecule, and also from a diameter and molecular weight of dye.

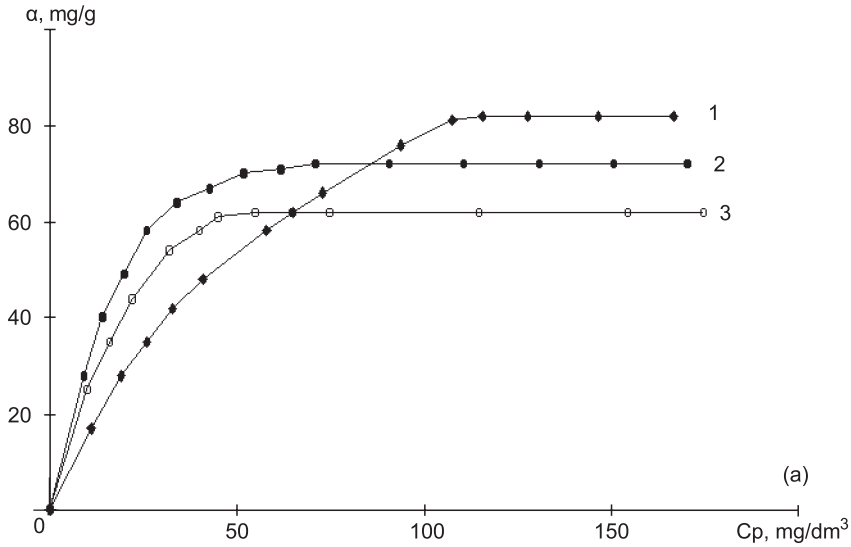

Figure 1. Sorption of dyes on ChCC (a) and ChaCC (b): (1) acid red (AR-2C); (2) direct light-blue (DB-K); (3) active bright-light-blue (ABB-2KT)

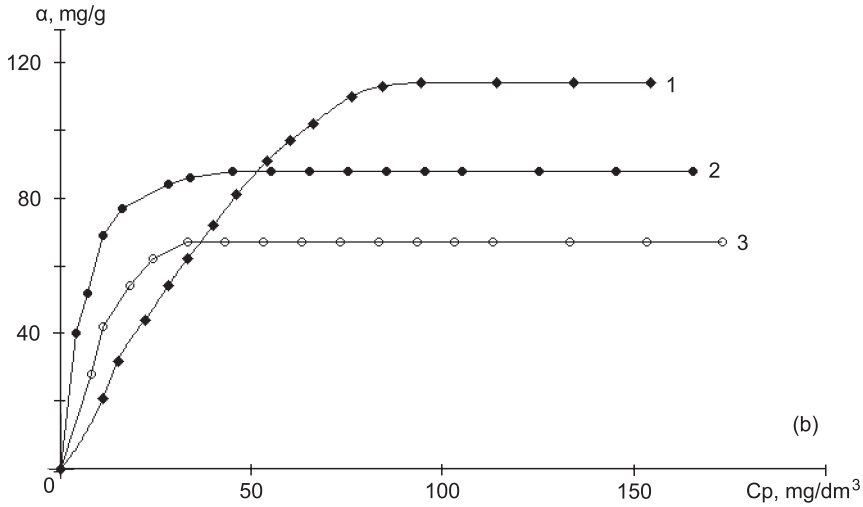

Figure 1. Sorption of dyes on ChCC (a) and ChaCC (b): (1) acid red (AR-2C); (2) direct light-blue (DB-K); (3) active bright-light-blue (ABB-2KT)

In the field of high equilibrum concentration both ChCC and ChaCC more effectively remove the direct and acid dyes from water solutions than the active ones. It is possible to explain the given fact by different abilities of dyes to association, and also by presence of meso-interstice in the structure of complexes.

The extraction of direct and acid dyes from water solutions occurs due to the sorption of their associative on complexes. Sorption of associative of acid dye occurs both in a superficial layer and inside the interstice of sorbent, and for direct dye the formation of associative only in a superficial layer is characteristic.

The active dye practically does not form associative. Sorption of active dye occurs at the expense of formation of chemical bonds between molecules of dye and functional groups of complexes. On initial sites of isotherm the lowered selectivity of complexes in relation to acid dye is characteristic. It is explained by specific interaction of dye with functional groups of complexes, which are accessible on a surface of walls interstice.

The results of research sorption of dyes also were submitted in coordinates Langmuir (Figure 2).

For all types of dyes the linear dependence is received, that proves an opportunity of application of the equation Langmuir for the description of the sorption process of dyes on complexes and allows to calculate constants of this equation. Constants α_∞ and K are listed in Table 1.

Table 1. Langmuir constants from Eqs. (1)

Complexes	α_∞, mg/g			K, dm^3/mg		
	DB-K	AR-2C	ABB-2KT	DB-K	AR-2C	ABB-2KT
ChCC	74	89	64	0,12	0,015	0,14
ChaCC	92	122	69	0,18	0,010	0,18

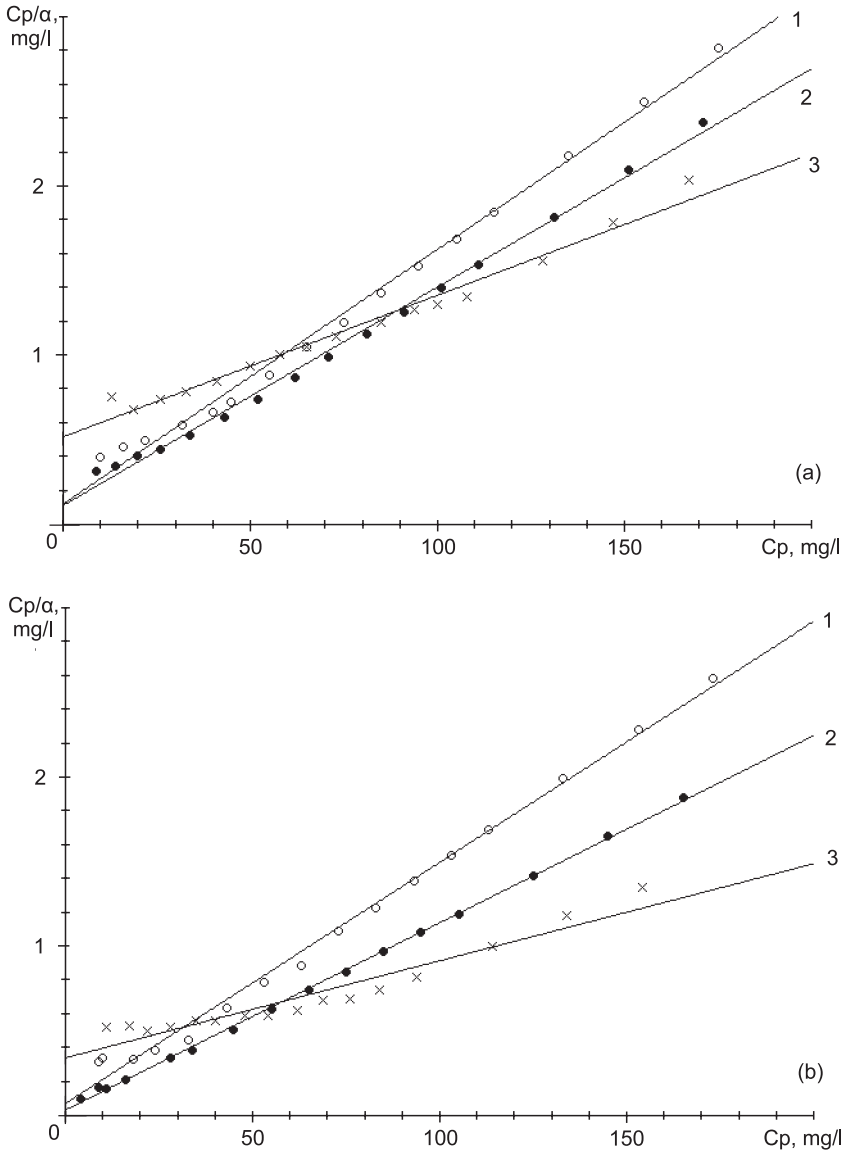

Figure 2. Langmuir dependences for dyes on ChCC (a) and ChaCC (b): (1) active bright-light-blue (ABB-2KT); (2) direct light-blue(DB-K); (3) acid red (AR-2C)

CONCLUSIONS

The sorption capacities of the dyes were 74, 89, 64 mg/g ChCC and 92, 122, 69 mg/g ChaCC for direct light-blue, acid red, active bright-light-blue respectively.

The experimentally received results show, that ChCC and ChaCC are good sorbents of direct and active dyes. ChaCC are more effective than ChCC for sorp-

tion of acid dyes. The opportunity of application of the equation Langmuir for the description of process sorption of dyes on complexes is shown. ChCC and ChaCC can be used as sorbent in columns for clearing waste water of dyes at the enterprises of light and chemical industries. Economically and ecologically it is more favourable to use ChCC, since at their reception process bioweight is processed by solutions of chemical solutions of low concentration at temperature about 60°C.

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FORMATION AND PROPERTIES OF COLLOIDS BASED ON THE COMPLEXATION BETWEEN NATURAL POLYELECTROLYTES

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INTRODUCTION

Polyelectrolyte complexes (PECs) have long been investigated in solution [1] or in interactions with various solid supports in the layer-by-layer approach [2]. But much less work was devoted to the elaboration of PECs in the colloidal domain. Buchhammer's group was interested in synthetic polyelectrolytes [3] and Mumper's group reported the elaboration of chitosan, a (1→4)-linked copolymer of 2-amino-2-deoxy-*β*-D-glucan) and 2-acetamido-2-deoxy-*β*-D-glucan based colloidal polyelectrolyte complexes [4], though no detailed investigation on the parameters controlling the interactions were reported. Several works reviewed by Liu et al. [5] report on the use of chitosan, with DNA as a gene delivery system. The use of this natural polymer is mainly motivated by its outstanding biological properties such as biodegradability [6] and biocompatibility [7].

Our work focused on the elaboration of positively and negatively charged colloidal PECs by complexation between chitosan as weak polybase (in excess) and dextran sulfate as strong polyacid (in default amount) [8]. Chitosan is an amphiphilic macromolecule whose degree of acetylation (DA), the molar ratio of acetylated to non-acetylated residues, is responsible for the balance between hydrophilic and hydrophobic interactions [9]. The formation of PEC particles from chitosan and dextran sulfate was expected to be highly dependent on the characteristics of both polysaccharides like charge density or chain length. Moreover, environmental parameters such as ionic strength or polymer concentration have been taken into consideration.

EXPERIMENTAL

Materials

Two weakly acetylated chitosans were used: one with a medium molecular weight (Aber technologies, batch A32E03, DA ~1%, M_w ~150 000 g mol^{-1}) and

the other with a relatively high molecular weight (France Chitine, batch 114, DA ~5%, M_w ~350 000 g.mol^{-1}). Prior to use, the polymers were purified according to [9]. Purified chitosans were *N*-acetylated in a hydro-alcoholic mixture at different DAs with acetic anhydride [10]. In addition, hydrolyses [11] were performed onto two chitosan samples having medium molecular weight and acetylation degrees of 1 and 15% to produce low molecular weight polymers. Dextran sulfate of ~5.10^3 (DS 5k), ~10^4 (DS 10k) and ~15.10^5 (DS 1500k) g.mol^{-1} weight average molecular weights were from Sigma and used as received. Sulfur content was determined for each sample by colloidal titration (see below) and water content was provided by the manufacturer.

Methods

Characterization of polyelectrolytes

Chitosan Degrees of acetylation were determined on purified chitosans by ^{1}H-NMR spectroscopy (Varian, 500 MHz), according to the method developed by Hirai et al. [12]. The weight average molecular weight (M_w), the z-average root-mean-square of the gyration radius ($R_{G,z}$) and polydispersity indexes (I_p) were measured by gel permeation coupled on line with a differential refractometer (Waters 410) and a multi-angle-laser-light-scattering (MALLS, Wyatt, Dawn DSP). A degassed 0.2M acetic acid/0.15M ammonium acetate buffer (pH = 4.5) was used as solvent and eluent. Refractive index increments (dn/dc) were determined independently for each sample in the same solvent with an interferometer (NFT Scan Ref)

Dextran sulfate. The molecular characteristics of the high molecular weight dextran sulfate (DS 500k) were determined by gel permeation chromatography. A 0.1N $NaNO_3$ solution adjusted at pH=7.0 was used as solvent and eluent. The degree of sulfation *i.e* the number of sulfate functions per glucoside units was evaluated by colloidal titration using toluidine blue and a UV/VIS spectrometer (μQuant, Bio-Tek instrument). The degrees of sulfation for each sample were determined as: 2.2 ± 0.2 (DS 5k), 2.4 ± 0.3 (DS 10k) and 2.2 ± 0.2 (DS 1500k).

Preparation of polyelectrolyte solutions. Chitosan was dissolved by adding a stoichiometric amount of 0.1N hydrochloric acid with respect to the free amino functions. Sodium chloride was added to vary the ionic strength of the solutions and pH was adjusted to pH=4.0 with 0.1N sodium hydroxide or 0.1N hydrochloric acid and the added amount was considered to calculate the final concentration and the total ionic strength. Dextran sulfate solutions, at different concentrations, were prepared in deionised water. Sodium chloride was added to obtain the required ionic strength and pH was adjusted at 4.0 with 0.1N hydrochloric acid. All solutions were filtered through 0.22μm pore size Millipore membranes before use.

Polyelectrolyte complexes formation. Polyelectrolyte complexations were performed at room temperature using chitosan as starting solution. Dextran sulfate solutions were added dropwise at a flow rate of 20 mL.hr^{-1}under moderate stirring. At defined molar mixing ratios (n^+/n^-) between cationic and anionic charged units, 2 mL aliquots were collected and left stand 12 hours prior further characterization. In most experiments, the initial concentration of chitosan glucosidic residues was set at 6.10^{-3} M corresponding to a mass concentration close

to 10^{-3} g/mL for low DAs and added dextran sulfate concentration was set at 1.2 10^{-3} M *i.e.* 5.10^{-4} g/mL.

Physicochemical characterization of the dispersions. Dynamic light scattering. measurements were carried out using a Coulter N4MD. Electrophoretic mobilities (μ_E) of the particles were determined at 25°C with a Malvern Zetasizer HS3000. Surface morphology and shape of particles were examined using scanning electron microscopy (Hitachi S800) operating at 15 eV.

RESULTS AND DISCUSSION

Polysaccharide characterization. All physicochemical data related to the chitosan samples are reported in Table 1. The complete characterization of chitosan samples were described in [9]. The conformation in solution of dextran sulfate (DS 500k) by static light scattering coupled with SEC, in 0.1 M $NaNO_3$, pH 7, gave the following molecular characteristics: $M_w = (1.51 \pm 0.20).10^6$ g/mol, $R_{G,z} = 72.5 \pm 3.0$ nm, $L_p = 1.6 \pm 0.3$ nm with a polydispersity index of 1.52 ± 0.09.

Colloidal PECs formation. In a first approach we used a slow dropwise addition of polyelectrolyte Data in Figure 1 show an increase of the mean diameter of the particles with the molecular weight of chitosan (DA=15%). Conversely, on increasing the molecular weight of DS, the mean diameter of the particles decreased. The values of polydispersity indexes followed the same trend as the sizes of the PEC *i.e.* high molecular weight chitosan led to the most polydisperse PECs. Since the chitosans of various molecular weights exhibited similar values of polydispersity indexes (Table 1), thus the chain length appeared to be the main parameter affecting the polydispersity of PEC, the high mobility of short chitosan chains being supposed to favor the formation of least polydisperse dispersions.

Table 1. Characteristics of Chitosans

DA (%)	Mw.10^5 (g/mol)	DP_w [a]	I_p [b]	$R_{G,z}$ (nm)
Low molecular weights				
1	0.13 ± 0.70	83 ± 4	1.25 ± 0.07	c
15	0.16 ± 0.80	96 ± 5	1.21 ± 0.06	c
Medium molecular weights				
1	1.28 ± 0.03	793 ± 19	1.64 ± 0.06	48.0 ± 1.4
15	1.39 ± 0.03	831 ± 18	1.43 ± 0.05	44.5 ± 1.5
24	1.42 ± 0.02	830 ± 12	1.36 ± 0.04	43.4 ± 1.7
51	1.58 ± 0.03	866 ± 16	1.35 ± 0.05	43.6 ± 2.0
71	1.88 ± 0.04	985 ± 21	1.26 ± 0.03	41.0 ± 2.0
High molecular weights				
5	3.50 ± 0.08	2145 ± 49	1.47 ± 0.04	94.5 ± 2.5
15	3.65 ± 0.09	2182 ± 54	1.41 ± 0.05	91.2 ± 2.4

[a] weight-average degree of polymerisation
[b] polydispersity index, $I_p = M_w/M_n$
[c] molecular weights are too low for determination of gyration radii

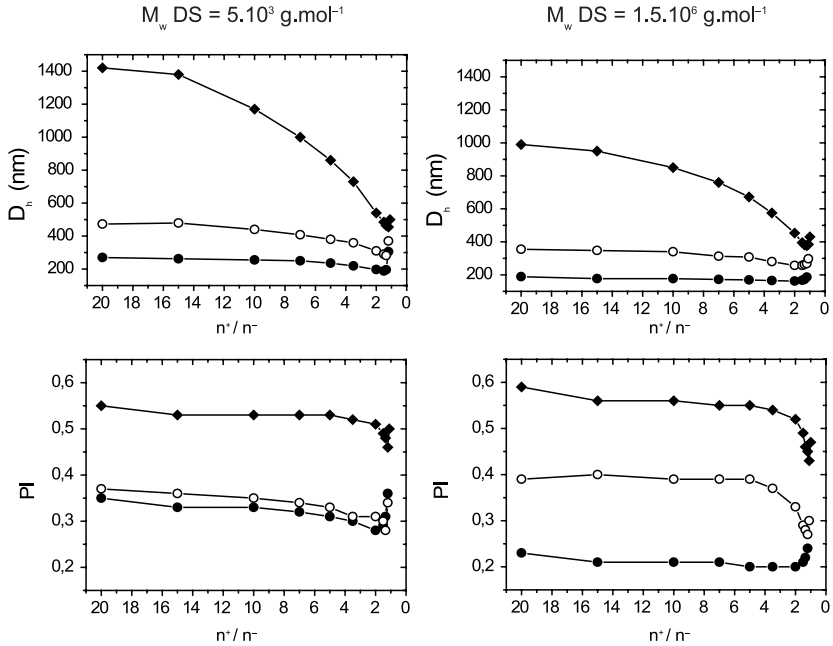

Figure 1. Influence of chitosan and dextran sulfate molecular weights on hydrodynamic diameters (D_h) and polydispersities (PI) of colloidal PECs. (●) DA=15% – low M_w, (○) DA=15% – medium M_w, (◆) DA=15% – high M_w

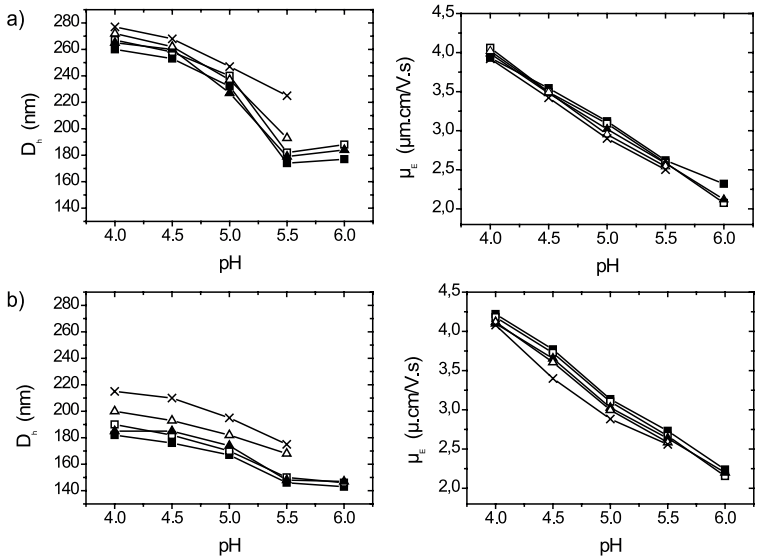

Figure 2. Sizes and electrophoretic mobilies of PEC particles washed twice as function of pH. Particles are based on chitosan (DA=15%, low molecular weight) complexed with DS 5k (a) or DS 500k (b) for various mixing ratios (n^+/n^-): (■) 10, (□) 5, (▲) 3.5, (△) 2, (×) 1.5

The degree of acetylation of chitosan (DA) is a means of modulating the charge density of the polycation. An increase of DA resulted in an increase in mean di-

ameter of the particles (data not shown), weakly charged chitosans favoring the formation of more swollen particles.

In Figure 2, is depicted a concomitant decrease of sizes and mobilities of colloidal complexes with increasing pH, resulting from the decrease of the thickness of the chitosan shell induced by deprotonation of glucosamine residues. For pH values higher than 6, the effective over-charge of the particles was reduced to such an extent that secondary aggregation occurred, impeding further measurements. If PEC particles displayed very close values of electrophoretic mobilities, whatever their composition, the secondary aggregation starting from pH=5.5 for n^+/n^-=1.5 and 2, evidenced lower amounts of excess chitosan surrounding PEC particles as n^+/n^- decreased. The structure of PEC particles (n^+/n^-=3) was examined by SEM at high magnification (Figure 3). DS 5k based particles displayed a spherical structure, although those obtained with DS 500k were less spherical and smaller, in accordance with the previously mentioned effect of DS chain length.

In a second approach, we used only a one-shot addition of polyelectrolyte. In fact, with a dropwise approach, flocculation occurs when n+/n– is close to 1 for both anionic and cationic particles. Addition of a large excess of titrant doesn't allow to dissolve the flocculate complexes.

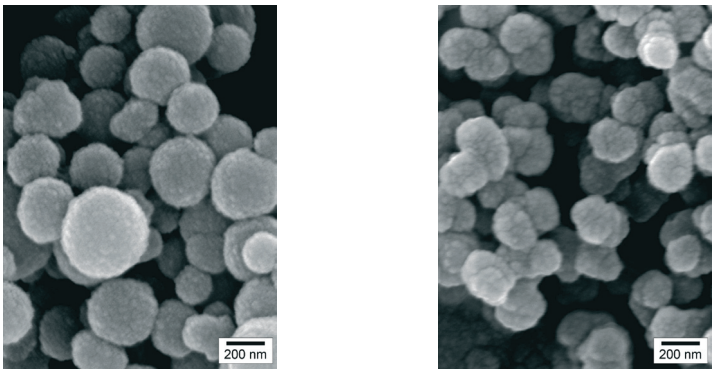

Figure 3. Scanning electron microphotographies of chitosan (low M_w, DA=15%) / dextran sulfate PEC particles (n^+/n^-=3) for two molecular weight dextran sulfate: (a) DS 5K, (b) DS 500k

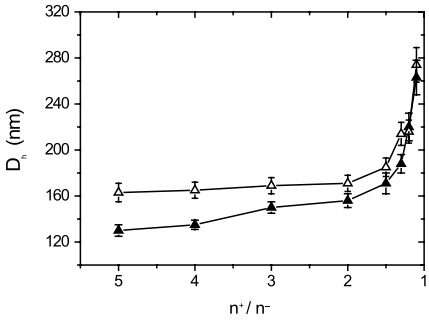

Figure 4. Effect of the mixing order on particles sizes with a one-shot addition ▲ dextran sulfate in chitosan △ chitosan in dextran sulfate (chitosan : DA=15%, M_w=15 000 g/mol and dextran sulfate : M_w=1 500 000 g/mol)

The figure 4 shows that's the effect of the mixing order on particles sizes with a one-shot addition induced a similar pattern as for the dropwise approach.

CONCLUSIONS

Positively charged colloidal PEC particles were obtained by dropwise addition of default amounts of dextran sulfate of various molecular weights, to chitosans different in molecular dimensions and degrees of acetylation. The main parameter controlling the size of the colloidal complexes was the molecular weight of chitosan and the molar ratios of DS to chitosan, suggesting that the relative higher reactivity of the strong polyanion had to be taken into account. The dropwise approach is limited by the flocculation at n^+/n^- ~1. The one-shot method is independent of the order of mixing. The formation of PECs from natural polysaccharides appears to be an efficient approach to obtain new materials via an environment friendly approach.

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INTERACTION STUDY BETWEEN COPPER II IONS AND CHITOSAN RESIDUES BY DFT CALCULATION

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Abstract: This study aimed with the interactions between one Cu^{2+} ion and one or several glucosamine monomer units. The geometry and the interaction energy of all chitosan oligomers complexes were computed using Density Functional Theory (DFT) methods. We investigated the interactions of Cu^{2+} with the different possible coordination sites of glucosamine and *N*-acetyl glucosamine. For every considered complex, the Cu^{2+} site was completed with H_2O and/or OH^- ligands to have a global neutral charge. The calculations confirmed that the most stable interactions involved the amino-nitrogen site in a pending complex. Another pending form was possible, involving the heterocyclic O site, but it was less favourable. Glucosamine could not act as a bidentate ligand and *N*-acetyl glucosamine was not a coordinate for Cu^{2+}.

Key words: Complexes, metal ions, molecular-modelling, glucosamine, *N*-acetyl-D-glucosamine.

Molecular modelling tools are very useful to understand interactions between molecules and/or ions. In this study we are focusing on the interactions between Cu^{2+} ions and oligomers of chitosan [1]. Chitosan chains form stable complexes with several metal ions. This feature is used both for depollution or for the development of new materials.

In chitosan we have two types of units, one of type A corresponds to glucosamine and the other, type B to *N*-acetylglucosamine. For monomers A and B, the different atoms were numbered as in figure 1.

Starting from previous works [1], a scan of the dihedral angle for one dimer, A-A, A-B, B-A and B-B, several complexes were considered:

One Cu^{2+} ion interacting with:
one monomer A or B.
two monomers A.
one dimer A-A.
two dimers A-A.
Two ions interacting with one dimer A-A

The formation energies (E) of the following reactions were considered:

$$[Cu(OH)_2(H_2O)_2] + A \xrightarrow{E} [Cu(OH)_2(H_2O)A_i] + H_2O$$

$$[Cu(OH)_2(H_2O)_2] + A \xrightarrow{E} [Cu(OH)_2A_{ij}] + 2H_2O$$

$$[Cu(OH)_2(H_2O)_2] + A\text{-}A \xrightarrow{E} [Cu(OH)_2(A_i\text{-}A_j)] + 2\,H_2O$$

For every model the conformations of minimal energy were computed using the Density Functional Method (DFT) [2, 3] (ADF software) [4]. Generalized Gradient Approximation (GGA) was set with the Becke gradient correction methods associated with the Perdew correlation term. The different atomic orbitals of models were described with the double Zeta atomic basis set (DZ) [5]. This method of calculation allowed a reliable description of the dative bond and weak interactions such as hydrogen bond. The geometry and the interaction energy of all complexes were computed using the Basis Set Superposition Error (BSSE). Results are reported on the table 1.

The analysis of the (E) energies, in the table 1, reveals some interesting results.

For the pending complexes formed with one monodentate monomer for one copper II ion, the most stable interaction occurs between the nitrogen atom of the amine group and the copper ion (complex noted ($A_{2'}$)). An interaction with the oxygen inserted in the sugar ring (complex ($A_{5'}$)) is also possible, but less stable, about 7 kJ.mol^{-1}. The other complexes formed with the other oxygen sites are energetically disfavoured.

All the bidentate complexes such as ($A_{2',3'}$) and ($A_{3',4'}$) exhibit very high energies and, then, cannot be formed.

The most stable complex involves two amine groups each one belonging to two different A monomers cis($A_{2'}$)($A_{2'}$). The trans conformation of this complex is less stable as its energy is 12.9 kJ.mol^{-1} higher.

The most stable B complex exhibits an energy difference higher than those of complex ($A_{2'}$), close to 70 kJ.mol^{-1}. As a consequence, these results confirm the absence of any interaction between Cu^{2+} and B monomers.

Cu^{2+} ion may interact with A-A dimers. We observe that the E energies of the formation reactions, are comprised between 51.9 and 151.5 kJ.mol^{-1}. These values show that these types of complexes are not probable.

In a last case, we considered one dimer A-A acting as a bridging ligand between two Cu^{2+} ions. The interaction energy for one Cu^{2+} was –89.7 kJ.mol^{-1}. This last value is found very close to –75.56 kJ.mol^{-1}, the interaction energy of one Cu^{2+} with one A, in the pending complex (A_2'). An analysis of the optimized geometry of the model reveals that there is no interaction and no close contact between the two ions. This model suggested that if the chitosan chain was saturated with copper II ions, every amino function would be interacting with one ion.

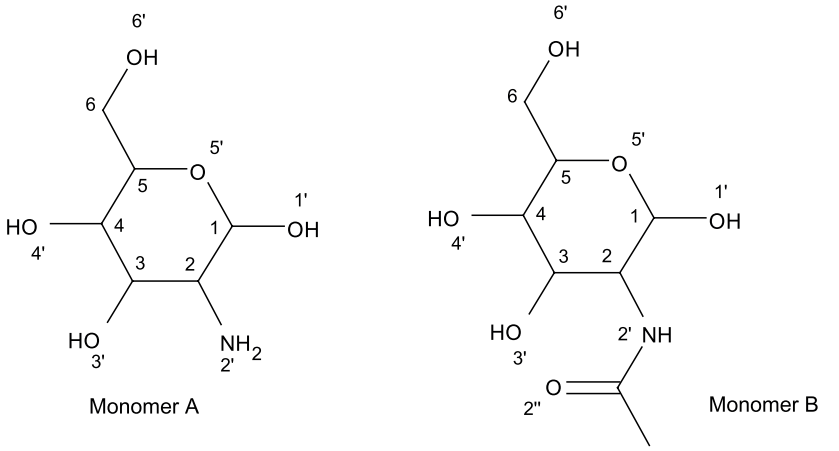
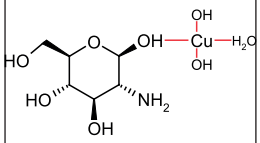
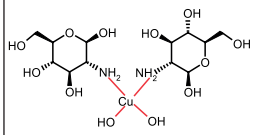
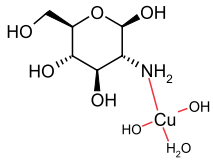
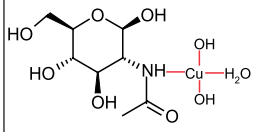
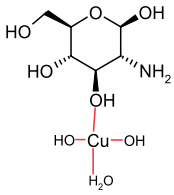
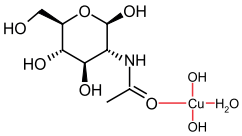
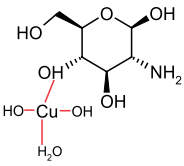
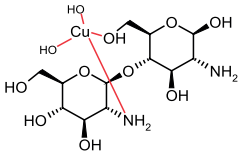
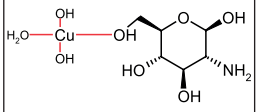
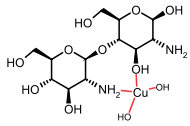
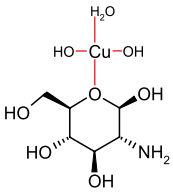
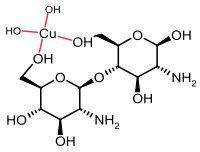
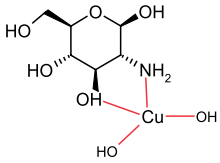
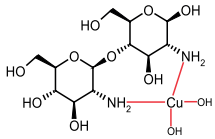
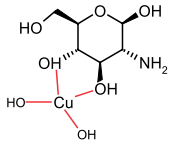
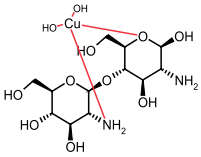
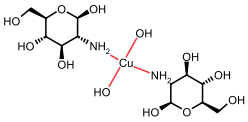
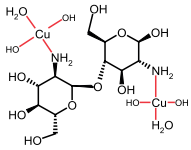


Figure 1. Numbering of heavy atoms for monomers A and B

Table 1. Energies (E) of formation reactions for all the studied cases

Structure	Complex name	Energy E (kJ. mol^{-1})	Structure	Complex name	Energy E (kJ. mol^{-1})
	$A_{1'}$	–1.87		$A_{2'}A_{2'}$ (trans)	–65.5
	$A_{2'}$	–75.6		$B_{2'}$	–5.31
	$A_{3'}$	–48.4		$B_{2''}$	–51.61
	$A_{4'}$	–35.6		$Cu(OH)_2$ $A_{2'}$---$A_{6'}$	+83.2

Structure	Complex name	Energy E (kJ. mol^{-1})	Structure	Complex name	Energy E (kJ. mol^{-1})
	$A_{6'}$	–7.49		$Cu(OH)_2$ $A_{2'}$---$A_{3'}$	+51.9
	$A_{5'}$	–68.9		$Cu(OH)_2$ $A_{6'}$---$A_{6'}$	+151.5
	$A_{2',3'}$	+26.9		$Cu(OH)_2$ $A_{2'}$---$A_{2'}$	+92.0
	$A_{3',4'}$	+90.0		$Cu(OH)_2$ $A_{2'}$---$A_{5'}$	+73.5
	$A_{2'}.A_{2'}$ (cis)	–78.4		Cu.$A_{2'}$---$A_{2'}$. Cu	–89.7 (by Cu^{2+})

CONCLUSIONS

This theoretical study is confirmed by experiments [6–9]. The most favourable site is the free amine function of D-glucosamine. No sites of the *N*-acetyl-D-glucosamine can coordinate with Cu^{2+} ions. The next step will be to investigate the bridging with Cu^{2+} of several long A chain using the QM/MM approach.

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ULTRASOUND ASSISTED DE-*N*-ACETYLATION OF *α*- AND *β*-CHITINS

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Abstract: In the heterogeneous de-*N*-acetylation process of α- and β-chitins, the action of low frequency (35 kHz) and low power (~2 W/cm^2) ultrasounds was applied. Reactions of de-*N*-acetylation were carried out in a flat-bottom flask with thermostatic jacket in the air and helium atmosphere at 30°C, and with chitin : alkali weight ratio equal 1:30 (relatively mild conditions). Degree of de-*N*-acetylation was determined by ^{13}C CP/MAS NMR and 1DUV methods. Intrinsic viscosities were determined by viscometry using Terbojevich's pair of Mark-Houwink-Kuhn-Sakurada equation constants. The results showed that the application of ultrasound during heterogeneous de-*N*-acetylation of chitin leads to the decrease in the crystallinity of the sample and to the decrease of degree of polymerization (air atmosphere) in comparison to the de-*N*-acetylation carried out without the ultrasound action.

Key words: *α*-chitin, *β*-chitin, ultrasound, de-*N*-acetylation, crystallinity

INTRODUCTION

Chitin, poly-(1→4)-2-acetamide-2-deoxy-D-glucopyranose, is the second most abundant polymer, after cellulose, occurring in nature [1]. This material consists mainly of one polymorph: *α*-chitin, in which the orientation of chain stacks is anti-parallel (↑↓), with all hydroxyl groups hydrogen-bounded. Less frequently used polymorph is *β*-chitin (i.e. from squid pens, spines of the diatoms), in which all chains are parallel to each other (↑↑) and there are no hydrogen bonds connecting chain stacks, what facilitates the swelling in water and/or penetration of chemical reagents. The third, rarely occurring polymorph of chitin is γ-chitin, composed of one anti-parallel and two parallel stacks per unit cell (↑↑↓) [2, 3]. The most stable among all the three polymorphs is *α*-chitin, which does not undergo any polymorph transition to another form, in opposite to *β*- and *γ*-chitins which can be readily converted to the *α*-form [3]. Thus, the *β* polymorph is reported to be transformed to *α*-chitin on swelling in 6M HCl or when the precipitation of chitin from formic acid is performed. In relatively mild conditions (preparation of chitin form native chitin) these polymorphs are stable [4].

Since the morphological structure is the main factor determining the chemical activity of chitin, it seems to be interesting to study the influence of the action of some mechanical, hydrodynamic or other forces on the morphological structure and reactivity of chitin materials obtained from different sources.

Thus, the aim of the present work is to investigate sonochemical effects on the de-*N*-acetylation process of α- and β-chitins. The choice of ultrasound field as the modificating factor is caused by its ability to induce high local mechanical and hydrodynamic forces (mainly as the result of cavitation effect), which can lead to the decrease in particle size (mechanical destruction) of solids, better mixing (emulsification) of reagents, and the activation of reactive centres in the liquid phase [5].

EXPERIMENTAL

Materials

Finely powdered α-chitin (particle size < 0.04 mm) and β-chitin (particle size < 0.1 mm) were obtained from Primex (Norway) and Heppe GmbH (Germany), respectively. Characterisation of the material was done by means of ^{13}C CP/MAS NMR (F_A of α- and β-chitins was in the range $0.95 \leq F_A \leq 1.00$), WAXS (wide angle X-ray spectroscopy) (crystallinity index, x_K) and viscometry techniques (described in detail below).

Determination of FA

F_A values of α- and β-chitin and de-*N*-acetylated (insoluble in 0.1M acetic acid) chitins were determined by ^{13}C CP/MAS NMR [6] on Brucker AMX 300 (75.47 MHz) instrument. F_A values of soluble (in 0.1M acetic acid) chitosans were determined by 1DUV (first derivative ultra-violet) method [7].

WAXS

X-ray diffraction patterns of starting chitin material, de-*N*-acteylated chitins and chitosans were obtained using diffractometer XPERT PRO type within the angle data range 5–35° by 0.1°. The method of Wakelin, Virgin and Crystal [8] was adopted for determination of the correlation crystallinity index.

Viscometry

For samples soluble in 0.1M CH_3COOH after de-*N*-acetylation, in order to determine values of intrinsic viscosity ([η]), the solvent system of Terbojevich et al. [9] consisting of 0.5MCH_3COOH/0.2MCH_3COONa was used at 25°C ± 0.02. Values of [η] were determined as the average from Huggins and Kraemer plot using flow times measured in Ubbelohde type viscometer. The kinetic energy correction was calculated and included in calculations of [η].

De-*N*-acetylation

Reactions of de-*N*-acetylation of chitins were carried out in a 500 mL flat-bottom flask with thermostatic jacket in relatively mild conditions, i.e. in 50% (w/w) NaOH water solution at 30°C (higher effectiveness of ultrasonic treatment at this relatively low temperature) for 6 h at weight chitin: NaOH ratio of 1:30 (5.00 g of chitin was taken for de-*N*-acetylation in each time). Additionally, the second step (3 or 6h, other conditions the same as above) of the process was done for de-*N*-acetylated α-chitin. Ultrasound assisted reactions of de-*N*-acetylation of chitins were carried at the same conditions, using low-power ultrasonic emitter:

Transsonic T420 characterised by applied frequency equal 35 kHz, and the sonic intensity ~2 W/cm^2. All reactions were carried out in air or helium atmosphere with constant rate (~100 rpm) of mechanical stirring. After de-*N*-acetylation, the stock solution was roughly neutralised with 80% water solution of CH_3COOH to pH ~9.0, and the resulting chitosan was filtered, washed with distilled water to neutral and finally with acetone and dried.

RESULTS AND DISCUSSION

FA changes

Values of F_A determined for insoluble in 0.1M CH_3COOH de-*N*-acetylated chitins (by ^{13}C CP/MAS NMR) and chitosans (by 1DUV) are collected in Table 1. It can be clearly seen that there was not possible to obtain fully soluble products from *α*-chitin in applied conditions after 6h of the reaction of de-*N*-acetylation, since F_A of these samples is much higher than 0.5. Though the ultrasound treatment caused decrease in F_A by 0.04, the determined value (0.66), however, is still too high to make the samples completely soluble.

In each case the influence of ultrasound treatment on F_A value is low and causes the decrease of F_A. This indicates that ultrasonic field does not accelerate the de-*N*-acetylation process itself and chemical effects are relatively week in the case of this process.

Table 1. F_A values of de-*N*-acetylated chitins and chitosans determined by ^{13}C CP/MAS NMR and 1DUV techniques, respectively

Form	De-*N*-acetylation			Solubility in 0.1 M CH_3COOH
	Time	Standard procedure	Ultrasound assisted	
α	6h	0.70	0.66	–
	2 × 6h	0.47	0.46	+
β	6h	0.48	0.44	+

Changes in degree of polymerisation (DP)

Simultaneously with de-*N*-acetylation, process of alkaline hydrolysis of chitin (chitosan) takes place in the system. Therefore, the changes in DP of resulting chitosans were studied. The values of [η] being the measure of viscosity average DP of obtained chitosans are collected in Table 2.

Table 2. Values of [η] (dL/g) for chitosans obtained in standard and ultrasound assisted de-*N*-acetylation in helium and air atmospheres. Solvent system: 0.5M CH_3COOH/0.2M CH_3COONa, T = 25°C [9]

Form	standard procedure		ultrasound assisted	
	helium	air	helium	air
α	6.4	7.0[a]	6.2	3.7[a]
β	6.1	–	6.1	

[a] – in 0.1M CH_3COOH/0.2M NaCl solvent system.

As it can be seen from Table 2, there is no effect of ultrasound on [η] values in helium atmosphere, what can be attributed to the absence of oxygen in this system what allowed to avoid the free radical degradation of de-*N*-acetylated chitins. The presence of oxygen in the system causes degradation of resulting chitosan, accelerated by the ultrasound action.

Changes in crystallinity

X-ray diffraction patterns provided the information about the relative changes of crystallinity of *α*- and β-chitin samples during de-*N*-acetylation. Figs. 1. a) and b) collect spectra for *α*- and β-chitin together with correlation crystallinity index (x_K) [8] calculated from the slope of the following equation:

$$I_{Wi} - I_{Ai} = x_K (I_{Ci} - I_{Ai}) + C \quad (1)$$

where I_{wi} is the intensity recorded for the considered sample at *i*- 2Θ angle, I_{Ci} and I_{Ai} are the intensities for the most crystalline and most amorphous samples, respectively, and C is the constant.

As it can be seen from diffraction patterns, the most crystalline is the mother *α*-chitin and the most amorphous is the mother *β*-chitin among all samples under investigation. Therefore, according to the procedure proposed by Wakelin, Virgin and Crystal [8], the relative crystallinity of *α*-chitin is established to be 100%, whereas for *β*-chitin it is 0%.

Diffraction patterns shown in Fig. 1.a) allowed to calculate the value of x_K for de-*N*-acetylated *α*-chitins in relation to the mother samples. The decrease of x_K is observed during de-*N*-acetylation. The additional slight (up to 8% after double 6h de-*N*-acetylation) decrease is caused by the ultrasonic treatment. This observation indicates that during the heterogeneous de-*N*-acetylation of *α*-chitin (up to ~60% extent of de-*N*-acetylation) the morphological structure of this form is being destroyed (what was expectable, cf. [10]), and the process is accelerated by the ultrasound action, proving that the mechanical forces present in the system due to the cavitation process are sufficiently high to decrease the crystallinity of de-*N*-acetylated *α*-chitin. The effect of ultrasound treatment is much higher in the case of *β*-chitin. The increase of the crystallinity index of chitosan obtained from *β*-chitin in comparison to the mother chitin sample is about 40% lower in the case of ultrasound assisted de-*N*-acetylation. This effect indicates that mechanical and hydrodynamic forces present in the system prevent ordering of de-*N*-acetylated *β*-chitin and/or chitosan chains. The increase in ordering of chains was also observed for chitosans with $F_A < 0.4$ obtained from *α*-chitin, after decrease of crystalinity index (up to F_A ~0.4, where the minimum of crystallinity index was observed) [10]. As this increase of crystallinity is probably caused by forming different morphological forms characteristic for chitosan [11], one may postulate, that crystalline structure of chitosan is much less stable than this in *α*-chitin, and ultrasound action may lead to obtain completely amorphous chitosan.

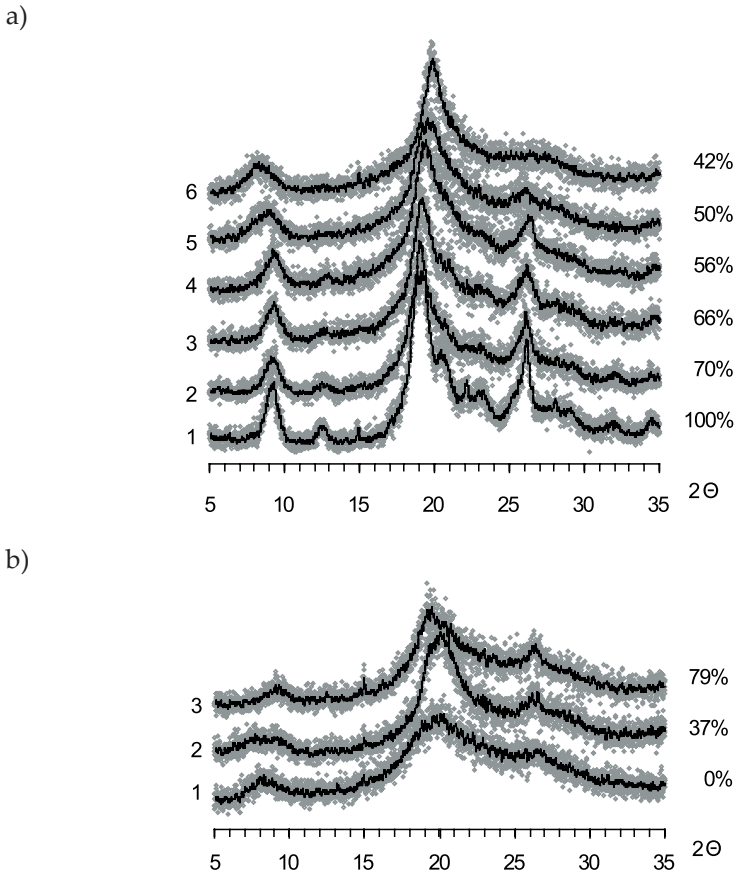

Figure 1. X-ray diffraction patterns for α-chitin (a): mother sample (1), sample de-*N*-acetylated 6 h without ultrasonic treatment (2) and with (3), sample de-*N*-acetylated 6 h plus additional 3 h with ultrasonic treatment (4), sample de-*N*-acetylated 6 h plus additional 6 h without ultrasonic treatment (5) and with (6), and for β-chitin (b): mother sample (1), de-*N*-acetylated through 6 h with ultrasonic treatment (2) and without (3). Values of x_K given in %

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CHAPTER VIII

FUNCTIONAL PROPERTIES OF CHITIN AND CHITOSAN

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CHITOSAN AS A POTENTIAL CARRIER FOR DRUG TARGETING ACROSS BODY BARRIERS

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Keywords: Chitosan, blood-brain barrier, drug delivery, nanoparticle

Abstract: In past years it was shown, that chitosan has a great potential as a drug delivery system across various body barriers. The present work investigates the possibility of chitosan as a transport carrier across the blood-brain barrier. Chitosan nanospheres labeled with fluorescent markers were used to study the efficiency and specificity of its uptake into cellular components of mouse brain by different routes of administration including intrahippocampal and intravenous application. Chitosan has been found to diffuse within the hippocampus. Applying fluorescence microscopic analysis chitosan was detected in high concentration in the hippocampal subfields CA1, CA2, CA3 and the dentate gyrus. We noticed, that chitosan was mostly taken up by pyramidal neurons. Nanoparticles were also detected in the vascular system of the brain regions inspected. Following intravenous injection chitosan was mainly taken up by neurons located in the hippocampal subfields CA1 to CA4 and dentate gyrus, but was detectable also in brain regions outside of the hippocampal formation.

INTRODUCTION

For a pharmaceutical or medical application of chitosan it is very important to have a defined product with defined charactersistics. In recent years chitosan particles were described as a drug delivery system across various body barriers, for instance the skin barrier for dermal application [1] or the intestine barrier for oral application of drugs [2]. Especially nanoparticles can penetrate the cell membrane and deliver active agents into the cells [3, 4]. For these different application fields it is necessary to modify chitosan in a way, that the best activity on the application area is achieved. Modifications are possible in size or surface conditions. Particles can be produced with a solid core or as capsule, as spherical particle or as fiber [1]. The activity of the transport system can be modified by varying the molecular weight and the degree of deacetylation or using chitosan derivatives. For producing the optimum active system these properties should be considered.

In the last years the mechanism of transport was discussed as an influence of chitosan on the surface charge on epithelial cells of intestine. That means the chitosan can change the binding properties of occludin and ZO-1 [5] and as a result the tight junctions are opening. In the present study we investigated whether the charge density of different chitosan particles was sufficient enough to open also the blood brain barrier.

Chitosan nanospheres labeled with fluorescent markers were used to study the efficiency and specificity of its uptake into cellular components of mouse brain using different routes of administration including intrahippocampal and intravenous application.

EXPERIMENTAL

Intrahippocampal administration of labeled chitosan

Mice received a stereotaxic injection of 1.6µl of labeled chitosan (0.1% in NaCl solution, labeled with Carbocyanine-3, Chitosan received from Heppe GmbH) into the hippocampus (AP = –1.7 mm, L = 1 mm, DV = 2 mm, relative to the bregma). Three days after injection the animals were transcardially perfused with phosphate-buffered 4% paraformaldehyde and 0.1% glutaraldehyde for the histochemical analysis and with 0.5% glutaraldehyde for electron microscopy, and brains were postfixed overnight in 4% paraformaldehyde.

Histochemistry: Following equilibration with 30% sucrose, brains were cut with a freezing microtome in 30µm thick sections and subjected to immunofluorescent labeling of neurons (e.g. parvalbumin), astroglia (e.g. GFAP) and microglia.

Electron microscopy: Coronal vibratome sections of the hippocampus were cut at a 50-µm thickness. To localize nanospheres, fluorescent thioflavin-S and carbocyanine-3 were photoconverted into electron-dense material using oxygen-enriched photoconversion in a closed conversion chamber. Tissue blocks were washed, osmificated, dehydrated, embedded in Ducurpan and semithin or ultrathin sections were prepared [6].

Intravenous injection of mice

Mice were injected intravenously (i.v.) with 100 µl of a 0.05% chitosan-carbocyanine-3 suspension in sodium chloride into the tail vein. 0.5, 2 and 5 hours after injection the mice were perfused using the same procedure as described above.

RESULTS AND DISCUSSION

Intrahippocampal administration

It was observed, that different chitosans demonstrate considerably different diffusion properties. Small variance in molecular mass and degree of deacetylation have a major impact on diffusion, uptake and accumulation properties. Here we present the results of that chitosan which shows currently the best diffusion properties.

After a direct administration the chitosan particles diffuse to different subregions of the hippocampus including into cells of the subregion CA1 to CA3. Figure 1A shows a whole section of the hippocampus. It was observed that the

particles diffuse through the hippocampus far away from the injection area. The accumulation occurred in neurons, preferentially in specific cells known as pyramidal neurons (see Fig. 1B).

Microscopical investigations were performed to prove, that the particles penetrate into the cells. By laser scanning microscopy we observed cells in different cutting planes. Surprisingly the particles were bigger than the original injected nanoparticles.

Electromicroscopic studies were performed in order to reveal the cause of this observation. Particles were found in 3 different stages in the cell (Fig 1C). In one stage, mostly 2 to 4 particles are surrounded by a membrane. In addition, there are also larger aggregates, in which particular particles are already visible. These aggregates are freely distributed within the cytoplasm or are attached to structures similar to lipids. On these structures the particles accumulate to larger extent comprising several few hundred particles, forming solid aggegates in which single particles are no longer visible.

Chitosan is mostly taken up by neuronal cells, as shown in figure 1B. We observed that chitosan was absorbed at a high percentage in parvalbumin – immunoreactive neurons known to mediate fast-firing responses. The particles were also observed in some other neurons. The uptake of particles by cells, which can be labeled with Calbindin binding proteins, demonstrated a region specific accumulation. Only Calbindin positive cells located in the hippocampal subregions CA1-CA3 were found to absorb chitosan, while corresponding cells in the dentate gyrus did not accumulate chitosan.

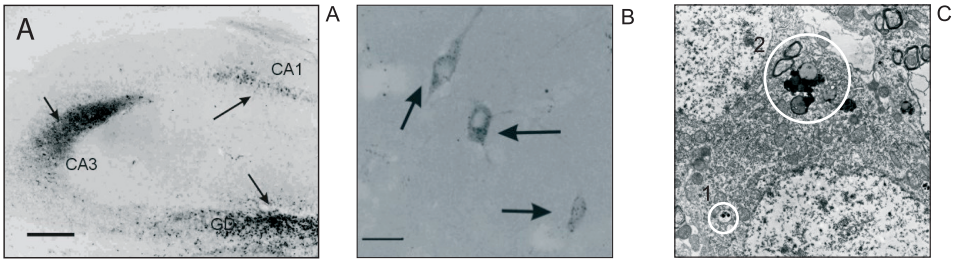

Figure 1. Fluorescence microscopy of the mouse hippocampus, 3 days after intrahippocampal injection of carbocyanin-3 labeled chitosan (dark black, A and B). Particles diffuse in the hippocampal subfields CA1, CA2 and CA3 and the dentate gyrus (GD; A). Particles accumulate mostly in pyramidal cell layers (indicated by arrows, images A, B). C. Ultrastructural image of a semithin section obtained from fixed and plastic embedded mouse CA3 region 3 days after intrahippocampal injection of fluorescent labeled chitosan and following photoconversion of carbocyanin-3. Representative adducts of photoconversion are indicated by circles. Circle 1 displays chitosan as small agglomerates of 2-4 particles, larger agglomerates are highlighted by circle 2. The biggest agglomerates represent hundreds of chitosanparticles. Scale bar: A = 200μm, B = 50μm, C = 2μm

The next step was to answer the question whether the particles may also accumulate on extracellular pathological structures in the brain, for example beta amyloid plaques one of the main features of Alzheimer's disease. We found, that chitosan also accumulates on plaques (Fig. 2A), whereas vessels, which interact with thioflavin-S, show rarely a second labeling with chitosan particles. These findings indicate that chitosan may exhibit a potential tool for in vivo-imaging of

beta-amyloid plaques as well as for selective drug targeting to sites of damage in Alzheimer's disease.

Following i.v. injection chitosan was mainly taken up by neurons located in the hippocampal subfields CA1 to CA4 and dentate gyrus(GD) (see Fig. 2B), but was detectable also in brain regions outside of the hippocampal formation. It was observed, that the particles were mostly taken up in pyramidal cell layers of the brain.

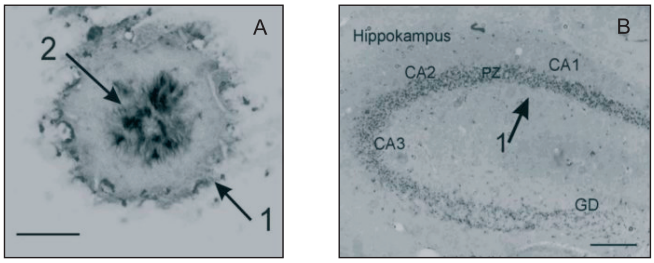

Figure 2. A: Native cutting section of brain from Tg2576 mices (17 month old), beta-amyloid plaques were labeled with thioflavin-S, chitosan particles are labeled with carbocyanin-3. Chitosan particles (arrow 1) accumulate on beta-amyloid plaques(arrow 2). Scale bar = 50 μm. B: shows fluorescence microscopic analysis of the mouse hippocampus 120 minutes after intravenous injection of carbocyanin-3 labeled chitosan. The highest concentration of labeled chitosan particles was also found in pyramidal cell layers. Scale bar: 200 μm

CONCLUSIONS

Chitosan is a potential transport system across the blood brain barrier. It can be used as a vehicle to transport drugs to specific cells in the brain and, consequently, to accumulate active agents on specific structures or in cells. In future studies we will test the usefulness of chitosan coupled with compounds of therapeutical or diagnostic interest to target damaged sites in neurodegenerative diseases, which will finally lead to novel therapeutic applications of chitosan in the medicine .

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MECHANISM OF WOUND HEALING BY CHITIN AND CHITOSAN

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We have investigated chitin and chitosan for veterinary application and also examined basic mechanism of wound healing acceleration by these materials for 18 years. Since 1985, we have been developing several materials from chitin and chitosan for clinical use in veterinary medicine. Now, we have more than 400,000 treated cases (most of them are dogs and cats) using with two types of veterinary remedies (Chitipack P (composite material of chitin and non-woven fabric of polyester) and Chitofine (chitosan fine powder)) in Japan. Chitipack P is usually used for hernia repair and Chitofine is for wound dressing. With developing chitin and chitosan materials for veterinary medicine, mechanism of wound healing acceleration is also investigated in various *in vitro* and *in vivo* studies. Main mechanisms are summarized as follows.

Complement activation [1]

Complement activation was examined by single radio-immunodiffusion using anti-human C3, C4, and C5. After incubation with chitosan particles and human serum, the concentration of C3 and C5 decreased, whereas it of C4 showed no change (Fig.1). In the alternative pathway of complement activation, complement component C4 does not activate. Therefore, chitosan activates complement via the alternative pathway. The concentration of C5a in the serum incubated with chitosan also estimated by RIA methods. In the human serum after incubation with chitosan particles, high concentration of C5a (over 400 ng/ml) was detected. C5a is the strongest anaphylatoxin, and acts as a chemoattractant for polymorphonuclear cells (PMNs) and activates vascular endotherium with contraction of vascular muscle. These effects cause strong vascular permeation with positive charged albumin and migration of numerous PMNs to chitosan. Most important mechanism of biological activation by chitosan is complement activation.

Chitosan also bound C3b (opsonin) and factor H (inhibitor of complement activation) as same as Zymosan. The detail of the data showed in another paper of the proceedings (Okamoto et al.).

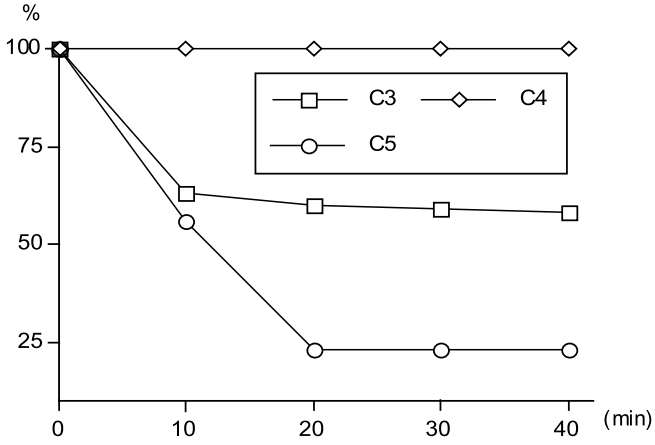

Figure 1. Complement activation by chitosan. Horizontal line shows incubation time. Vertical line means complement activation. Concentration of each complement component decreases by activation with chitosan

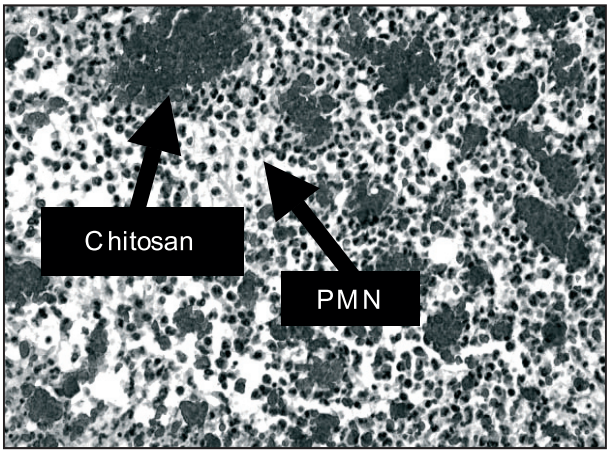

Figure 2. Histological findings on 7 days post-injection of chitosan suspension to canine subcutaneous tissue Numerous PMNs migrated to and around chitosan particles. One of the messengers for PMNs migration to the chitosan particles is C5a

Biological activation of migrated PMN [2]

Migrated PMNs produced super oxide (Table 1), and arachidonic acids (Table 2) such as PGE_2, LTB_4 and LTC_4, after attachment to the chitin and chitosan particles. Super oxide production from PMNs was estimated using chemiluminescence reader. Arachidonic acids were measured by EIA methods using commercial kits. PMNs recognize chitin and chitosan particles only existing fresh serum from the results of the serum free and the heat-treated serum. To recognize the particles by PMNs, opsonization by C3b in fresh serum is essentially needed. The arachidonic acids, especially PGE_2 and LTB_4 were much produced from PMNs. PGE_2 induces increasing of vascular permiabilty like C5a, and LTB_4

enhances PMN migration from vessels. These productive substances act as biological activator and killer of invader such as bacteria.

Table 1. Effect of chitin and chitosan on super oxide production from PMNs*

Stimulating factor	Fresh canine serum	Heat-treated canine serum	Serum free
Zymosan	537.4 ± 22.3	215.0 ± 5.3	122.4 ± 12.9
Chitin	156.0 ± 12.3	2.4 ± 1.5	1.7 ± 0.3
Chitosan	269.8 ± 27.3	1.4 ± 0.7	1.5 ± 0.3

* The maximum chemiluminescence (LU) per 1,000 PMNs was measured using an LB-9505C Multi-Biolumat (Berthold, Germany). Data are displayed as the mean ± s.d. (n=6)

Table 2. Arachidonic acid synthesis and release from canine PMNs due to stimulation by chitin and chitosan

Stimulating substance*	Substance produced**		
	LTB_4	LTC_4	PGE_2
Chitin	8300 ± 1500	64 ± 24	3700 ± 1000
Chitosan	12000 ± 2500	200 ± 32	5200 ± 3000
Latex beads	150 ± 20	73 ± 16	60 ± 15
Control	16 ± 5	19 ± 7	21 ± 11

* The control was Hank's buffer solution, the culture solution.

** The values show the mean ± S.D. (pg/ml) for the concentration of the substances produced (leukotoriene B4 and C4, and prostaglandin E2) in the supernatant when canine PMNs and the stimulating substance were mixed and incubated

Direct effect of chitin and chitosan to fibroblast and vascular endothelial cell [3, 4]

Chitin and chitosan stimulate collagenase production from fibroblasts. Collagenase activity was measured by RIA method for estimating prolyl-hydroxylase activity in granulating tissue regenerated recovering from the chitin-nonwoven fabric of polyester (chitin-NWF) or the chitosan-NWF administrated wounds (Table 3). Migration of fibroblasts and vascular endothelial cells were measured by Boyden chamber method. Human umbilical endothelial cells (HUVEC) and mouse fibroblast cell line (3T6) were used for a direct migratory assay (Table 4).

Collagenase activity was much higher in the chitin-NWF than others. Chitosan did not activate collagenase. It is well recognized chitosan induces much granulating tissue formation than chitin. We have many experiences of clinical cases with excessive granulating tissue formation in wound resulting long-term administration of chitosan. Chitin also regenerates granulating tissue in wound, however no excessive tissue regenerates. From the results of prolyl-hydroxylase production, degradation of tissue collagen will more strictly occur by chitin administration.

Table 3. Prolyl-hydroxylase activities in regenerated granulating tissues by chitin or chitosan administered wounds

Stimulating substance*	Concentration of prolyl-hydroxylase (dpm/μg of protein)		
	Day 4	Day 7	Day 11
Chitosan-NWF	1.8 ± 0.2	5.0 ± 4.0	9.5 ± 3.0
Chitin-NWF	1.0 ± 0.2	9.0 ± 1.0	9.5 ± 2.0
Control (NWF)	1.8 ± 0.2	6.0 ± 4.0	8.3 ± 6.0

* Each stimulating substance was implanted in back of mice surgically, and was recovered on day 4, day 7 and day 11 after implantation

From the results of direct migratory activities of HUVEC and 3T6 to chitin and its derivatives, chitin and chitosan found to enhance HUVEC migration, however inhibit 3T6 migration. The data well explain clinical response of wound by chitin or chitosan administration. Chitin and chitosan regenerate vascular rich granulation in the wound. In contrast, 3T6 did not enhance migratory activity by chitin or chitosan, the results well explained for no scar formation observed after wound healing using chitin or chitosan in the treatment.

Table 4. Direct migratory activities of vascular endothelial cells (HUVEC) and fibroblasts (3T6) to chitin and its derivatives

Stimulating substance**	Number of migrating cells	
	HUVEC	3T6
Control	240 ± 5	55 ± 5
Chitin	340 ± 25 *	35 ± 8*
Chitosan	290 ± 5*	20 ± 5*
GlcNAc	260 ± 20*	75 ± 5
GlcN	150 ± 20*	40 ± 8*
NACOS	220 ± 10	60 ± 10
COS	240 ± 10	40 ± 10

* There are significant difference between control and other substance ($p<0.05$). **Concentration of each stimulating substance was 1 mg/ml.

Analgesic effect [5]

Chitin absorbs bradykinin, most painful one, but chitosan do not. Chitosan elevates pH by absorbing protons, but chitin does not.

Re-epithelization

In final process of wound healing, we have recognized from the results of many clinical cases, chitin induces good re-epithelization, but chitosan do not. Chitosan stimulates granulating tissue formation continuously, and excessive granulation inhibits keratinocyte migration on the regenerated tissue. The mechanism of re-epithelization also showed in another paper of the proceedings (Okamura et al.).

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MECHANISM OF COMPLEMENT ACTIVATION BY CHITIN AND CHITOSAN

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Key words: amino group, chitin, chitosan, complement, degree of deacetylation

INTRODUCTION

We have investigated wound healing acceleration by chitin and chitosan on various point of views [1]. When we administered subcutaneously a large amount of chitosan for treatment of wound in the dogs, a lethal hemorrhage-pneumonia developed [2]. This phenomenon resembles adult respiratory distress syndrome (ARDS) in human, which is characterized by complement activation [3]. We found that chitin and chitosan activated the alternative pathway of complement *in vitro* [4–6] and *in vivo* [7]. In this paper, we interpret mechanism of complement activation by chitin and chitosan

MATERIALS

Homogeneous and heterogeneous partially acetylated chitosans

Chitosan powder was prepared from commercial chitosan flake (Koyo Chemical Co. Ltd, Tokyo, Japan) with degree of deacetylation (DDA) 82% and molecular weight approximately 80,000. Homogeneous partially acetylated chitosan was prepared by the method of Hirano *et al.* [8]. The DDAs of final products were 82, 50, 42, 24, and 14, respectively. Heterogeneous partially acetylated chitosan was prepared by the method of Miya *et al.* [9]. The DDAs of final products were 100, 53, 40, 33, and 14, respectively. We named homogeneous and heterogeneous partially acetylated chitosans to oDAC and eDAC, respectively. All samples were sterilized by ethylene oxide gas and then suspended or dissolved (oDAC 50 and 42) in saline at 10 mg/ml.

Soluble and non-water soluble chito-oligosaccharides (COSs)

Two type of water soluble COSs (oligo 1: MW 3,000–30,000, Wako Pure Chemicals, Ltd, Osaka, Japan; oligo 2: MW 216-1204, Yaizu Suisankagaku Industries Co. Ltd, Shizuoka, Japan) were prepared. Non-water soluble COSs were prepared by a modified version of the method described by Muraki *et al.* [10]. DPs 44, 71, 91 and 165 were recovered and selected for the study. These were insoluble in water, so each sample was suspended in saline at 10 mg/ml.

Other reagents

D-glucosamine HCl (Yaizu Suisankagaku Industries Co. Ltd, Shizuoka, Japan) was dissolved by saline at 10 mg/ml. Latex beads (particle mean diameters: 1.0 μm (latex 1) and 2.8 μm (latex 2)) were purchased from Polyscience Inc. (USA), and suspended in saline at 10 mg/ml, respectively. Zymosan A (lot. 49H0557) was purchased from Sigma Chemical Co. (St. Lous, USA). Zymosan was tereated by the previous methods (Suzuki et al. 2000), and then stocked at –80°C before use.

Anti-serum

Goat Anti-Dog C3 was purchased from the ICN Pharmaceuticals, Inc. (Ohio, USA). Mouse monoclonal anti-human C3b-alpha and anti-human factor H were purchased from Progen Biotechnik GmbH (Heidelberg, Germany) and Calbiochem (Darmstadt, Germany), respectively. FITC-rabbit anti-mouse IgG (H+L) was purchased from Zymed Laboratories, Inc. (California, USA).

METHODS

Blood collection

Three healthy beagle dogs were used for the collection of normal plasma. Blood samples were collected from the jugular vein into a heparinized sterile syringe, were portioned into sample tubes on ice, and were centrifuged at 4°C and 1200 g for 5 min. Plasma was separated in micro tubes and was frozen at –80°C until use.

C3 assay

The sample used for the C3 assay was the supernatant separated by centrifugation at 1,500 rpm for 5 min at 4°C after incubation with 100 μl of dog plasma and 100 μl of reagents for 20 min or 40 min at 37°C. Each assay was performed in triplicate. The supernatant was separated into micro tubes and was frozen at –80°C until use.

The details of the SRID method were described previously [4]. In brief, the solid gel plate including anti-dog C3 serum was cut into pieces (1×1 cm^2) and a hole was made in the center of each piece. Next, each supernatant was added to a gel and incubated at 4°C for 48 hr. After incubation, the size of the precipitation ring was measured under a stereoscopic microscope. Then the C3 value was calculated as follows: Ring size = π (Ra^2 –Rb^2), where Ra is the radius of the ring diameter and Rb is the radius of the central hole diameter. Each ring size (C3 value) was converted into a percentage by each base value ring size (100%).

C3b and factor H detection

C3b and factor H were detected by inmunofluorescence method [5]. In brief, chitosan and zymosan suspensions (100 μl) were incubated with 100 μl of human plasma (positive sample) or saline (negative sample) in a water bath at 37°C for 20 min. Then each sample was centrifuged 1,500 rpm for 5 min at 4°C. After removal of the supernatant, the pellet was washed three times with saline, then the pellet was incubated with mouse monoclonal anti-human C3b-alpha for 1 h on ice and washed by the same procedure. Subsequently, the pellet was incubated with FITC-rabbit anti-mouse IgG (H+L) and washed again by the same procedure. Finally, the pellets were resuspended in 20 μl of saline and scanned by a Confocal Laser Scanning Microscope (CLSM).

The digital images taken by CLSM were analyzed using computer image analyzer software (Adope Photoshop 3.0 for Macintosh, Adobe system, Tokyo). Each image was analyzed on the green setting. The fluorescence intensity (FI) was obtained by subtracting FI of the negative sample from FI of the positive sample. FI values of 200,000 pixels were randomly collected from 10 field in a CLSM image and image analysis was performed using Adobe Photoshop 3.0 for macintosh.

RESULTS AND DISCUSSION

Influence of physico-chemical properties of chitin and chitosan on complement activation

C3 activations by oDAC and eDAC are shown Figs. 1 and 2, respectively. In oDACs except for oDACs 50 and 42, C3 activation increased in depending manner of deacetylation degree. In eDACs, C3 activations were about 30% in all samples independent of degree of deacetylation. Water soluble materials, oDACs 50 and 42, chitosan oligosaccharides and D-glucosamine, did not induced C3 activation. Latex beads, which were a physical control, also did induced C3 activation. The present results indicated that water insoluble materials were necessary to activate C3. Moreover, amino residue was found to be important to activate C3.

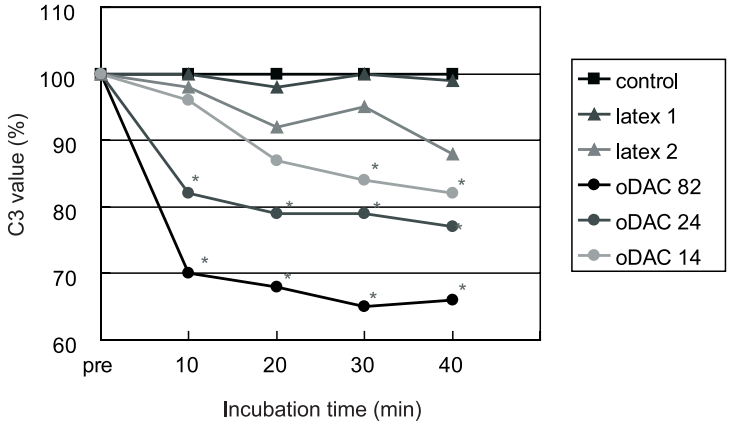

Figure 1.

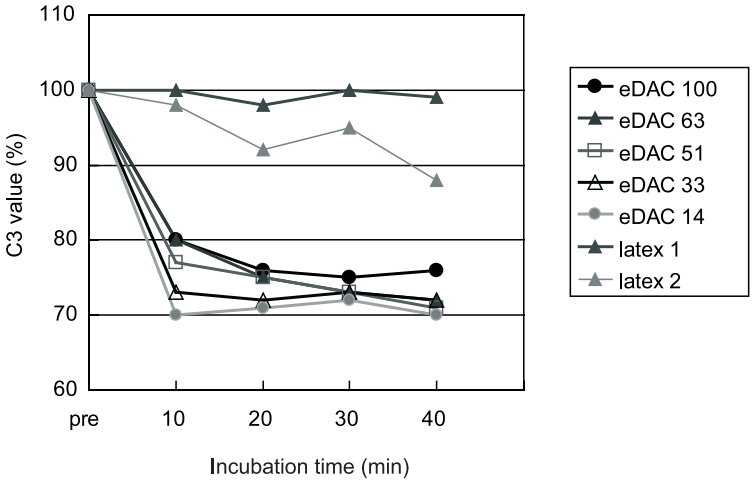

Figure 2.

Influence of the chain length of chitosan on complement activation

C3 activation by COSs is shown in Fig. 3. C3 was less activated by all COSs compared with the chitosan positive control. C3 activation by COSs was increased in a chain length dependent manner. By regression analysis, the correlation between the number of NH_2 group of COSs and the % C3 activation after 20 min of incubation (Fig. 4) gave the following equation ($y = 0.0865\ x + 10.751$, $R^2 = 0.9916$, $P < 0.01$). The extent of C3 activation by the reagents was significantly correlated with the number of NH_2 groups.

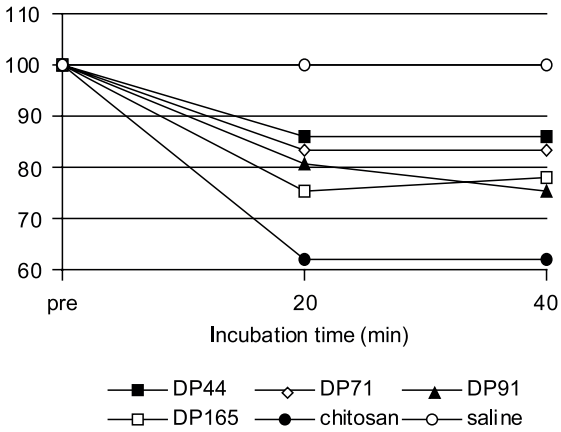

Figure 3.

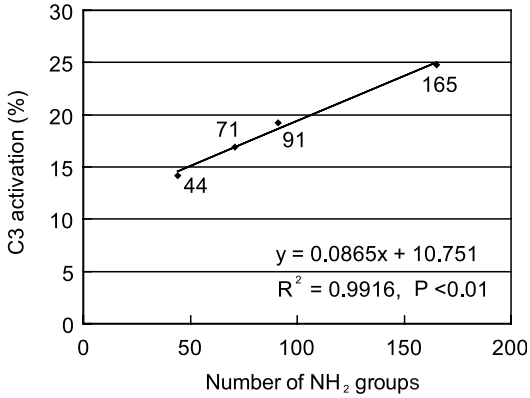

Figure 4.

C3b and factor H bindings

Bindings of C3b and factor H were clearly detected on chitosan (DAC 82) particles as well as on zymosan particles. The fluorescence intensities (FI) of chitosan and zymosan were similar (Table 1). From these results, chitosan binds C3b and factor H in the same way same as zymosan.

Table 1. Image analysis os fluorescence intensity (FI)

	DAC 82	Zymosan
C3b	19.9	17.5
Factor H	18.5	20.4

CONCLUSIONS

Chitosan activates complement in an NH_2 group-dependent fashion. After activation of C3, C3b is produced and effectively binds to chitosan with factor H. The mechanism of complement activation by chitosan seems to closely resemble that for zymosan.

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FUNCTIONALIZATION OF FIBRE SURFACES BY THIN LAYERS OF CHITOSAN AND THE ANTIMICROBIAL ACTIVITY

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Abstract: Results on the permanent fixation of chitosan on cotton surfaces using different chemical bond formation are presented. The antimicrobial activity of those finished textiles has been determined using laser nephelometry and tetrazolium compounds against some selected bacteria and fungi.

INTRODUCTION

An increasing demand develops for imparting active agents to textile materials (fabrics and non-wovens) by chemical ways in order to create additional properties ("functional textiles"). With synthetic fibres this may create a better hydrophilic behaviour (water retention, sweat transport ...). On natural fibres this could mean the anchoring of bacteriostatic or odour binding agents and similar. An advantageous strategy is to rely on existing fibre types and to modify only the surface thus retaining the well known mechanical properties of the bulk fibre. In addition such a strategy imparts more flexibility to the textile finishing industry [1].

So an important task for research and development lies in the evaluation of methods how to anchor such biopolymers permanently onto fibre surfaces in a way that the biopolymers retain their bulk beneficial properties of action [2, 3].

The physicochemical interactions used for permanent fibre surface modification are summarized in Table 1.

One interesting scope for textile finishes lies in the hygienic area. The use of antimicrobial agents on textiles dates back to antiquity, when the ancient Egyptians used spices and herbs to preserve mummy warps. In 1935, Domagk, a German scientist, developed an important class of antimicrobial agents based on quaternary ammonium salts. In the last few decades, the prevention of microbial attack on textiles has become increasingly important to consumers and textile producers in order to create new fields of application. The major classes of antimicrobial agents for textiles include organo-metallics, phenols, quaternary ammonium salts, and organosilicones. These finishes should be durable, have se-

lective activity towards undesirable organisms, be compatible with other finishes and dyes, and be nontoxic to humans [4].

Table 1. Interactions of (derivatisized) biopolymers for permanent anchoring on fibres

Modes of anchoring	Fibre type				
	CO/CV	WO	PA	PES	PAN
Cross linker (resin finish)	y	n	n	n	n
Ionic interaction	n	y	y	n	y
Covalent bonding	y	y	y	n	n
van der Waal Interaction	n	n	y	y	y

y: possible interaction, n: no or weak interaction

Chitosan being a polycationic polymer with specific structure and properties [5] is known to exhibit antimicrobial activity and thus constitutes a potential candidate for textile treatment. It is obtained commercially from shrimp and crab shell chitin (a *N*-acetylglucosamine polymer) by alkaline deacetylation in various grades [5, 6]. Chitosan is relatively inexpensive and non toxic and possesses reactive amino groups. The antimicrobial activity of chitosan against different groups of microorganisms, such as bacteria and fungi, has received considerable attention in recent years. Due to the variability of chitosan preparations some of the antimicrobial tests performed up to now seem to be not conclusive so new testing methods have to be introduced. Some of them are described in this paper too.

In this work.chemical bond formation via different anchor groups is compared regarding to add-on achieved and the antimicrobial activity of modified textiles exemplarily investigated.

MATERIALS AND METHODS

Methods and materials used are described in detail in [7]. Only specific data are given here.

Chitosan with average molecular weight of $1{,}36.10^5$ Da and degree of deacetylation of 85% with code name S 85/60 /A1 (Heppe GmbH, Germany) was used and cotton web (ECE) S/400 (TESTEX, Germany) of 102 $g.m^{-2}$.

2,3,5-Triphenyltetrazolium chloride (TTC) (Merck), cyanuric chloride (Fluka), propane-1,2,3-tricarboxylic acid (PTCA) (Merck), sodium acetate trihydrate (Merck, Germany), butane 1,2,3,4 tetracarboxylic acid (BTCA) (Aldrich) and 2,4-dichloro-6-methoxy-s-triazine were used.

Escherichia coli (*E.Coli*) DSM 498, *Candida albicans* DSM 11225 and *Candida krusei* ATCC 6258. Details of their cultivation, inoculation are described in [7].

This actively growing broth culture was then adjusted with sterile saline to a final working concentration of 6×10^7 colony forming units (cfu) per mL. The cell cultures of *Candida* species were counted using CASY® 1 and adjusted to a final working concentration of 6×10^5 cells per mL in SGB.

Preparation and treatment of cotton with chitosan

A scoured and bleached 100% cotton fabric was used in this investigation. Chitosan was dissolved in an acetic acid solution (mass concentration of 1%). Cotton fabrics were padded twice with chitosan solution (1%) to a wet pickup of 100%. The padded fabrics were then dried at 80°C for 5 minutes and cured at 90-170 °C for 3 minutes depending on the anchor group type. For the crosslinking of chitosan to cotton fabrics different anchors were used: cyanuric chloride, (2,4-dichloro-6-methoxy-1,3,5-triazine), (propane 1,2,3-tricarboxylic acid) and (butane 1,2,3,4-tetracarboxylic acid).The concentration range of each anchor was from 5 and 8 x 10^{-4} mol/g.fibre. The fabric was immersed in an aqueous solution containing the crosslinking agent. In case of carboxylic acids 0.6 mole sodium acetate trihydrate to 1 mole anchor respectively was used as a catalyst. The fabrics were padded in this solution to a wet pick-up of 100%. The padded fabrics were dried at 80 °C for 5 minutes and cured at 90–170°C for 3 minutes.

Measurement of antimicrobial activity of chitosan

Agar dilution method

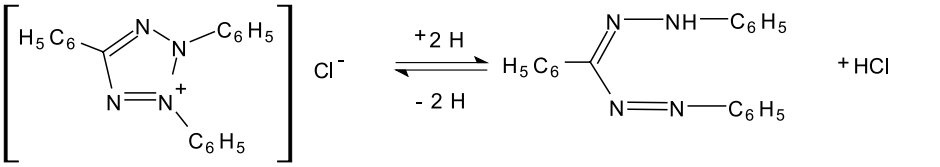
Stock solution of chitosan was prepared (1% in acetic acid), then 2 fold serial dilution was prepared using SI medium. Before sterilizing, the pH of the broth was adjusted to 6.0 with 1.0 N NaOH. After cooling the broths were inoculated with cells of *E.Coli* (1x10^7 cfu / mL) and incubated at 37°C. After certain periods of time (1 h,2 h and 24 h) surviving cells were counted by spreading on nutrient agar plates. The counting of colony forming units (cfu) was done in triplicate each time. The initial cell concentration was determined by a similar spread plate method.

Laser nephelometry in microtiter plate method

The method used to determine the antimycotic influence of chitosan was a turbidimetric method using laser nephelometer NEPHELOstar Galaxy® (BMG LABTECH GmbH, Germany) as described in [7] This method uses Sabouraud-glucose-bouillon as medium and 3x10^5 cells/mL as an inoculum.

Measurement of antibacterial activity of cotton finished with chitosan

TTC- test method:



2,3,5-triphenyl-2H-tetrazol-3-ium 1-phenyl-2-[(Z)-phenyl(phenylhydrazono)methyl]diazene

The tetrazolium/formazan couple is a special redox system acting as proton acceptor or as oxidant [8] and serves as indicating system for the determination of the viability of microorganisms and can be used on surfaces. To do this test circular swatches of test and control textile materials were cut in the same diam-

eter of 3.8 ± 0.1 cm. The number of swatches to be used was 6. Both of the test and control swatches were sterilized at 110°C. All swatches were stacked in 40 mL nutrient broth medium containing 10 μL of (10^8 cfu/mL) *E.coli* as an inoculum volume, then all flasks were incubated with shaking at 37°C/200 rpm for 3–4 h, then 1 mL from each flask containing the test fabrics finished with chitosan and the control was added to sterilized test tubes containing 100 μL TTC (0.5%). All tubes were incubated at 37°C for 20 minutes. The resulted formazan was centrifuged at 4000 rpm/3 min. followed by decantation of the supernatants. The pellets obtained were resuspended and centifugated again in ethanol. The red formazan solution obtained at the end which indicated the activity and viablility of the cells was measured by photometer at 480 nm.

RESULTS AND DISCUSSION

Prior to anchoring experiments some antimicrobial results on chitosan dissolved at pH 6 are shown in Fig. 1. These conditions have been choosen because some work in the literature presumably has only measured the effect of standard acetic acid solvent on bateriae. Here it can be seen that at high dilution (Fig. 1, left side vs. control) the inhibitory effect of the chitosan used on *E.Coli* is valid under almost neutral conditions.

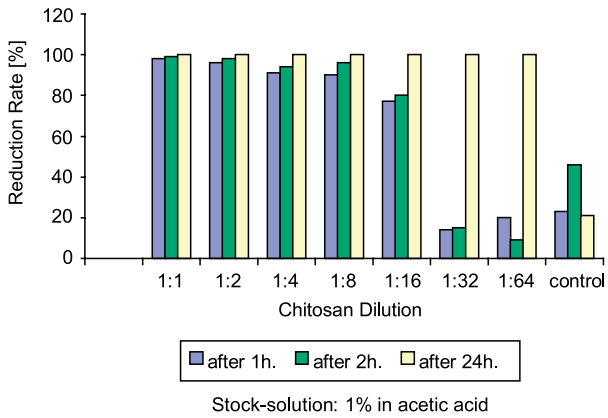


Figure 1. Influence of dissolved chitosan on the reduction rate of *E.Coli* (pH adjusted to 6; 25°C)

Fig. 2 represents the antimycotic effect measured by the laser nephelometric procedure. Against control showing a rather rapid increase of cells medium concentrations of chitosan inhibit fungus growth effectively for long terms.

Following these studies investigation dealt with the permanent fixation of chitosan onto cotton using different anchor molecules for chemical bond formation. Anchoring amount varies since it is expected that only few anchoring points are tolerable in order to have high activity of the chitosan polymer chain. But fixation points enough for giving permanent treatment have to be created. The reactive triazines were expected to give high fixation rates, the cyanuric chloride possibly leading to more crosslinking than the methoxydichloro compound. Polycarbonic acids bind via intermediate anhydride formation [9]. Though much more expensive they have been choosen because of their low toxicity. Figs 3-6

show the percent add-on achieved by the treatment conditions used. The amount of anchoring chemical is always calculated as being equivalent to only a part of reactive amino groups of chitosan (<<50% of stoichiometric).

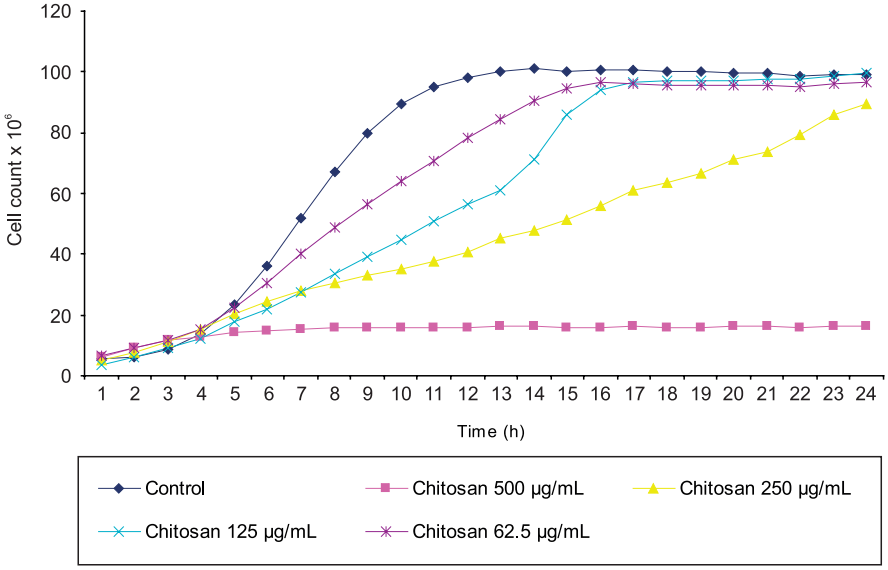


Figure 2. Influence of Chitosan on *Candida krusei*

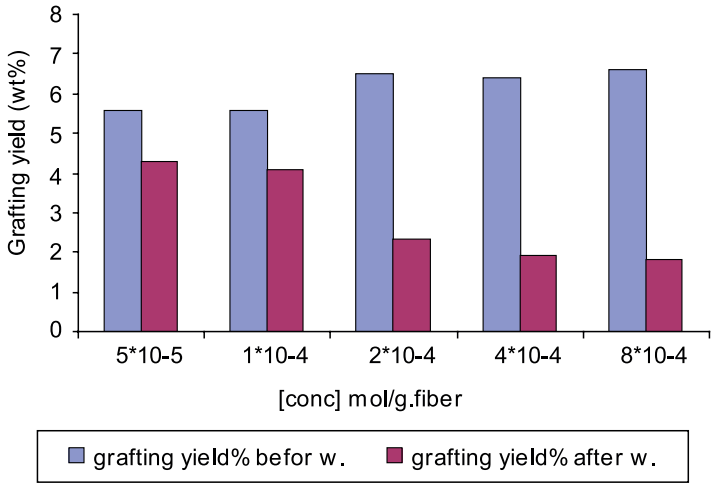


Figure 3. Grafting yield of chitosan on cotton with cyanuric chloride anchoring cotton

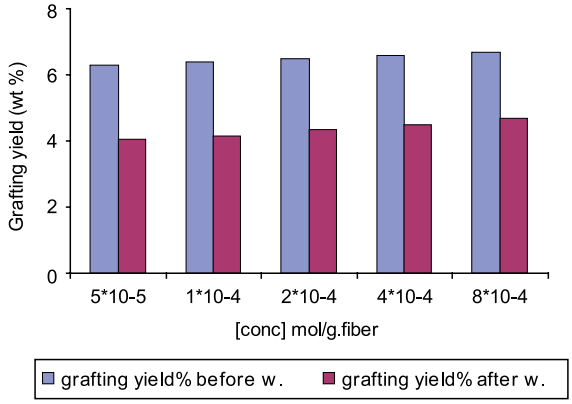


Figure 4. Effect of 2,4-dichloro-6-methoxy-1,3,5-triazine as an anchor group on the grafting yield of chitosan on cotton

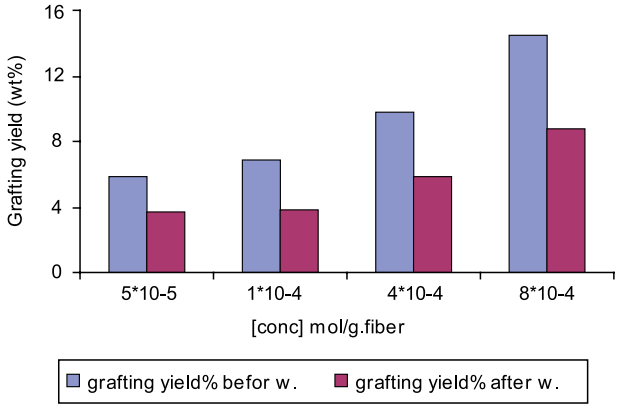


Figure 5. Add-on of chitosan using propane 1,2,3 tricarboxylic acid as an anchor group

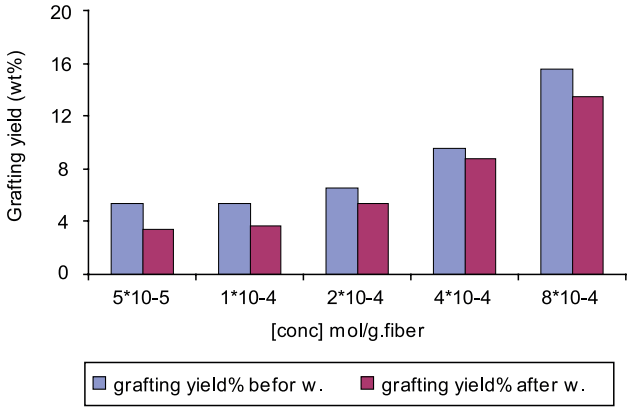


Figure 6. Effect of butane-1,2,3,4 tetracarboxylic acid as an anchor group on the grafting yield of chitosan on cotton

As can be seen usually a higher amount of anchoring chemical gives higher fixation values booth directly after fixation and after washing as well. The (relative) loss on washing -meaning removal of unbound chitosan - is disappointingly high when using cyanuric chloride under the fixation conditions described. In the other cases add-on after washing remains in practical ranges. It must be noted here that add-ons of > 5% of chitosan with rather high molecular weight as used means a very stiff product of low textile character.

Figs. 7 and 8 give an example of antibacterial action of treated fabrics followed by the TTC-method described. The lower the formazane absorption the more bacteriae have ceased metabolism on the finished sample thus prooving the existence of at least a bacteriostatic surface.

Even if there is a distinct difference in grafting yield using the different anchors the antimicrobial suppression seems to be similar. As far as textile properties have to be retained add-ons of less than 5% seem to be reasonable.

It could thus be shown that chitosan imparts antimicrobial activity to cotton fabrics even when tested under nearly neutral pH-conditions. The finish will be durable because of chemical bonding. Future tasks will be aimed to test against more microbe types and in introducing chitosans of different molecular weight, the lower ones preferred because of processing behaviour and to the treatment of other fibre types.

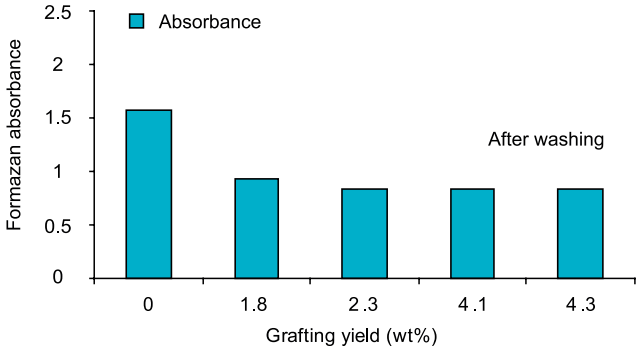


Figure 7. Effect of grafting yield [w-%] of chitosan on the reduction of *E. Coli* after washing using the TTC test method. Anchor: Cyanuric chloride

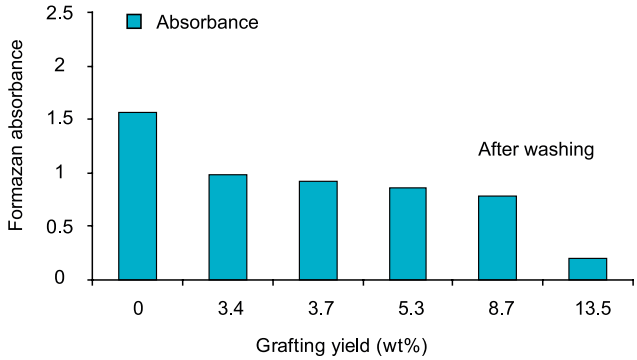


Figure 8. Effect of grafting yield [w-%] of chitosan on the reduction of *E.Coli* after washing using the TTC test method, Anchor: Butane 1, 2, 3, 4 tetracarboxylic acid

ACKNOWLEDGMENT

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PREPARATION AND CHARACTERIZATION OF CHITIN GELS – A COMPARATIVE STUDY

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INTRODUCTION

Thermoreversible gelation of chitin solutions [1], coagulation from an alcoholic solution [2, 3], or gelation by non-solvent addition [4] is well documented in the literature. It is believed that gelation of chitin occurs on decreasing the solvent power by destroying the $[DMAc\text{-}Li]^+$ or $[NMP\text{-}Li]^+$ complex that solubilizes chitin.

In this study, physical properties of chitin gels obtained by nonsolvent addition and gels prepared by heating to the gelation point followed by nonsolvent addition were investigated. The effects of the type of solvent and the addition of organic acids to the solvent were also studied.

EXPERIMENTAL

Chitin solutions with 0.5% (w/w) concentration were formed in DMAc-5%LiCl and NMP-5%LiCl solvent systems. Ascorbic acid (AA), maleic acid (MA) and oxalic acid (OA) were added to these solutions at a concentration of 0.005% (w/w).

In the first method, gels were formed by heating the solutions to the gelation temperature and then adding the nonsolvent ethanol. In the second method, gelation was induced by ethanol addition at room temperature.

FTIR spectra of the dried gels were taken using a Mattson 5000 Satellite FTIR spectrophotometer.

Mechanical analysis of the gels swollen in ethanol was carried out using a Llyod LRX 5K instrument with a 5000 N cell. The gels whose diameter to length ratio (D/L) was 0.75 were compressed in ethanol at 2 mm/min.

The swelling tests were carried out on gels (D/L = 0.33) in ethanol and in pH=7.4 phosphate buffer solution at 37°C. The swelling ratio (Q) was calculated as the ratio of the swollen weight to the dry weight.

RESULTS AND DISCUSSION

Gel Formation

Gelation of chitin solutions by nonsolvent addition was previously studied by our group and the effect of polymer concentration and the type of nonsolvent on gel and bead formation was reported in detail [4].

In this study, gels were formed from chitin solutions at room temperature upon ethanol addition. As a second approach, chitin solutions were heated to the gelation temperature and then further treated with ethanol to obtain stable gels at room temperature (Figure 1). Gels formed by two different methods were compared to each other with respect to their FTIR spectra, mechanical strength, and swelling properties.

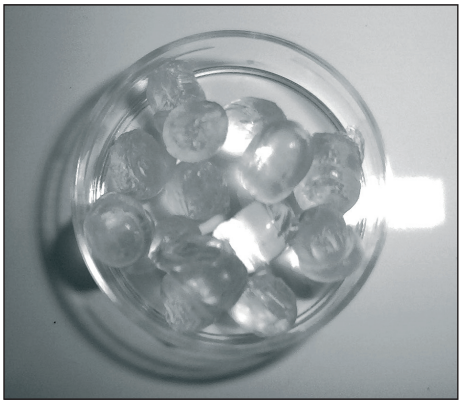

Figure 1. Chitin gel formed from 0.5%w/w solution of DMAc-5%LiCl by heating to gelation temperature followed by ethanol addition

Table 1 gives Young's Modulus (E) values, swelling indices (Q) and the gelation temperatures (T_{gel}) of chitin gels formed by heating to the gelation temperature followed by ethanol addition. The gelation temperature (134°C) for the NMP-LiCl solution of chitin is much higher than that of the DMAc-LiCl solution of the biopolymer (115°C). This suggests that the $[NMP\text{-}Li]^+$ complex is a stronger one than the $[DMAc\text{-}Li]^+$ complex since gelation of chitin solutions is believed to arise from the decomposition of the solvent complex and hence decrease of the solvent power.

Table 1. Young's Modulus (E) Values and Swelling Indices (Q) and Gelation Temperatures (T_{gel}) of chitin gels formed by heating to gelation temperature followed by ethanol addition

Sample	Chi/NMP-5%LiCl	Chi/DMAc-5%LiCl	Chi/DMAc-5%LiCl/MA	Chi/DMAc-5%LiCl/OA	Chi/DMAc-5%LiCl/AA
E^* (MPa)	0.051	0.042	0.066	0.041	0.028
T_{gel} (°C)	134	115	112	110	97
$Q_{ethanol}$	1.4	1.3	1.8	1.5	1.5
Q^{**}_{PB}	2.6	2.1	2.8	2.4	2.2

* E values determined in ethanol
** Q in phosphate buffer pH=7.4

Addition of organic acids to the gelation system resulted in lower gelation temperatures (Table 1). This should be related to the complexation ability of the organic acid with the $[DMAc\text{-}Li]^+$ system. The competition of the carbonyl group of the acid with that of DMAc weakens the $[DMAc\text{-}Li]^+$ complex, so it decomposes at a lower temperature. The color change in the solutions is an evidence for the complex formation between the organic acid and the solvent during heating. While all chitin solutions in DMAc-LiCl, whether containing an organic acid or not were colorless before the heat treatment, the ones containing an acid change color and form a colored gel at the gelation temperature. The gel formed in the presence of AA was yellow, and the others were brownish. After the gels were treated with ethanol, they all became transparent as a result of solvent exchange.

FTIR Spectroscopy

Chitin gels obtained were characterized by FTIR spectrometry. Figure 2 shows FTIR spectra of (a) raw chitin, (b) chitin gel regenerated from DMAc/LiCl solution and (c) chitin gel regenerated from NMP/LiCl. In the FTIR spectrum of raw chitin, free O-H groups absorb strongly at 3450 cm^{-1}, and H-bonded O-H groups are observed to absorb at 3264 cm^{-1} forming a shoulder. In the FTIR spectra of the gels regenerated either from DMAc/LiCl or NMP/LiCl solutions, it can be observed that absorption of intermolecularly hydrogen bonded O-H group increases at the expense of the free hydroxyl band, as a consequence of gel formation.

FTIR spectra of gels prepared in the presence of organic acids were identical with those of gels obtained in their absence, indicating no chemical change upon treatment with organic acids.

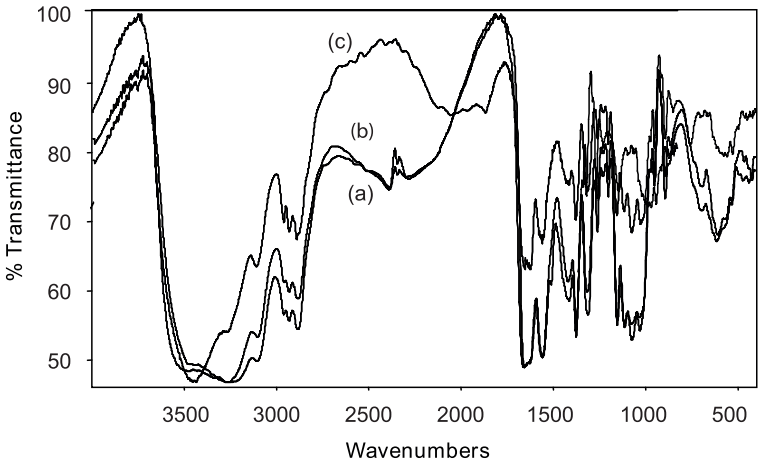

Figure 2. FTIR spectra of chitin gel formed from 0.5%w/w solution of (a) Chi/NMP-5%LiCl (b) Chi/DMAc-5%LiCl and (c) original chitin sample

Mechanical Analysis

Gels formed by nonsolvent addition only and without any heat treatment did no have as regular shapes as the ones formed by heating to the gelation temperature. Therefore, no compression tests could be applied on these samples. The gels formed by heating, followed by nonsolvent addition, could easily be obtained in

the form of regular cylinders in ethanol. The presence of MA in the DMAc- 5% LiCl solvent system led to the formation of a stronger network. This gel had the highest Young's modulus, E = 0.068 MPa, among the ones studied. The presence of AA, on the other hand, resulted in a weaker gel with E = 0.028 MPa. When mechanical stabilities and gelation temperatures of acid containing DMAc-LiCl based systems are compared to each other, it can be noted that a higher gelation temperature results in a more stable gel, indicating a more compact network formation. This, in turn should be related to the degree and the nature of intermolecular interactions and the amount of polymer taking part in gel formation. The system with AA has the lowest gelation temperature. This indicates that AA has the highest complexation ability with the $[DMAc\text{-}Li]^+$ system. This could have led to a smaller fraction of chitin molecules to participate in gel formation leading to a weaker gel. When two different solvent systems, NMP-LiCl and DMAc-LiCl, are compared to each other a similar trend to exhibit a better mechanical strength with higher gelation temperature can be observed.

Swelling Properties

Chitin gel obtained from NMP-LiCl solution swells considerably more than the one derived from DMAc-LiCl system as shown in Table 1. When the gels formed in acid containing solvents are compared to each other, it can be observed that the gel formed in the MA containing system resulted in a gel swelling more than the others both in ethanol and in phosphate buffer. The gel from the AA containing system swells the least. The swelling tests confirm the above given discussion that a smaller amount of polymer should be involved during the formation of the mechanically weaker gels. The network elasticity of the gel-AA, for example, should be less than the others due to the smaller amount of polymer involved in the gel formation. A similar observation was made with the swelling behavior of chitin beads formed from different concentrations of chitin solutions [4].

CONCLUSION

Heating the chitin solution to the gelation temperature before nonsolvent addition results in stronger gels than by preparing similar gels at room temperature. Addition of an organic acid causes the T_{gel}, E, and Q values of the gels to decrease in the order gel-MA>gel-OA>gel-AA. Experimental results indicate that the gelation mechanism in the presence of these acids involve complex formation between the acids and the $[DMAc\text{-}Li]^+$ system which plays a role in the amount of polymer forming the gel. A smaller amount of chitin involved in gel formation results in weaker gels having lower Young's Modulus (E) values and smaller swelling indices (Q).

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GENOME PROTECTION PROPERTIES OF THE CHITIN-CONTAINING PREPARATION MYCOTON

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Key words: chitin-containing material, radioprotection, DNA single-strand breaks

INTRODUCTION

Huge intelligent, material and human resources are spent in order to health protection and to treatment numerous diseases. At the same time less efforts are done for prophylaxis of diseases and the genetic bases of health protection against unfavorable effects of external factors. It is well known that many environmental factors increase quantity of breaks in DNA strands. Chemical pollutions, heightened background radiation, X-rays and UV-irradiation are the most dangerous. Accumulation of genetical errors is the result of senescence. The reparation systems of a living cell are not always capable to eliminate disturbance in a genome. Protection for genetic material against mutations will allow resolving many problems of health maintenance and increasing the duration of active living. Chitin-containing materials have genoprotector properties against irradiation and chemical mutagens. These materials have the huge potential to be used for protection the molecular and genetical basics of health. Mycoton is one of a number such materials. This preparation was obtained from *Higher Basidiomycetes*. Chemical composition of Mycoton is very favorable for the usage in medicine. Each of its components – chitin, glucans and melanins, are well known in medicine as biologically active substances. Mycoton has high sorption capacity to different heavy metals and radionuclides, increase the quantity of free radicals and eliminate metabolic shifts. Chitin has no toxicity of even in high doses. Glucans from higher fungi are well known in medicine as potent immunomodulators and a lot of glucan drugs with the oncostatic effect are on sale. Melanins are one of the most powerful bioprotectors. They have strong antioxidant properties.

The purpose of our investigation was to study the capacity of Mycoton to protect the genome after a chronic or acute irradiation and chemical mutagens in the Ames tests [1].

MATERIALS AND METHODS

The capacity of chitin-containing drug Mycoton to protect a genetic material from destructive effect of penetrating radiation and chemical mutagens was studied. We investigated this drug influence on death rate and birthrate of linear mice. A rate of mortality two groups of animals we analyzed in experiments:

1) Inbred mice CC57W/mv – 80 animals in control group, 79 exposured mice, 92 – exposured mice with addition Mycoton to standard diet; 2) inbred mice BALB/c – 105 animals in control group, 105 mice with addition Mycoton to standard diet. The birthrate was studied for 15 control females and for 21 females receiving Mycoton (line C57Bl/6 and line BALB/c).

DNA damages were induced by γ-radiation and chemical mutagens. Linear mice were irradiated in acute (4 hours with dose level 1.9 Sv) and chronic experiment (6 months with dose level 2.4 Sv). Nuclear fuel materials from Unit 4 of Chernobyl Nuclear Power Plant was used as radiators. This nuclear materials were placed under cages with experimental animals. The chronically irradiated mice daily received Mycoton with meal in a dose 25 mg/kg of live weight. The quantities of DNA single-strand breaks (DNA SSB) in pools of lymphocytes, splenocytes and hepatocytes from linear mice CC57W/mv were estimated in one month after acute irradiation. Electrophoretic motility of phage λ DNA in presence an oxidation reaction of benzidine (Bz) was also investigated *in vitro* in the Ames tests. In these experiments as a mean to protect the DNA the soluble components of Mycoton in different concentration were used.

Isolation of lymphocytes (from a peripheral blood), splenocytes and hepatocytes (from cell suspension from these organs) were executed by centrifugation (400 G, 25 min) in a density gradient of ficol-verografinum.

A newly developed fast and sensitive microplate assay (Fast micromrthod) for unwind and labeling DNA by a fluorescent dye PycoGreen (Leiden, Netherland) was used [2]. Measurements of fluorescence were performed for one hour at 480 nm excitation and 520 nm emission using a fluorescense ELISA plate reader (Fluoroskan Tecan, Austria). The effects determined are expressed as strand scission factors (SSF) and were calculated after 20 min/ of denaturation by equation:

$$SSF = \log (\% \text{ dsDNA in sample}/\% \text{ ds DNA in control})$$

The results were analyzed by one-way analysis of variance (ANOVA), followed by Sach [3]. P values < 0.05 were considered to be significant.

RESULTS

Chronic γ-irradiation increases mortality rate for linear mice CC57W/mv from 12.5% to 15.3%. Mycoton addition to the meal reduces this parameter twice – up to 7.6%. It is interesting that Mycoton reduced not only mortality rate of

irradiated mice, but also reduced this parameter of non irradiated mice as well, decreasing natural losses of experimental animals. Mycoton capacity to reduce the natural death rate was confirmed in similar experiment on BALB/c mice. In was shown that the death rate is decreases from 7.6% till 2.9% in this case.

Mouse females with Mycoton in standard diet during 80 days on the average gave birth to more babies. In a line BALB/c with Mycoton the birthrate was increased from 46,7% to 52.4%, in a line C57BL/6 – from 26.7% to 52.4%.

It's known that ionizing radiation in doses superior 0.5–1.0 Gy is one of the main causes of structural damages in DNA of multicellular organismes. These damages can arbitrarily be sectioned into two groups: singly damaged site base modification – single-strand breaks (alkaline-labile sites), including basic sites; and locally multiply damaged sites (clustered lesions), double-strand breaks (DSB), intermolecular cross-links. Ratio of quantity of SSB to DSB of DNA can corresponded values 10–50 depending on conditions of irradiation and kind of radiation. The formation of local multiple damages on the DNA sites play a dominating role in radiation loss, originating of chromosomal, gene mutations and neoplastic transformation of cells [4]. The information about quantity of DNA damages after irradiation in low doses is not established. Usually the researchers manipulate by the outcomes of the high dose of irradiation to extrapolate into the effects of low doses. It's shown that hydroxyl radicals and other free forms of oxygen played the main role in formation of DNA SSB in the irradiated cells [5]. It was demonstrated that the considerable proportion (60–80%) of DNA damages, induced *in vivo* by ionizing radiation with small linear power transmission, are the outcome of indirect effect of irradiation [6, 7, 8]. During evolution of multicellular organisms were allotted by molecular protective systems from oxidants. Most active components of such systems are enzymatic and low molecular weight antioxidants [9]. Mycoton has potent antioxidant capacities. Therefore we investigated it in different models as a potential means for the DNA protection against radiation. Mycoton capacity to protect DNA against irradiation is demonstrated by the data in the Table 1.

Table 1. Mycoton influence on quantity of DNA sigle-strand breaks in lymphocytes, splenocytes and hepatocytes of inbred mice

Groups of experimental mice CC57W/mv	Strand scission factors (SSF)		
	Lymphocytes	Splenocytes	Hepatocytes
1. Intact mice (control group)	0.0152 ± 0.0017	0.0208 ± 0.0029	0.0200 ± 0.0031
2. Exposured mice (2.4 Sv per 6 months)	0.0322 ± 0.0041	0.0334 ± 0.0040	0.0439 ± 0.0058
Exposured mice with Mycoton in standart diet	0.0041 ± 0.0060	0.0308 ± 0.0039	0.0099 ± 0.0012
3 Exposured mice (1.9 Sv per 4 hours)	0.0435 ±0.0052*	0.0550 ± 0.0068*	0.0510 ±0.0064*
Exposured mice with Mycoton in standart diet	0.0168 ± 0.0023	0.0294 ± 0.0042	0.0351 ± 0.0046

$p < 0.05$

These data show that the irradiation of experimental animal induces increase of DNA SSB in different cells irrespectively of time of accumulated common dose. Mycoton addition in standard diet decreases DNA SSB. So this drug has properties to protect genome.

We investigated the influencing of Mycoton (M) (from 0.034 up to 10 mkg/ml) on a phage λ DNA (6 mkg/ml) after demolishing it by benzidine (Bz) (5 x 10^{-6} M) in medium with H_2O_2 (10^{-3}M) and peroxidase (P) (5 x 10^{-10} M) at pH 5.5 during 30 min at 30°C in the classic Ames tests (Fig. 1).

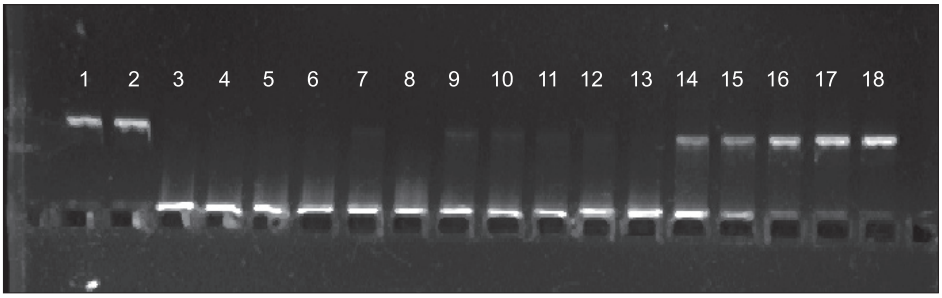

Figure 1. Electrophoregram of a phage λ DNA

1. DNA; 2. DNA, P, H_2O_2. 3. DNA, P, H_2O_2, Bz; 4. + M (0,034 mkg/ml); 5. + M (0,051 mkg/ml); 6. + M (0,077 mkg/ml);7. + M (0,12 mkg/ml); 8. + M (0,17 mkg/ml); 9. + M (0,26 mkg/ml); 10. + M (0,39 mkg/ml); 11. + M (0,59 mkg/ml); 12. + M (0,88 mkg/ml); 13. + M (1,3 mkg/ml); 14. + M (2,0 mkg/ml); 15. + M (3,0 mkg/ml); 16. + M (4,4 mkg/ml); 17. + M (6,7 mkg/ml); 18. + M (10 mkg/ml).

It was shown that incubation of this phage λ DNA with products of benzidine oxidation by H_2O_2 caused strong fragmentation of DNA in electrophoregram (Fig.1). The decontamination of toxiferous products of benzidine oxidation took place after addition to incubating medium the water soluble component of Mycoton. The effect is well seen at concentration of Mycoton 2 mgk/ml, and at 10 mgk/ml influencing the toxic products of benzidine is completely neutralized.

Antimutagen property of Mycoton on the strains *Salmonella typhimurium* TA-100 and TA-98 were studied in the Ames tests also. The revertantes appeared in the first strain after mutagen influence as a result of replacement of basis pairs. Mutagen cause the DNA damages such as a frameshift of a genetical code in the second strain [10].

The metabolic activation of an indirect carcinogenesis caused by benzidine induces formation electrophylic radical reaction products. This reaction can be the result of implement by hydroxylation on nitrogen or aromatic ring, and also can be an outcome of peroxidase oxidation by different hemoproteins – cytochrome P-450, iodperoxidase, prostaglandinesynthetase, myeloproxodase etc. In this connection we studied genoprotective property of Mycoton in process of peroxidative path of metabolic activation of benzidine by horse-radish peroxidase and other hemoproteins. Mycoton twice reduces spontaneous occurrence of his+revertans without a mutagen (up 65 ± 5 to 30 ± 5 per a plate). Peroxidase oxidants of benzidine induce growth of his^+revertants up to 300 per a plate with the strain *S. typhimurium* TA-100 and up to 460 for *S. typhimurium* TA-98 with

benzidine (10^{-5} M). The increased concentration of Mycoton considerably reduced quantity of mutant colonies induced by operating of benzidine with both strains. Mycoton in concentration 0.2 mg/ml reduces quantity of his+revertants on the strain *S. typhimurium* TA-100 up to a spontaneous level, and on the strain *S. typhimurium* TA-98 up to 220 ± 10 per a plate. It is possible to say that antimutagen influence of Mycoton in the greater degree prevents mutations for the strain *S. typhimurium* TA-100 and defends DNA from replacement of bases pairs.

So, Mycoton, is highly active antioxidant which causes the significant decrease of mutagenic properties of benzidine and formation of mutations such as a frameshift and replacement of DNA bases pairs for microorganisms.

CONCLUSIONS

The chitin-containing preparation Mycoton may be considered as a promising component of obligatory genoprotective measures when performing radiation-dangerous works and works with chemical mutagens also as a preventive agent for wide circles of population under conditions of many unfavorable factors.

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CHITOSAN-BASED BIOACTIVE SUPPLEMENTS

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The application of agents of natural origin for the prophylaxis of many diseases and enhanced effectiveness of their treatment is a certain step in the evolution of the society's views on human health. To a great extent this is the result of multidisciplinary scientific researches and people's awareness about them.

At present considerable amounts of knowledge has been accumulated concerning an in-depth and comprehensive analysis of all the potentials of natural therapy, which recognizes the Nature's healing power.

Bioactive nutritional supplements are additional measures toward the promotion of health.

The results of research conducted by the Institute of Nutrition of the Russian Academy of Medical Sciences have indicated certain misbalance in the nutritional status of Russia's population that manifest themselves in the excessive consumption of animal fats, the deficiency of food fibers, polyunsaturated fatty acids, the majority of vitamins, certain mineral substances, mainly calcium, iron, trace substances (iodine, fluorine, selenium, zinc) [1].

The consequence of the food pattern misbalances found is the wide-spread occurrence of various forms of obesity, which entails a number of such diseases as atherosclerosis, coronary heart disease, essential hypertension etc.

One of effective ways of addressing the problem of correcting the population's nutrition is the wide use of bioactive nutritional supplements.

Bioactive nutritional supplements are concentrates of natural bioactive substances (or substances identical to them) intended for direct consumption or for introducing into foodstuff [2].

Aquatic organisms are the sources of unique complexes of bioactive substances – proteins, amino acids, DNA, calcium- and phosphorus-containing mineral components, iodine, polyunsaturated fatty acids, chitin and other components [3].

Today, experts around the world demonstrate a keen interest in preparations based on chitin and chitosan of Crustacea in connection with great opportunities for their use in various areas. This is also related to the biological properties of these biopolymers, which are of natural origin, their biological compatibility and decomposability down to substances that are common for the organism [4].

The use of chitosan as a bioactive food supplement has been approved in many countries of the world. The combination of such properties of chitosan

as harmlessness and biological activity is important for its use as a food supplement. Great importance is attributed to the lipotropic effect of chitosan as an important factor that contributes to fighting cardiovascular diseases.

Sorption properties and ability to restore the microbial flora of the intestines are highly useful qualities of chitosan when used for nutritional purposes.

The enhanced curative effect of chitosan-based preparations is due to its specific properties. Thus, polyfractional chitosan deacetylated to 75–95% containing from water-soluble fractions to fractions with molecular weight of 250–450 kDa brings about prolonged action of various medications owing to its complex-forming properties.

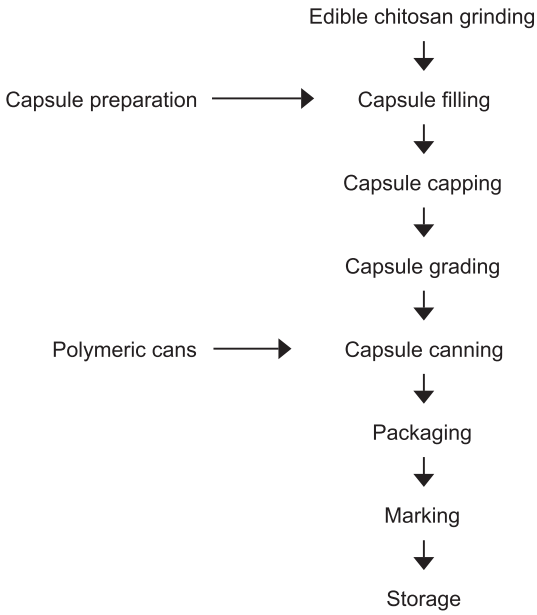
The above-mentioned data about the properties of chitsan have been the grounds for the formulation of a number of chitosan-based bioactive preparations. The VNIRO* personnel have formulated the bioactive nutritional supplement "Chitan" based on alimentary chitosan, the characteristics of which are provided in Table 1.

Table 1. Characteristics of chitosan obtained from crab shell

	Water content by weight, %	Mineral substance content by weight, %	Insoluble substance content by weight, %	Kinematic viscosity, cSt	Deacetylation degree, %	pH of aqueous solution
Edible chitosan	9.2	0.3	0.1	500	87	7.1

The process chart for the "Chitan" production comprises the following stages:

A package of standard documents for the product has been drawn up, and hygienic certificate has been obtained.



Based on the results of clinical tests of "Chitan", the Institute of Nutrition of the Russian Academy of Medical Sciences recommended using this bioactive supplement for preventive and curative purposes in treating large intestine and biliary dyskinesia, fat metabolism misbalances, cardiovascular disease and other disorders, arterial hypertension in combination with excessive weight.

Brown algae are a source of natural bioactive substances that have a wide range of effects upon the human organism: they are capable of reducing the arterial blood pressure, enhancing the organism's resistance to infectious diseases, regulating the amount of lipids in blood as well as of cholesterol in plasma [5].

Brown algae are regarded as full source of mineral elements for humans, as it contains practically all biogenic elements necessary for a living organism for normal vital activity: iron, copper, magnesium, cobalt, zinc, manganese, molybdenum, sodium, potassium, and calcium. Selenium contained in brown algae is of great importance as it participates in metabolic processes and produces a special effect on the iodine fixation [6]. The iodine content of 0.1% with the total content of mineral substances of about 30% has allowed many species of brown algae to be used for the production therapeutic products and bioactive food supplements, which are a source of natural organic and inorganic iodine [7].

In recent decades a significant portion of Russia's population has experienced the iodine deficiency, which is why a particular emphasis is placed on formulating iodine-containing bioactive food supplements.

Taking the above into account, the VNIRO specialists have developed a process for producing a preparation called "Polychit" to address the problem of iodine deficiency as well as to enrich bioactive food supplements. Edible chitosan and laminaria japonica are the base of this preparation (Table 2).

Table 2. Composition of bioactive nutritional supplement "Polychit"

	Edible chitosan	Laminaria	Powdered sugar	Citric acid
Component content,%	55	25	10	10

The bioactive nutritional supplement "Poluchit" is produced in the form of capsules and tablets.

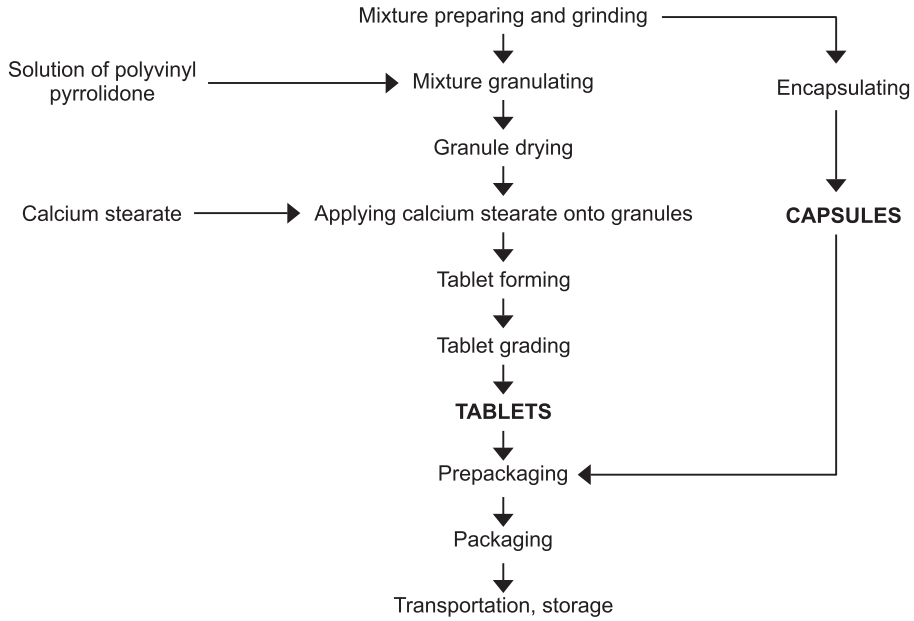
On the basis of research conducted the standard technical documents for this product have been drawn up, and the registration certificate has been obtained.

In recent years a great importance has been attached to the problem of obesity, one of the most serious diseases, which progresses rapidly and manifests itself in increased fat content in the human organism.

The use of bioactive food supplements is one of simple and available methods of fatness control [3].

The review of publications and patents associated with formulating of a new generation of bioactive supplements for fatness control has indicated a poor choice in the market.

The process chart for the production of the bioactive nutritional supplement "Poluchit" is shown below:



In this connection, the objective of our further research was the formulation of a product that would help lose weight and, at the same time, replenish the lack of vitamins.

In formulating a chitosan-based product, only natural, mainly vegetable components were used, which were characterized by high biological activity and did not produce pronounced side effects.

The preliminary stage of research made it possible to select a composition of components that produced, to a varying degree, the "weight loss" effect.

Thus, some components become turgescent (corn silk, laminaria etc.) occupying a large volume and producing the satiation effect. As a result, the appetite decreases, and, consequently, the food consumption is reduced by up to 30%.

The components of another series (green tea, mountain ash etc.) cause fat decomposition in the fatty tissue, are able to slow down the action of ferments digesting proteins and fats, and thereby reduce their absorption in the intestines and protect the body against fat accumulation in tissues.

The inclusion in the bioactive supplement formulation of some components (mountain ash etc.) that are rich in vitamins C, P, E, K, B, folic acid, manganese, iron, zinc, copper, magnesium ensures the enhanced biological value of the product.

As a result, the bioactive nutritional supplement being formulated reduces the potential of fat formation out of food components consumed by people, and help decompose the fat accumulated in tissues.

Research in this direction is underway.

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STUDIES ON THE INNOCUOUSNESS OF CHITOSAN AND ITS SHORT-CHAIN DERIVATIVE CHITODEXTRIN™ (LIBRACOL™) GENERATED BY ENZYMATIC HYDROLYSIS IN HUMAN SUBJECTS

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Abstract: Due to its beneficial activity on lipid disorders, chitosan is seriously considered as a potential ingredient of functional foods. In animal studies, chitosan forms of intermediate molecular weight were shown to be more effective than high molecular weight forms in lowering plasma cholesterol. Here, we present the results of clinical studies of the innocuousness of chitosan added as a supplement to the unrestricted diet of female and male volunteers. Two types of chitosan differing by their molecular weight, 240 kDa and 30 kDa (Libracol™) were used. A proprietary procedure to generate the 30 kDa form by enzymatic hydrolysis and a procedure of recovery of the hydrolysate fully compatible with applications to human diets were used. Monitoring of a series of physiological, biochemical and clinical tests was used to assess the effects. No untoward effects of either form of chitosan were noted on the neurological, cardio-vascular, respiratory, urinary, hepatic, digestive and circulatory systems. Some minor discomforts were observed such as belching and some cases of bloating. However, the ingestion of chitosan did not cause any major digestive problems. There were no changes in a series of clinical and biochemical tests. The plasma levels of the lipo- and water-soluble vitamins were not affected. The diet supplement of chitosan 240 kDa was associated with a significant decrease in plasma cholesterol levels. A phase II clinical study is under way to test the Libracol™ (30 kDa form) efficacy in lowering plasma cholesterol.

INTRODUCTION

Chitosan is a cationic polysaccharide produced by partial or total deacetylation of chitin from crustacean shells. We have previously demonstrated the hypocholesterolemic effect of chitosan in rats [1]. In addition to its lowering effect on plasma cholesterol level *per se* [2, 3], chitosan had the property of blocking the increase in plasma cholesterol level in rats fed a high cholesterol diet. The hypocholesteroleamic properties of chitosan compared favorably with those of cholestyramine [1, 3]. Although cholestyramine is an efficient agent to eliminate bile acids, its use is not without secondary effects. In addition to its foul taste,

particularly repulsive to young and adult patients, cholestyramine is a plastic-type polymer made of cross-linked polystyrene that causes irritation of the intestinal mucosa and bleeding [4–7].

INITIAL CLINICAL STUDY ON THE INNOCUOUSNESS OF CHITOSAN

One objective of our first clinical trial was to assess the innocuousness of chitosan (240 kDa) added to the otherwise unrestricted diet of human subjects; a second objective was to assess the claim that chitosan reduced the levels of plasma cholesterol in humans. The following criteria were used to select volunteers: good health, age 18 to 65 years, satisfy the criteria of exclusion and sign an informed consent form. The criteria of exclusion were: taking medications, birth control pills, alcohol or drugs; acute or chronic pathologies; becoming sick during the study either as a result of ingesting chitosan or for other reasons; presenting symptoms that could indicate the development of a pathological condition; developing an intolerance to chitosan; pregnancy; allergic reactions to sea foods or crustaceans. The study received the approval of the local Institutional Ethics Committee.

Source and product description

Chitosan, technically designated as *encapsulating grade* was obtained from Vanson (Eugene, OR). The product had a viscosity of 180 cps, a degree of deacetylation of 82% and a molecular weight 240 kDa. The material was found to be free of pathogens by microbiological assays. It was encapsulated in size 00 gelatin capsules (400 mg of chitosan/capsule). The volunteers took daily 1.6 g of chitosan during the first month of the study (2 capsules at lunch and 2 capsules at supper). The dose was raised to 2.4 g of chitosan/day (2 capsules at breakfast, 2 capsules at lunch and 2 capsules at supper). During the third month of the study, the volunteers took 3.2 g of chitosan (2 capsules at breakfast, 3 capsules at lunch and 3 capsules at supper).

RESULTS

The study initially included 32 healthy volunteers aged 34.4 ± 1.73 (average ± SEM) years. One woman was excluded from the study for reasons unrelated to the ingestion of chitosan.

Clinical/serological results

Neurological system No participants complained of alteration of consciousness, dizziness, blurred vision, diplopia, paresis or paresthesia; we thus concluded that the ingestion of chitosan did not affect the neurological system.

Cardiovascular system We did not detect any retrosternal pain, palpitations, syncope or suddent onset of dyspania.

Respiratory system No modifications were found that could be related to some side effects of chitosan. We did not observe any signs of dyspnea, coughing or expectoration.

Digestive system Eleven subjects reported a feeling of bloating, and 8 reported that their appetite was increased, whereas 1 subject reported the opposite. Ten subjects reported an increased frequency of stools, 4 others noted softer stools whereas 5 additional volunteers noted firmer stools but not constipation. When the dose of chitosan was increased to 3.2 g per day, 6 out of 7 subjects noted firmer stools and they complained of constipation. Amongst the latter subjects, 3 of them reported episodes of rectal bleeding. One subject had had episodes of rectal bleeding before taking chitosan. The 2 other subjects did not experience repeated bleeding during the course of the study. We do not think that these symptoms are related to the ingestion of chitosan because the episodes did not increase during the course of the protocol or with higher doses of chitosan. However, individuals who have a tendency to suffer from rectal bleeding should abstain from taking chitosan

Urinary system No subjects had any urinary infections during the study. The levels of creatinine and electrolytes remained unchanged (Table 1), which is consistent with the interpretation that chitosan ingestion has no effect on the urinary and the kidney functions.

Menses Three subjects reported a two-to-three day shorter menstrual cycle whereas 1 participant noted a cycle increased by 7 days. One subject noted a more abundant menstruation. These cases could not be accurately validated. There was no increase or decrease of abdominal pain during menstruation.

Pancreatic function This function was assessed by monitoring the levels of glycemia and, amylase activity. There were no significant changes during and post-study (Table 1).

In Summary our observations showed that chitosan can cause some side effects that are generally minor and restricted to the digestive system. No serious pathologies were noted during the three-month course of the study and these are corroborated by the results of the clinical analyses to be described below.

Serum proteins Serum albumin and proteins were monitored throughout the study to make sure that the ingestion of chitosan did not interfere with protein absorption. Table 1 shows that chitosan did not affect protein absorption in this group of subjects.

Hepatic and post-hepatic functions Diagnostic enzyme activities, namely ALT, AST and alkaline phosphatase were monitored throughout the course of the study. Results (Table 1) clearly indicated the absence of any significant changes in all cases. In addition there were no changes in the serum levels of bilirubin. These data is consistent with our statement that chitosan had no deleterious effects on liver and post-liver functions.

Blood count Blood cell count was determined at the beginning of the study and at each visit thereafter. Results showed that there were no changes. Furthermore, hemoglobin concentration and parameters of blood coagulation (TP, PTT) were unchanged.

Blood pH The monitoring of pH balance, creatinine and urea done at every visit showed no changes in these parameters. These observations were also true for bicarbonate (Table 1), demonstrating that chitosan ingestion did not cause blood acidosis or alkalosis.

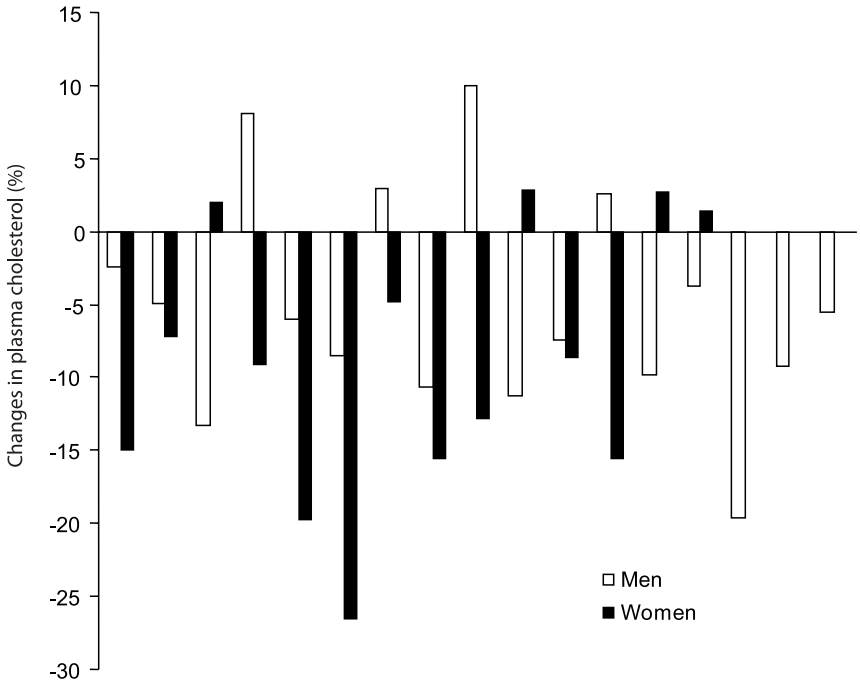

Figure 1. **Effects of chitosan on total plasma cholesterol levels in fourteen female and seventeen male subjects.** Chitosan was added to the diet healthy human subjects in regimens of 1.6, 2.4 and 3.2 g/day for the first, second and third month, respectively. Data are represented as the changes (%) of plasma cholesterol levels of each subject at the end of the study relative to the levels before taking chitosan. Results are expressed as the percentage of changes between plasma levels before and after the addition of chitosan to the diet. The data clearly indicate that the levels of plasma cholesterol decreased in the case of 22 individuals, increased in 2 individuals and remained unchanged in the case of the other 7 subjects, based on the fact that the changes were less than 3% which is within the accepted margin of intra-assay variations. Analysis of the data for the 31 individuals using Student's t-test showed a significant decrease in plasma cholesterol levels ($p = 0.004$).

Blood glucose Results indicated the absence of any significant changes in blood glucose during these periods of observations (Table 1).

Iron and ferritin There were no significant changes in the levels of total iron. The serum reserve of iron was assessed by measuring the levels of ferritin. The results revealed no significant changes (Table 1), suggesting that chitosan did not affect iron metabolism. A word of caution ought to be added to the effect that the ingestion of chitosan decreased the plasma levels in women with initially low levels of plasma iron. We strongly recommend that in these cases, the ingestion of chitosan be supplemented with a source of iron.

Fecal fats Quantitative analyses of fecal fats during the course of the study showed an absence of increased excretion of fats. As a preliminary conclusion, the results suggest that chitosan did not interfere with fat absorption.

Serum triglycerides Results showed no significant decrease and the values remained within the normal limits (Table 1).

Lipoproteins Serum levels of HDL were monitored during and after the study was completed. Results showed an absence of significant changes (Table 1).

Total plasma cholesterol The effects of the chitosan supplement on plasma cholesterol were monitored on a monthly basis. There was, in most cases, a progressive decrease in plasma cholesterol levels as a function of time and quantities of chitosan supplement (not shown). Figure 1 show the effects of chitosan ingestion over the 3 month-period of the study.

Plasma cholesterol levels post-chitosan ingestion The plasma cholesterol levels returned towards pre-study values 1 month after cessation of chitosan ingestion (results not shown).

SECOND STUDY: INNOCUOUSNESS OF CHITODEXTRIN™ (LIBRACOL™)

The efficacy of chitosan as a hypocholesterolemic agent depends in part on its molecular weight and its degree of deacetylation [1]. We have developed a proprietary enzymatic method to hydrolyze chitosan and a new method to isolate large quantities of a food compatible 30 kDa chitosan deacetylated to 93% (Chitodextrin™). This product is produced and marketed by Magistral Biotech Inc. (Blainville, QC) under the trade name of Libracol-HEP-30. Animal studies (unpublished data) have shown that the efficacy of Chitodextrin™ in lowering plasma cholesterol is better than that of the 240 kDa chitosan used in our initial study. Since the characteristics of Libracol-HEP-30 differ from those of the 240 kDa chitosan used in the first study, the objective of this report is to present the results of a Phase I clinical study to investigate the safety of this new product.

Protocol: Eighty three male and female volunteers were recruited using the same exclusion criteria as for the first study. Chitodextrin™ capsules were randomly distributed as follows: Group 1: Placebo, 3.6 g/day (21 participants); Group 2: Chitodextrin™, 3.6 g/day (21 participants); Group 3: Chitodextrin™ 2.4 g/day + Placebo 1.2 g/day (21 participants); Group 4: Chitodextrin™ 1.2 g/day + Placebo 2.6 g/day (20 participants). All capsules weighed 400 mg and were identical in apparence, color and smell. The placebo contained microcrystalline cellulose (Solka-Floc™). The study extended for 8 weeks. Physical examinations and biochemical tests were performed to insure a close monitoring of any changes, at time 0, 8 weeks and 4 weeks post-study.

RESULTS

Data collected during this study showed that patients who took Chitodextrin™ had no clinically significant side effects. The patient questionnaire addressed specific points such as asthenia, dizziness, headache, thirst, dryness of mouth, appetite, dyspepsia, abdominal pain, rectal bleeding, diarrhea, constipation, changes in stool frequency and volume and color, heart palpitations, thoracic pains, dyspnea, cough, expectoration, cramps, myalgia, articular pain, urinary symptoms, change in sexual activity, menstrual regularity, hot flashes, excessive sweating, hair loss, cold extremities, change in skin color, etc.

Neurological and Cardiovascular systems No noticeable neurological symptoms and no cardiovascular signs or symptoms attributable to Chitodextrin™ were observed.

Respiratory system During follow-up there were no significant cutaneous, or pulmonary, changes. Six volunteers taking placebo, 3 from group 2 and 1 from group 4 noticed dryness of the mouth during the study; however, the difference between groups was not significant. Digestive system 2 patients from group 2 and 3 patients from group 3 had an improvement in stool quality. These volunteers had softer stools than before the study, at which point they had been very hard. Two patients from group 2 and 1 patient from group 1 had an increase in the frequency of stools. Six patients from group 2, three patients from group 3 and 1 patient from group 1 experienced nausea and dyspepsia after taking the capsules, however these patients were taking the capsules before meals. These symptoms disappeared when the patients took their capsules with meals. Two patients from group 4 noticed increased eructation and rectal gas with Chitodextrin™, which vanished when they stopped taking it. Two patients from group 3 and 1 patient from group 1 had diffuse and crampiform abdominal pains which varied with time throughout the study. Physical examinations showed these patients to be perfectly normal. There was 1 case of proctorrhagia (1 time only) in 1 patient from group 4. However, at the end of the study, this subject notified investigators that he had experienced proctorrhagia before enrolment. He also had a history of internal haemorrhoids. Only 2 patients from group 2 experienced mottled stools during the study, in contrast to the study described above in the first part of this article, in which several subjects had mottled stools.

Urinary and Gynecology systems There were no urological and gynaecological symptoms or signs associated with the use of Chitodextrin™. Three patients (2 men, 1 from group 3 and 1 from group 2, and 1 woman from group 2) noticed an increase of sexual activity during that study. No changes in the menstrual cycle in women participating in this study were reported.

Biochemical tests

The analysis of laboratory data showed no significant differences between the four groups (Table 2). Complete blood counts were conducted on all subjects using markers of infection, inflammation, or hematologic disorders. There were no significant differences between the 4 groups. Hemoglobin, iron, ferritin and the percent saturation of transferrin did not show any changes in subjects taking Chitodextrin™. The marker of coagulation PTT was not modified in subjects taking Chitodextrin™, suggesting that vitamin K concentrations were preserved during the study. Markers of nutritional status, such as serum proteins, albumin, calcium, magnesium, phosphorus and hemoglobin, did not change during the study. Hepatic functions were not affected: the enzymes ALT, AST, PAL, GGT, and bilirubin remained unchanged. Urea, creatinine and serum electrolytes were not affected. Thyroid activity remained stable during the study with no change in TSH. Lipid soluble vitamins vitamin D, vitamin E, and retinol showed no significant changes in subjects taking Chitodextrin™; levels of these vitamins were similar between all 4 groups. These results, especially those concerning vitamin

D, present evidence that Chitodextrin™ does not interfere with the intestinal absorption of lipid-soluble vitamins.

DISCUSSION AND CONCLUSIONS

Several clinical studies have shown that daily ingestion of chitosan has very few or no side effects. Macchi [8] reported that adding chitosan to the diet of 30 volunteers for 30 day did not produce any significant side effects. Another study revealed that chitosan did not induce changes in the hematologic parameters of 40 obese adults [9]. In an 8-week study of 51 obese women, Wuolijuki et al [10] reported that a daily ingestion of chitosan did not alter the levels of iron, transferrin and lipid soluble vitamins, such as vitamins A and E. Pittler et al. [11] noted that vitamins A, D, E and beta-carotene were not affected by chitosan. Our two studies showed no significant side effects associated with the ingestion of chitosan or Chitodextrin™. During our initial study, a decrease in the level of vitamin D was observed (not shown), which was attributed to normal seasonal variations. During the second study, the addition of a placebo group clearly showed that reductions in vitamin D between October and January were similar for all subjects, placebo and experimental. In agreement with our results on the 30 kDa form, Jing et al [12] reported no significant side effects in using a chitosan molecule (a 27 kDa chitosan form deacetylated to 89%) similar to our product in 80 patients with renal failure. Furthermore, their chitosan reduced plasma cholesterol up to 40%. These authors thus validate our data in animals on the efficacy of this type of molecule. Our preliminary results showed significant decreases in the levels of plasma total- and LDL-cholesterol in eleven hypercholesterolaemic volunteers taking the Chitodextrin™ 30 kDa form (not shown). A phase II clinical study is under way to fully test the efficacy of the Chitodextrin™ 30 kDa form in lowering plasma cholesterol. Our data lead us to conclude that Chitodextrin™, a form of chitosan with a molecular weight of ~30 kDa and a degree of deacetylation of ~93%, can be safely given on a daily basis up to a dose of 3.6 g per day. This product should be taken with meals.

Abbreviations: AST: Aspartate transaminase; ALP: Alkaline phosphatase; ALT: Alanine transaminase; IU: International units; l: liter; TP: Time for prothrombin coagulation; Apo B: Apoprotein B; PTT: Time for partially activated tromboplastin coagulation; TIBC: Total iron binding capacity; TSH: Thyrotropin; n.d.: not determined; ANOVA: Analysis of variance; n.s: non significant.

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Table 1. Parameters studied after Chitosan (240 kDa) ingestion

	Month 0 (Beginning)	Month 3 (End)	ANOVA **(0 *vs* 3)**	Month 4 (Post-study)	ANOVA **(0 *vs* 4)**	Normal range	Units
HDL	1.34 ± 0.06	1.23 ± 0.05	n.s.	1.28 ± 0.06	n.s.	>0.9	mmol/l
TG	1.18 ± 0.12	1.18 ± 0.14	n.s.	1.23 ± 0.15	n.s.	0.6 – 2.3	mmol/l
AST	24.19 ± 1.13	24.10 ± 1.09	n.s.	23.00 ± 1.09	n.s.	14 – 50	IU/l
ALP	63.94 ±3.80	63.39 ± 4.47	n.s.	62.90 ± 3.61	n.s.	38 – 126	IU/l
ALT	29.52 ± 2.00	30.07 ± 2.01	n.s.	28.20 ± 1.89	n.s.	21 – 72	IU/l
Creatinine	79.29 ± 2.96	78.65 ±2.16	n.s.	80.87 ± 2.35	n.s.	62 – 121	µmol/l
Amylase	50.97 ± 2.73	51.39 ± 2.41	n.s.	50.00 ± 2.35	n.s.	30 – 110	IU/l
Billirubin	12.80 ± 0.77	11.64 ± 0.72	n.s.	12.23 ± 0.62	n.s.	3.4 – 17	µmol/l
Sodium	141.49 ± 0.25	143.13 ± 0.43	n.s.	141.23 ± 0.24	n.s.	137 – 149	mmol/l
Potassium	4.14 ± 0.05	4.22 ± 0.05	n.s.	4.04 ± 0.04	n.s.	3.7 – 5.4	mmol/l
Calcium	2.35 ± 0.01	2.35 ± 0.07	n.s.	2.38 ± 0.01	n.s.	2.10 – 2.54	mmol/l
Iron	23.03 ± 1.26	17.99 ± 1.44	n.s.	18.72 ± 1.40	n.s.	8.8 – 31.5	µmol/l
Ferritin	90.36 ± 12.63	74.68 ± 11.19	n.s.	71.07 ± 9.74	n.s.	14 – 179	µg/ml
Bicarbonate	27.34 ± 0.37	27.03 ± 0.39	n.s.	27.40 ± 0.30	n.s.	24 – 28	mmol/l
Serum proteins	74.20 ± 0.53	73.16 ± 0.61	n.s.	73.20 ± 0.68	n.s.	60 – 80	g/l
Urea	4.19 ± 0.15	4.36 ± 0.17	n.s.	4.39 ± 0.19	n.s.	3.2 – 7.5	mmol/l
Chloride	103.00 ± 0.29	114.35 ± 9.59	n.s.	102.81 ± 0.32	n.s.	98 – 107	mmol/l
Glucose	4.63 ± 0.08	4.67 ± 0.07	n.s.	4.63 ± 0.07	n.s.	3.3 – 6.1	mmol/l
Serum albumin	44.16 ± 0.42	44.25 ± 0.45	n.s.	43.42 ± 0.47	n.s.	35 – 50	g/l
Carotenoids	3.88 ± 0.21	3.26 ± 0.21	n.s.	3.64 ± 0.21	n.s.	0.9 – 3.7	µmol/l
Tocopherol	34.11 ± 6.00	29.00 ± 10.54	n.s.	29.44 ± 3.23	n.s.	11.6 – 46.4	µmol/l
Retinol	2.22 ± 0.09	2.38 ± 0.11	n.s.	2.26 ± 0.11	n.s.	1.05 – 2.27	µmol/l
Ascorbate	71.87 ± 3.69	70.50 ± 3.92	n.s.	72.61 ± 3.22	n.s.	30 – 114	µmol/l
TP	0.97 ± 0.01	1.00 ± 0.01	n.s.	n.d.	n.s.	0.88 – 1.12	IU
PTT	32.00 ± 0.70	33.50 ± 0.72	n.s.	n.d.	n.s.	25.0 – 37.0	seconds
pCO_2	51.8 ± 1.41	49.9 ± 1.10	n.s.	51.3 ± 1.18	n.s.	41.0 – 51.0	mm of Hg
pH	7.34 ± 0.01	7.34 ± 0.03	n.s.	7.34 ± 0.01	n.s.	7.32 – 7.42	–

n. s. – non significant

Table 2. Parameters studied after 8 weeks of Placebo and/or Chitodextrin ingestio

Biochemical Tests	Placebo* mean ± SD	Chitodextrin* (3.6 g/day) mean ± SD	p value	Chitodextrin* (2.4 g/day) mean ± SD	p value	Chitodextrin* (1.2 g/day) mean ± SD	p value	Clinical values (normal range)
Albumin	43 ± 3	43 ± 3	0,11	44 ± 3	0,07	43 ± 3	0,09	35 – 50 g/l
Alkaline phosphatase	79.1 ± 16.7	97.5 ± 9.2	0,37	82.2 ± 13.1	0,40	80 ± 13	0,42	38 – 126 IU/l
ALT	31 ± 10	30 ± 10	0,16	30 ± 10	0,37	35 ± 16	0,22	21 – 72 IU/l
Apo B	0.82 ± .20	0.86 ± .23	0,28	0.89 ± .29	0,19	0.81 ± .18	0,43	0.6–1.6 g/l
AST	26 ± 7	24 ± 5.4	0,15	25 ± 7	0,32	28 ± 9	0,29	14 – 50 IU/l
Billirubin (total)	6 ± 4	8 ± 4	0,16	9 ± 7	0,06	8 ± 4	0,09	3.4 – 17 mmol/l
Billirubin (Conjugated)	2 ± 0	2 ± 0	1,0	2 ± 0	1,0	2 ± 0	1,0	0 – 3.4
Calcium	2.36 ± .11	2.34 ± .09	0,24	2.40 ± .09	0,17	2.39 ± .08	0,20	2.10 – 2.54 mmol/l
Chloride	108 ± 3	108 ± 3	0,46	108 ± 3	0,21	107 ± 3	0,07	98 – 107 mmol/l
Creatinine	72 ± 14	76 ± 11	0,12	81 ± 15	0,03	77 ± 11	0,09	62 – 121 μmol/l
Ferritin	80 ± 59	76 ± 64	0,45	50 ± 61	0,08	71 ± 71	0,36	14 – 179 mg/ml
Gamma GT (GGT)	40 ± 30	31 ± 11	0,11	42 ± 33	0,41	32 ± 22	0,19	8 – 78 IU/l
Globulins	31 ± 3.1	31 ± 2.4	0,48	31 ± 4.2	0,28	32 ± 2.7	0,13	16 – 42 g/l
Glycemia	5.1 ± .6	5.2 ± .9	0,41	4.9 ± .5	0,08	5.0 ± .4	0,16	3.3 – 6.1 mmol/l
Haemoglobin	145 ± 13	143 ± 15	0,34	144 ± 12	0,42	143 ± 15	0,37	120 – 180 g/l
Iron	19 ± 6.3	18 ± 7.1	0,29	19 ± 8.2	0,18	17 ± 6.6	0,16	8.8 – 31.5 mmol/l
Lactate dehydrogenase	415 ± 64	430 ± 103	0,30	418 ± 58	0,43	406 ± 70	0,35	300 – 600 IU/l
Magnesium	0.82 ± .06	0.79 ± .06	0,07	0.80 ± .05	0,17	0.80 ± .06	0,16	0.7 – 0.9 mmol/l
Phosphorous	1.05 ± .15	1.13 ± .29	0,13	1.09 ± .11	0,16	1.11 ± .14	0,11	0.8 – 1.45 mmol/l
Potassium	4.4 ± .4	4.4 ± .2	0,42	4.4 ± .3	0,38	4.4 ± .3	0,37	3.7 – 5.4 mmol/l
Proteins (total)	73 ± 5	74 ± 4	0,30	75 ± 5	0,14	75 ± 3	0,08	60 – 80 g/l
PTT	29.5 ± 2.4	28.6 ± 2.2	0,18	29.9 ± 1.4	0,23	28.2 ± 2.5	0,06	25–35 sec
Red blood cell count	4.71 ± .54	4.68 ± .44	0,43	4.70 ± .39	0,42	4.84 ± .45	0,22	3.8–5.9 x 10^12/l
Retinol	1.19 ± .39	1.36 ± .44	0,41	1.47 ± .57	0,41	1.43 ± .46	0,21	1.05 – 2.27 mmol/l
Sodium	142 ± 2	143 ± 2	0,17	143 ± 2	0,32	143 ± 2	0,27	137 – 149 mmol/l
TIBC	72 ± 10	72 ± 8	0,43	76 ± 7	0,09	77 ± 9	0,07	40 – 80 μmol/l
TSH	1.84 ± .88	1.92 ± 1.85	0,44	1.69 ± .89	0,30	1.96 ± 2.06	0,38	0.5 – 5 mU/l
Urea	5.14 ± 1.73	5.05 ± 1.36	0,43	4.16 ± .97	0,02	4.31 ± .80	0,03	3.2 – 7.5 mmol/l
Uric acid	258 ± 71	286 ± 89	0,15	296 ± 86	0,08	279 ± 61	0,17	150 – 415 μmol/l
Vitamin D	39.6 ± 19	49.2 ± 22.4	0,26	61.2 ± 5.7	0,38	65.3 ± 57.2	0,38	30 – 127 nmol/l
Vitamin E	18.± 1 6.2	17.7 ± 6.2	0,39	18.5 ± 7.2	0,88	19.5 ± 12	0,72	18 – 29 mmol/l

* Mean ± SD

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INFLUENCE OF FOOD ADDITIVES ON THE ANTIBACTERIAL EFFECT AND THE BINDING OF CHITOSAN ON *STAPHYLOCOCCUS AUREUS*

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Abstract: In order to assess the potential use of chitosan as biopreservatives, we have investigated the influence of common food additives on the inhibitory activity of chitosan against *Staphylococcus aureus.* The inhibitory activity of low-molecular-weight chitosan was determined in presence of sucrose, ethanol, NaCl and organic acids; acetic, lactic, and citric acid respectively. The addition of sucrose and Nacl at a concentration of 2% decreased the inhibitory effect of chitosan. Among the organic acids tested, acetic acid and lactic acid enhanced the inhibitory activity of chitosan, while citric acid showed the adverse effect. The influence of these additives on the binding capacity was investigated by using FITC-chitosan [2] on the bacterial cells [3]. These results indicate that although the natural compound chitosan has potential for use as a preservative, its antibacterial activity may be affected by several food additives.

INTRODUCTION

Consumer concerns regarding food safety and demands for foods without chemical preservatives are growing. In developing countries, large-scale food preparation and heat-and-eat convenience foods increase the risk of proliferation of the microorganisms that cause food-borne illness. Since the 1990s, the antimicrobial activity of chitosan against food-borne pathogens has attracted much research attention . Chitosan is a nontoxic, biodegradable polysaccharide that is a potential food biopreservative to replace chemical preservatives. However, too little is known about the factors that affect the antimicrobial activity of chitosan in foods. Therefore, we investigated the influence of the most widely used food additives on the inhibitory activity of low-molecular-weight chitosan against *Staphylococcus aureus* to assess its activity in foods.

MATERIAL AND METHODS

Chitosan Preparation

Low molecular weight chitosans of MW 3,000 and 50,000 were obtained from Amicogen Inc.(Jinju, Korea). These chitosans had minimum degree of deacety-

lation 95%. Stock solution containing 5,000 ppm of each chitosans in 0.5% acetic acid was sterili zed by membrane filtration.

Strains and culture condition

Staphylococcus aureus KCTC1916 were purchased from the Korean Collection for Type Cultures (Daejon,Korea) and subcultured twice in TS broth for 16 h at 30°C for all experiments**.**

Inhibition Test

Sucrose, ethanol, and NaCl were added to TS broth containing 100 ppm chitosan (MW 3,000) to determine the influence of these additives on the activity of chitosan. To examine the influence of organic acids, 0.1% acetic, lactic, and citric acids were added.

Binding of FITC-labeled chitosan to the cells

RESULTS

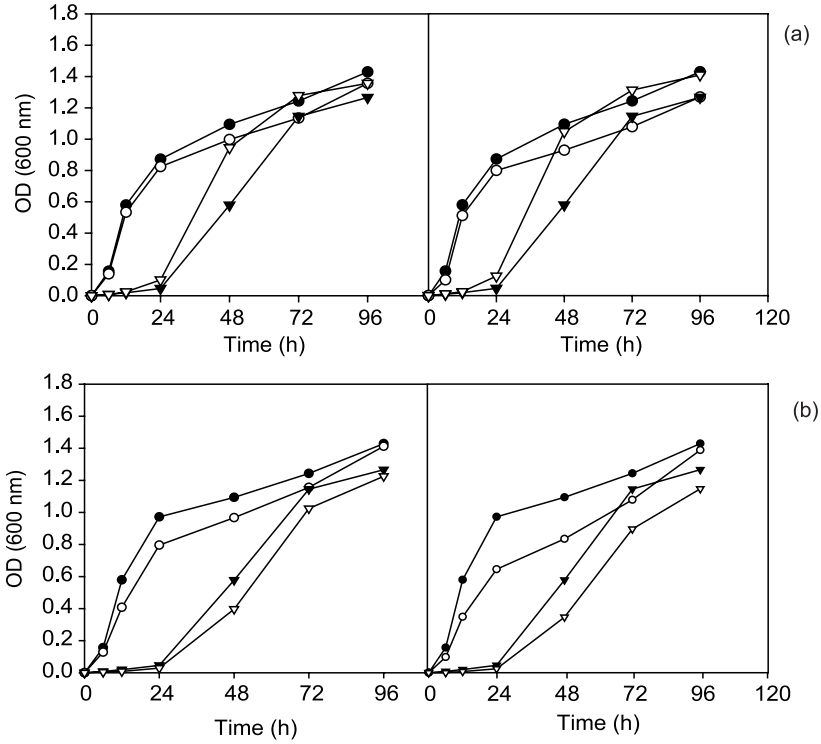

Figure 1. Inhibition of *S.aureus* by 100ppm of 3kDa chitosan with 2% (left side) and 4% (right side) sucrose(a), ethanol(b) (●: control ○: sucrose, ethanol and NaCl added ▼: chitosan added ▽: sucrose, ethanol and NaCl and chitosan added)

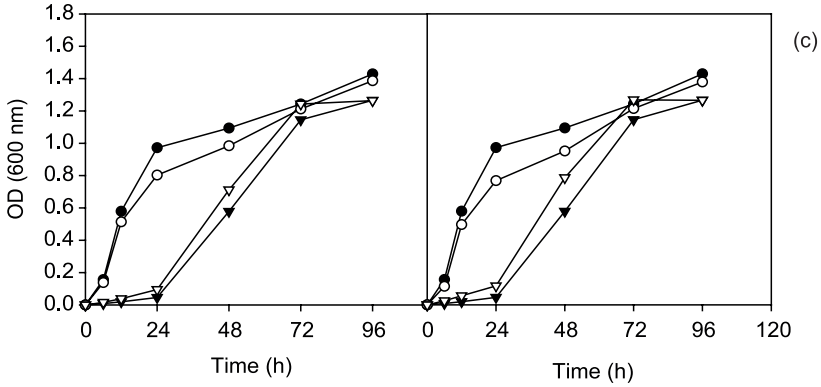

Figure 1. Inhibition of *S.aureus* by 100ppm of 3kDa chitosan with 2% (left side) and 4% (right side) NaCl(c) (●: control ○: sucrose, ethanol and NaCl added ▼: chitosan added ▽: sucrose, ethanol and NaCl and chitosan added)

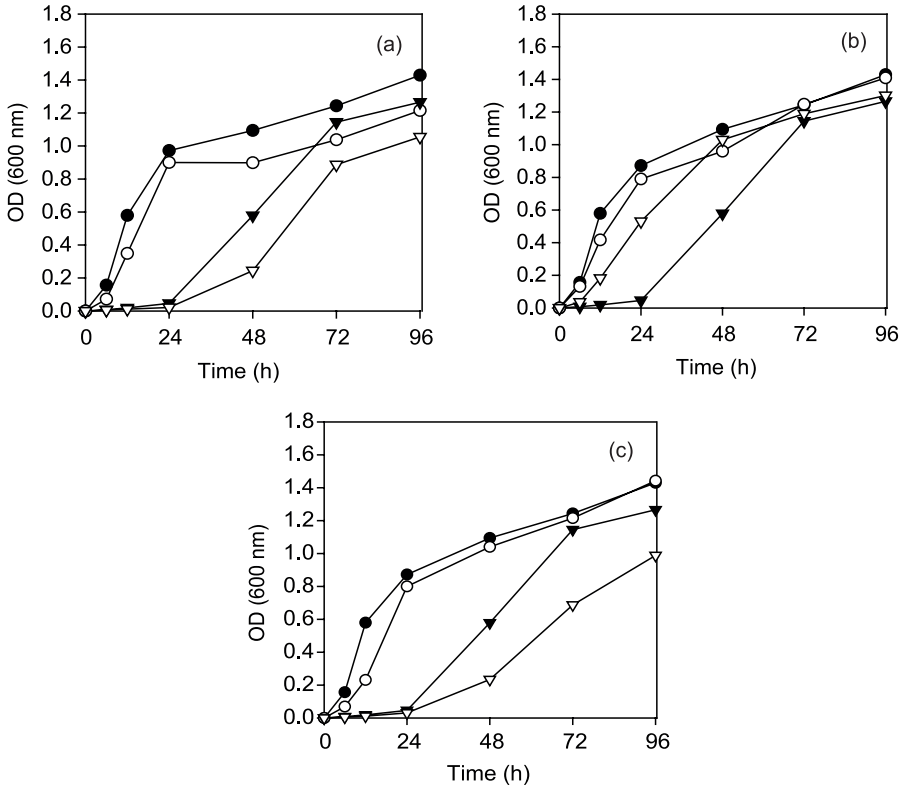

Figure 2. Inhibition of *S.aureus* by 100ppm of 3kDa chitosan with 0.1% acetic acid (a), citric acid (b) and lactic acid (c) (●: control ○: acetic acid, lactic acid and citric acid added ▼:chitosan added ▽: acetic acid, lactic acid, citric acid and chitosan added)

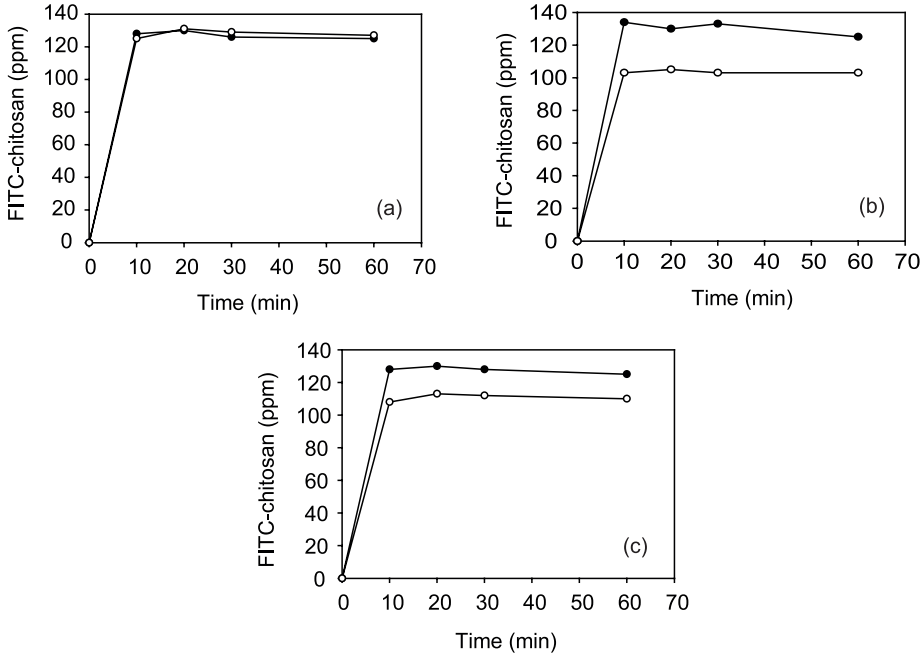

Figure 3. Effect of 2% sucrose (a), ethanol (b) and NaCl (c) treatments one the binding of 100ppm of 3kda FITC-chitosan to the non-growing cells of *S.aureus*.(●: FITC-chitosan treated ○: sucrose, ethanol and NaCl treated)

CONCLUSIONS

Although the natural compound chitosan has potential for use as a preservative, few attempts have been made to determine its antimicrobial activity in foods. Owing to the extremely complex nature of foods, it is necessary to investigate the factors that affect the activity of chitosan in foods. The results presented here demonstrate that common food additives, such as sucrose, ethanol, NaCl, and organic acids, have considerable effects on its antibacterial activity.

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EFFECTS OF WATER-SOLUBLE HIGH MOLECULAR WEIGHT CHITOSAN FEED ADDITIVES ON THE PHYSCOCHEMICAL PROPERTIES OF OLIVE FLOUNDER *(PARALICHTHYS OLIVACEUS)*

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INTRODUCTION

The worldwide production of cultured fish increases every year. However, the most troublesome problems in fish culture are fish diseases such as bacterial pathogens (*Vibrio anguillarum*, *V. salmonicida*, *Edwardsiella tarda*, *Streptococcal*, etc.), parasitic infections (*Scuticoci liatida ciliates*, etc.) and viral diseases (*Lympocytes disease*, etc.). Besides, overcrowding tends to produce poor physiological environment for fish and to increase susceptibility to infections. Immunostimulants are useful to control fish diseases in fish culture. Many kinds of immunostimulants such as glucan, chitin, lactoferrin, levamisole, vitamins B and C, growth hormone and prolactin have been reported. Actually, immunostimulants can reduce the losses caused by disease in aquaculture; however, they may not be effective against all diseases. For the effective use of immunostimulants, the timing, dosages, method of administration and the physiological condition of fish need to be taken into consideration. Chitosan has been known to display biological activities such as immunoadjuvant activity and protective activity against disease infection. Therefore, this research was performed to develop a new fish additives which consist of water-soluble chitosan and to improve the cultivation technology that is effective in prevention and treatment of various fish diseases, and to produce a high quality fish in nutritionally and functionally.

EXPERIMENTAL

Experimental fish. Young Olive flounder, *Paralichthys olivaceus* was obtained from Gochang, Korea. The average length and weight of the fish were 4.5 ± 0.4

cm and 3.2 ± 0.2 g respectively. They were randomly distributed into the water tank (1,000 m3) for fish culture. The water temperature was maintained at 22 ± 0.1

Water-soluble high molecular weight Chitosan-based feed additive. The water-soluble high molecular weight chitosan (Mw 300,000 degree of deacetylation 98%) sample is from Webiotech Co., Korea. The amount of WSC was formulated to contain 0.2 (C200) and 0.4% (C400) based on the commercial pellet feed. The control group was fed commercial pellet without WSC.

Fish sampling. Ten fish were sampled every 3 month during culture and stored at –40°C in a freezer for analysis.

Analysis of protein and lipid. Crude protein content was determined by the Kjeldahl method using Auto Kjeldahl System (Kjeltec Switzerland), lipid content was determined by the Soxhlet extraction method.

Analysis of polyunsaturated fatty acid (PUFA). For analysis of PUFA, the crude fat of the freeze-dried flesh was extracted using a Soxhlet apparatus with ethyl ether as an extraction solvent for 6 hr.

Fatty acid composition was analyzed by using a gas chromatography (Vatian 3800; arian Inc., walnut Creek, CA). The column used was a Supelcowax 10 fused-silica capillary column (30m × 0.25 mm I.d.; Supelco, Bellefonte, PA) .

Amino acids were analyzed after hydrolysis of dried sample using a HPLC (JASCO, PU-980, Tokyo, Japan) with a 851-AS auto sampler, a 3.9 × 150 mm internal diameter Waters pico-tag column and UV-975 UV/VIS detector.

Drip loss was determined after thawing freshly frozen flesh that has been stored for 1 week at –30°C. Ten flesh pieces were placed in a freezer (–20°C) for 3 days, and then the thawing was conducted by placing a frozen flesh in a refrigerator (5°C) for 24 hr. They were then drained according to procedure 963.18 (AOAC 2000) with some modification. Drip loss was calculated as follows:

Drip loss (%) = Weight after thawing-weight after draining/Weight after thawing x 100%

Rheological properties of flesh was measured using a rheometer (model CSL100, TA Instruments Co., New Castle, DE) with frequency ramp (1-10 rad/s) at the strain of 0.3% and at 25°C.

The microstructure of flesh was observed by scanning electronic microscope (S-2389N, Hitachi, Japan).

RESULTS AND DISCUSSION

This study was carried out to find out the effect of water-soluble high molecular weight chitosan-based feed additives on growth, chemical composition, textural and ultrastructural properties of Olive flounder *Paralichthys olivaceus* cultivated for 12 months. The levels of the WSCFA investigated were 0.2 and 0.4%. Some essential amino acids contents increased as the level of WSCFA increased,

while the death rate of fish decreased. The eicosapentaenoic acid and docosahexanoic acid contents were also increased by about 1.5 and 1.2 times after 12 month evaluation, respectively. The textural study of the flesh showed that the G′ (storage modulus) increased with the increase of WSCFA level. When the flesh were frozen and thawed, the drip loss was decreased by 12.4 and 16.9% for the additives of 0.2 and 0.4%, respectively. Good correlation was observed between the drip loss and G′ values. The ultrastructure of the flesh after freezing and thawing showed a less-ruptured structure by ice crystals for the fish fed with WSCFA.

CONCLUSIONS

The value of current fish culture appears to be enhanced by using the feed additives, which protect fish diseases and produce high quality fish, in Korean flatfish culture. The cultivation technology of high quality fish with high DHA and essential amino acids contents and the stability of freezing and thawing, appears to have high possibility of aaplication to fresh flesh products market, which is suitable for new fish distribution system. This research is extensively in process to apply these environmental friendly and natural additives for fish feed to the culture of other fish kinds.

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ANTIMICROBIAL PROPERTIES OF SELECTED CHITOSAN FORMS AGAINST MICROFLORA EXISTING ON CHEESE

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INTRODUCTION

Chitosan, a copolymer consisting of *β*-(1,4)-D-glucosamine and *N*-acetyl-*β*-(1,4)-D-glucosamine units, is a polysaccharide prepared by deacetylation of chitin, which is one of the most abundant natural polymers in living organisms such as crustacea, insects and fungi. Physico-chemical and biochemical modification of chitosan, especially its molecular and supermolecular structure allows to obtain new forms of the polymer with unique properties like microcrystalline chitosan, chitosan salts and modified chitosan salts. Chitosan exhibits antibacterial, antifungal and antiviral properties. Chitosan has been proved to be non-toxic, biodegradable, bioactive and biocompatible [1–2]. Combining poly lactic acid (PLA) with selected chitosan forms, which reveal low oxygen permeability rate make possible to produce biobased packaging materials, important for the microbial stability of the cheese and oxidative deterioration processes in the cheese.

EXPERIMENTAL

Materials

Chitosan forms used in experiments (Table 1).

Table 1. Characteristic of the chitosan useful forms used in the bioactivity tests

Symbol of preparation	Type of preparation	Polymer concentration (%)	$\overline{M}_v$,kD	DD ,%
M	microcrystalline chitosan	2.75	110.0	82.2
MGS	modified glutamate salt	5.64		
MLS	modified lactate salt	4.80		

Microorganisms (IBT collection at BioCentrum Technical University of Denmark)
- yeast strains – *Kluyveromyces marxianus IBT 443, Debaryomyces hansenii IBT 152*
- mould strains – *Penicillium commune IBT 1025, Penicillium roqueforti IBT 12845, Penicillium nalgiovense IBT 12105,Penicillium caseifulvum IBT 15151*

Semi hard cheese "Łaski" – 45% fat content made by Okręgowa Spółdzielnia Mleczarska, Łask, Poland;

Agar, BTL sp. z.o. Łódź, Poland; Tween-80, POCh, Gliwice, Poland; Yeast Extract, Difco; Czapek dox broth, Difco; Yeast Broth, Difco; $ZnSO_4 \times 7H_2O$, POCh, Gliwice, Poland; $CuSO_4 \times 5H_2O$,POCh, Gliwice, Poland.

Microbial tests

Bioactivity of chitosan was tested according to direct method on the base of method received from Centre for Advanced Food Studies (LMC), Denmark.

On the surface of the nutrient medium, chitosan preparates were evenly distributed in the amount of 0.5–2.0 g. The medium-containing plates were inoculated in three points of the surface with the fungi in the amount of 0.11 g calculate on the dry weight. Plates without chitosan were used as control. The plates were incubated at 5, 15 and 25°C for 5–14 days. Afterr that time the diameters in two direction were measured for each colony. The relative growth inhibition was calculated form :

$$D = \frac{D_{control} - D_{active_compound}}{D_{control}} \cdot 100\%$$

Were $D_{control}$ is the average diameter of the control. $D_{active_compound}$ is the average diameter of the colonies with chitosan.

For the inoculation of the samples a solution was prepared containing:0.5% Tween-80 and 0.5% agar. After sterilization and cooling down to 45°C the solution was inoculated with spores of fungi and the suspension density was fixed at 10^6–10^7 cells/ml.

RESULTS AND DISCUSSION

The impact was investigated of the amount and type of chitosan on the inhibition of fungi growth. The chitosan preparates (microcrystalline chitosan – MCCh, modified glutamate and lactate salt) were deposited on the medium.

Results the relative growth ihibition are presented in Tables 2–3. Figure 1 illustrate the fungi growth *P. roqueforti* and *P. nalgiovense* on selected preparations in comparison with the growth on medium without chitosan. All attached photos show the growth after 5 days incubation at 25°.

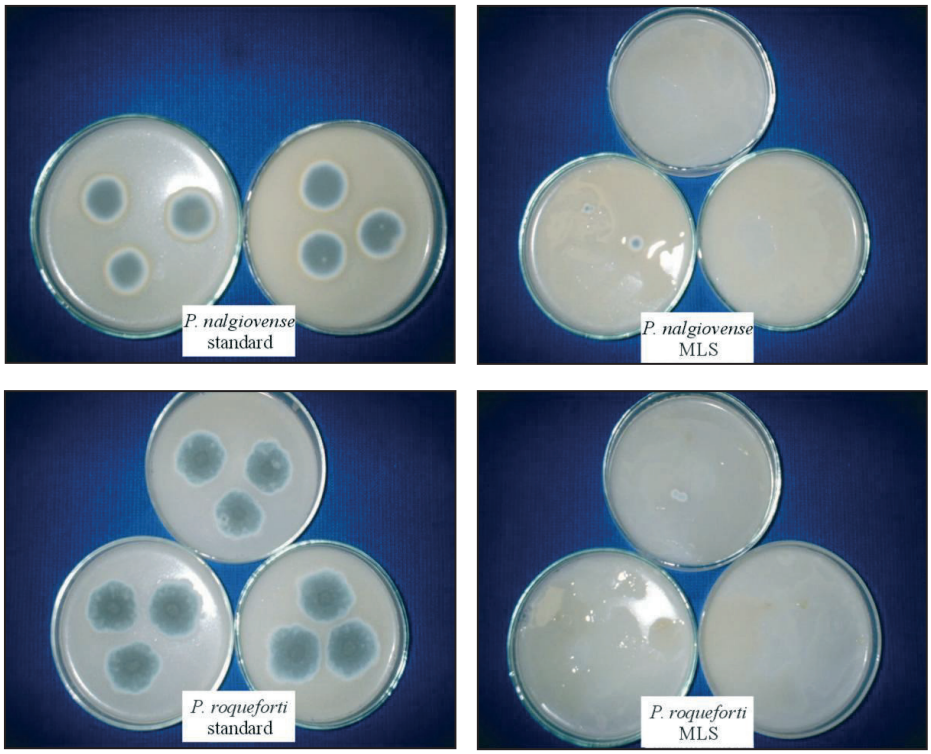

Figure 1. Impact of modified chitosan lactate salt (MLS) on growth inhibition of *P. nalgiovense* and *P. roqueforti* (direct method) – comparison with a control

Table 2. Impact of chitosan preparation kind and amount on growth inhibition of moulds related to time and temperature of incubation (direct method)

Strain	Symbol of preparation	Relative inhibition (%)		
		Temperature (°C)		
		5	15	25
		Time (days)		
		14	5	5
Penicillium commune IBT 10253	M	25	0	45
	MGS	46	50	50
	MLS	63	42	76
Penicillium roqueforti IBT 12845	M	100	100	37
	MGS	100	100	100
	MLS	100	94	97
Penicillium nalgiovense IBT 12105	M	77	77	51
	MGS	100	100	57
	MLS	83	85	73
Penicillium caseifulvum IBT 15151	M	19	35	39
	MGS	30	23	47
	MLS	56	45	44

Table 3. Impact of chitosan preparation kind and amount on growth inhibition of yeast related to time and temperature of incubation (direct method).

Strain	Symbol of preparation	Relative inhibition (%)		
		Temperature (°C)		
		5	15	25
		Time (days)		
		14	5	5
Kluyveromyces marxianus IBT 443	M	100	100	68
	MGS	100	100	100
	MLS	87	86	87
Debaryomyces hansenii IBT 152	M	86	100	100
	MGS	100	100	100
	MLS	82	89	93

CONCLUSIONS

It was found that the relative inhibition in case of various useful chitosan preparations depended upon the incubation time, kind of tested microorganism and type of preparate on the plate.

All chitosan forms tested revealed biological activity within the investigated incubation time and temperature. Amongst the tested microorganisms the highest relative inhibition (80–100%) was found for *P. roqueforti* and *P. nalgiovense* and for *K. marxianus* and *D. hansenii*, all yeast and fungi associated with the cheese microflora.

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IMMUNOMODULATORY ACTIVITY OF A CHITOSAN HYDROLYSATE BY ORAL ADMINISTRATION IN MICE

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Abstract: Cellulase was used to degrade shrimp chitosan to obtain a water-soluble hydrolysate, and the immunoactivity of this hydrolysate was further evaluated by oral administration into Balb/c mice. The hydrolytic activity of cellulase on chitin/chitosan increased with increasing deacetylation degree (DD) of chitosan, with DD95 being the most easily hydrolyzed. The optimal reaction temperature and pH for cellulase digestion of chitosan were 55°C and pH 5.2, respectively. During cellulase digestion of chitosan, the 9-h hydrolysate had the highest enhancing effect on the proliferation of a human hybridoma cell HB4C5. Three doses of 1 mg, 10 mg and 50 mg per mouse of this hydrolysate powder were administrated into Balb/c mice and the feeding test continued for 4 weeks. The phagocytic activity of the peritoneal macrophage, and the proliferation of the concanavanin A (ConA) –induced T-cells of spleen and Peyer's patch were significantly increased for the 3 experimental groups fed with hydrolysate. Furthermore, the proliferation rates of the T-cells increased significantly with the increasing of feeding dose. However, the proliferation of the liposaccharide (LPS)-induced B-cell of spleen and Peyer's patch were significantly suppressed if the mouse was fed with 50 mg hydrolysate. The concentrations of IgM, IgG and IgA in serum were significantly increased for the group fed with 50 mg hydrolysate.

Key word: Chitosan hydrolysate, Oral feeding test, Peyer's patch, Lymphocytes

INTRODUCTION

Chitin (poly-*β*-(1→4)-*N*-acetyl-D-glucosamine) and chitosan (a partially or fully deacetylated chitin) have various physiological activities. They can accelerate wound healing in animals [1], activate macrophages, complement proteins and cytotoxic T lymphocytes [2, 3] and protect animals from pathogen infections [4, 5]. However, the water-insolubility of chitin and chitosan make them disadvantageous as immunotherapeutic agents. The water-soluble chitooligosaccharides and *N*-acetyl chitooligosaccharides, in particular, the hexamers of *N*-acetyl chitohexaose and chitohexaose, have recently attracted much attention for their immune-enchancing effects [6, 7] and antitumor activity [8, 9]. However, the very high cost of these hexamers limits their application. A water-soluble chitosanolytic product with immunoactivity merits developed.

Although various acids, such as HCl, HF, phosphoric acid and nitrous acid, have been used to hydrolyze chitin or chitosan [10–14], enzymatic digestion be-

comes more popular due to its mild reaction condition and good reproduction [15–17]. Cellulase has been demonstrated to hydrolyze chitosan for the preparation of chitooligosaccharide with strong antibacterial activity [18]. In this study we investigated first the effects of the deacetylation degree (DD) of chitosan, and the reaction pH and temperature on the hydrolytic activity of cellulase on chitosan in order to find the optimal hydrolytic conditions for cellulase degradation of chitosan. Then, a water-soluble chitosan hydrolysate with immunoactivity was prepared. The effects of this hydrolysate on the cell proliferation and antibody secretion of lymphocytes, and phagocytic activity of macrophage were evaluated in vivo by oral administration into Bulb/c mice.

EXPERIMENTAL

Preparation of colloid chitin and chitosan

Chitin was isolated from shrimp (*Solenocera melotho*) shells [19]. This chitin powder was added to 12 N HCl (25 g of chitin per 500 mL of HCl) and stirred in the ice bath for 1 h. After filtration through a glass cloth, the filtrate was added with an equal amount of deionized water and centrifuged (3000 × g, 30 min). The precipitate of colloid chitin was collected and washed with deionized water until the effluent became neutral.

The chitin powder was deacetylated with 40% NaOH at 100°C for 45 min, 1 h and 2.5 h to obtain chitosan with DD of 50%, 65% and 75% (DD50, DD65 and DD75), respectively. For the preparation of chitosan with 95% deacetylation (DD95), chitin was added to 50% NaOH and heated at 140°C for 1 h. The DD of chitosan was measured by a colloid titration method [20].

Cellulase digestion of colloid chitin and chitosans with various DD

The chitosan solutions and a colloid chitin suspension (5%, wt/vol) were hydrolyzed with cellulase (10 U/mL) in 0.5 N acetic acid- bicarbonate buffer (pH 5.2) at 55°C with 125 rpm for 24 h. At intervals, 3 mL of the reaction medium was sampled, boiled for 15 min, adjusted to pH 7.0 and then centrifuged (12,000 × g, 30 min). The content of the reducing sugar in the supernatant was analyzed using Somogyi method [21].

Production of chitosan hydrolysate with immunoactivity

DD95 was added into 2000 mL acetic acid-bicarbonate buffer (pH 5.2) containing cellulase (10 U/mL). After digestion at 55°C with 125 rpm for 9 h, the hydrolysate was neutralized and centrifuged (12,000 × g, 30 min). The supernatant was freeze-dried.

Administration of chitosan hydrolysate to mice and antibody measurement

Forty-eight female BALB/c mice (4-week old) were used. They were fed *ad libitum* for 1 week, and then divided randomly into 4 groups of 10 animals: one control group orally administrated with saline and 3 experimental groups orally administrated with chitosan hydrolysate at dosages of 1, 10, and 50 mg per mouse per day, respectively. The mice were administrated 5 days per week and last for 4 weeks. After 4 weeks of feeding, the mice were sacrificed, and their

blood and spleens were obtained. The amounts of total IgA, IgM and IgG in sera were determined, according to the method of Tsai and Yu [22].

Phagocytosis of peritoneal macrophage

The peritoneal macrophages (1×10^6 cells/mL) were incubated with *Saccharomyces cervisiae* CCRC21607 cells (1×10^7 cells/mL), which were pre-incubated in 10% FCS at 37°C for 30 min, at 37°C for 1 h. The phagocytosis of macrophages was then microscopically assessed after H&E staining.

Lymphocyte proliferation assay

One hundred μL of the spleen or Peyer's patch lymphocyte suspension (2×10^6 cells/mL) were placed on a 96-well microplate, and followed by the addition of 20 μL of lipopolysaccharide (LPS, 30 μg/mL, Sigma) or concanavalin A (Con A, 20 μg/mL, Sigma) in triplicate. After incubation at 37°C for 48 h, the viable cells were measured by MTT assay [23]. Calculation of lymphocyte proliferation was done: proliferation rate (%) = $[T_{ConA\ or\ LPS} / T] \times 100$, where $Exp._{ConA\ or\ LPS}$ is optical density value of test well contained ConA or LPS; T is that of test well without ConA or LPS.

RESULTS AND DISCUSSION

In this study we first investigate the factors of chitin/chitosan DD, reaction pH and temperature on the hydrolytic activity of cellulase. Two different grades of cellulase products were used, one of which being in a reagent grade from Sigma, and the other being in a technical grade with a bulk package from Challenge, Taiwan. Irrespective of the big difference in price for these two cellulase products, they showed similar results on the degradation of chitosan. The hydrolytic activity of cellulase on chitosan increased as the chitosan DD increased. The colloid chitin was hardly hydrolyzed by cellulase. This demonstrates that the cellulase used in this study may also act on chitosan. This is in contrast to lysozyme whose activity decreased as chitosan DD increased [24]. The optimal pH value and temperature for these two products of cellulase to degrade chitosan were pH 5.2 and 55°C, respectively.

The product of cellulase from Challenge was chosen in the following study. During cellulase digestion of chitosan, the 9-h hydrolysate showed the highest increasing effect on the proliferation of a human hybridoma cell HB4C5. After oral administration of this chitosan hydrolyste into mice for 4 weeks, the phagocytic activity of the peritoneal macrophage of mice fed with chitosan hydrolysate at dosage of 1, 10, or 50 mg/mouse/day was significantly higher than the control group.

In vivo the chitosan hydrolysate significantly increased the Con A- induced proliferation of both spleen and Peyer's patch lymphocytes. The proliferation of LPS-induced lymphocytes was not affected by this chitosan hydrolysate, except for the high dose group (50 mg) of mice, where some suppressive effect was observed. After 4 weeks of feeding, the levels of serum IgM, IgA, and IgG of 50 mg-fed group were significantly higher than those of the control group. In addi-

tion, the IgM content of 10 mg-fed group was also significantly higher than those of the control group.

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IMMUNOMODULATORY EFFECTS OF VARIOUS CHITOSANOLYTIC PRODUCTS ON MURINE MACROPHAGE CELL RAW264.7

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Abstract: A water-soluble hydrolysate with immunomodulatory activity was obtained by cellulase degradation of shrimp chitosan. A low-molecular-weight chitosan (LMWC) and the chitooligosaccharide mixture were further separated from this hydrolysate. The immunomodulatory effects of these products on the murine macrophage RAW264.7 is evaluated. All tested samples did not affect the proliferation of RAW264.7; while in the presence of interferon-γ the hydrolysate and the chitooligosaccharide mixture stimulated the NO production and phagocytic activity of RAW264.7 However, the hydrolysate and chitooligosaccharide decreased the oxidative burst of RAW264.7. In the presence of the hydrolysate and chitooligosaccharide RAW264.7 cells increased in size and changed from macrophage-like to dendritic-like cells.

Key words: Chitosan hydrolysate, Chitooligosaccharide, LMWC, RAW264.7

INTRODUCTION

Chitin (poly-*β*-(1→4)-*N*-acetyl-D-glucosamine) and chitosan (a partially or fully deacetylated chitin) have immunomodulatory activity [1–7] and protect animals from pathogen infections [8–10]. However, the water-insolubility of chitin and chitosan make them disadvantageous as immunotherapeutic agents. In a previous study a water-soluble chitosan hydrolysate with immunoactivity was obtained by cellulase degradation of chitosan [11]. In vitro and in vivo this hydrolysate could enhance the cell proliferation and the antibody production. In this study, a low-molecular-weight chitosan (LMWC) with molecular mass of 20.0 kDa and a chitooligosaccharide mixture containing sugars with degree of polymerization (DP) of 1–8 were separated from this chitosan hydrolysate. The effects of the hydrolysate and its separated components on the cell proliferation, phagocytic activity, production of nitric oxide (NO) and superoxide anion of a mouse monocyte-macrophage, RAW 264.7 cell, were investigated.

EXPERIMENTAL

Preparation of chitosan

The chitin powder was deacetylated with 50% NaOH (1 g of chitin per 13 mL of NaOH) and heated at 140°C in an oil bath for 1 h to obtain chitosan with DD of 95%, as measured by a colloid titration method [12].

Production of chitosan hydrolysate with immunoactivity and component separation

DD95 (100 g) was added to 2000 mL acetic acid-bicarbonate buffer (pH 5.2), containg cellulase (10 U/mL). After digestion at 55°C with 125 rpm for 9 h, the hydrolysate was neutralized and centrifuged (12,000 × g, 30 min). The supernatant was added to an equal volume of methanol to separate the low-molecular-weight chtitosan (LMWC) and the chitooligosaccharides with DPs of less than 16, according to the protocol of Muraki et al [13].

RAW264.7 cell proliferation measurement

The murine macrophage cell, RAW264.7, was cultured in an RPMI 1640 medium containing glutamine at 37°C for 2 days. After adjusting the cell density to 1×10^5 cells/mL, 100 μL of cell suspension were added to the well of the microtiter plate, and then, 20 μL of various concentrations of the filter-sterilized samples of chitosan hydrolysate, LMWC and a chitooligosaccharide mixture were added. After incubation at 37°C for 2 days, the proliferation of cell was counted using MTT assay, as described by Hansen et al. (1989) [14].

Phagocytic activity measurement

The phagocytosis of the RAW264.7 cell was analyzed by incubating the cells (1×10^6 cells/mL) at 37°C for 30 min with *Saccharomyces cervisiae* CCRC21607 (1×10^7 cells/mL), which had been opsonized by incubation in 10% FCS at 37°C for 30 min. Using H&E staining the phagocytic cell was microscopically observed.

Nitric oxide (NO) assay

The NO content in cell cultures was measured by the Griess reaction [15].

NBT reduction assay

RAW264.7 cells were incubated with various concentrations of samples in a 96-well flat bottom plate (2×10^5 cells/mL) at 37°C for 2 days. Then the cells were re-incubated with 2 mg/mL of NBT and 1 mg/mL of 12-o-tetradecanoylphorbol 13-acetate at 37°C for 30 min. After centrifugation, the cells were resuspended in 200 μL of DMSO, and the absorbance at 570 nm was determined [16]. NBT reduction ratio (%) = $[Exp._{Sample} / T._{Control}] \times 100$, where $Exp._{Sample}$ is optical density value of test well treated with various concentrations of samples; $T._{Control}$ is that of test well without sample treatment.

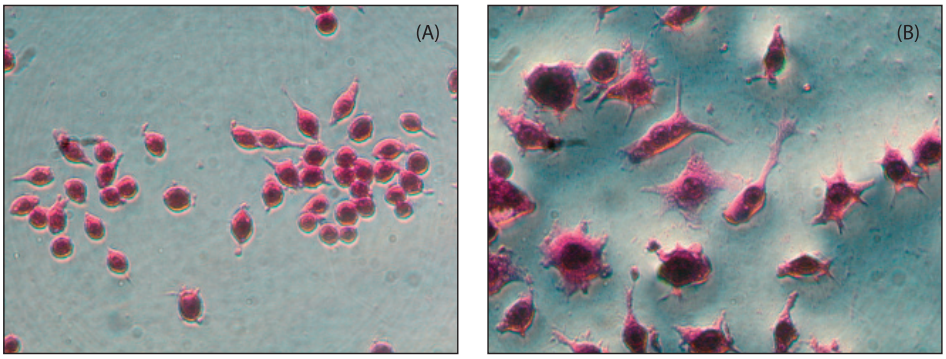
Morphological changes

Ten μL of RAW264 7 cells (1×10^6 cells/mL) were added on the surface of a glass slide. One hundred μL of the filter-sterilized chitosan hydrolysate, LMWC and a chitooligosaccharide mixture were added. After incubation at 37°C for 2 days,

the glass slides were fixed in ice-cold methanol for 30 min. Slides were then washed and stained with Hematoxylin and Eosin (H&E) reagents. The morphology of cells on the slide was observed under a microscope with a digital camera.

RESULTS AND DISCUSSION

An immuno-enhancing hydrolysate was obtained by cellulase digestion of chitosan. The hydrolysate was further separated into a low-molecular-weight chitosan (LMWC) with a molecular mass of 20.0 kDa and a chitooligosaccharide mixture containing sugars with 1~8 degree of polymerization. All of the tested samples (chitosan hydrolysate, the chitooligosaccharide and LMWC) did not significantly affect the proliferation of RAW264.7 cells; while the hydrolysate and chitooligosaccharide could increase the phagocyte activity of RAW264.7 cells by 3.0 and 2.8 times, respectively. In the presence of IFN-γ, both of the hydrolysate and chitooligosaccharide showed a significantly increased NO secretion as compared to control cultures, and this enhancing effect was dose dependent. At the concentration of 3 mg/mL, the hydrolysate and chitooligosaccharide increased the NO production of RAW264.7 cells by 2.0 and 2.2 times, respectively; while LMWC had little effect on NO production. This demonstrated that the molecular size of chitosan might determine its effect on the NO secretion of macrophages, and the small molecular chitosan (e.g. chitooligosaccharide) showed the enhancing effect. However, the hydrolysate and chitooligosaccharide exhibited dose-dependent suppression on the production of superoxide anion by RAW264.7 (NBT reduction assay). At the same time, the apparently morphological changes of RAW264.7 cells were observed (Fig.1). Both hydrolysate and chitooligosaccharide caused the RAW264.7 cells becoming more dendritic-like cells (Fig. 1, B and C), which showed stronger antigen-presenting capability and the superoxide-generating ability was suppressed. The data from the suppression effect on superoxide production and the morphological observation indicated that both hydrolysate and chitooligosaccharide induced the differentiation of RAW264.7 cell line into dendritic-like cells. The effect of the tested samples on the changes in the CD markers of RAW264.7 cells merits further study to confirm our hypothesis.



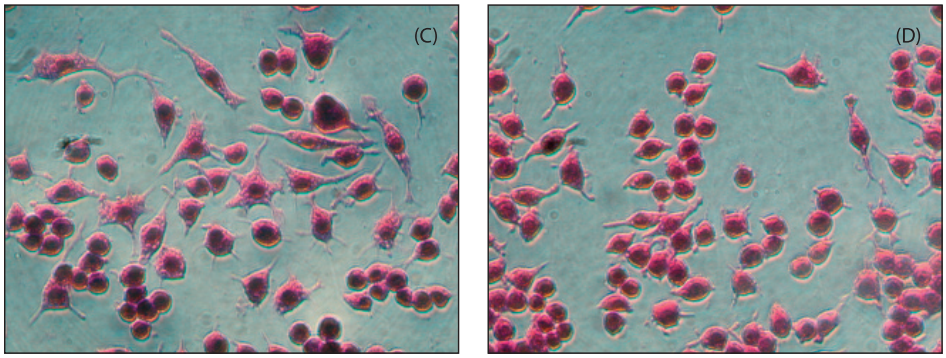


Figure 1. The morphological changes of RAW264.7 cells stained by H&E method after treatment with samples for 2 days. (A) Untreated RAW264.7 cells (control). (B) Hydrolysates-treated RAW264.7 cells. (C) Chitooligosaccharide-treated RAW264.7 cells. (D) Low-molecular-weight chitosan (LMWC)-treated RAW264.7 cells

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THE CHITOSAN AS A REMOVAL AGENT OF THE NITROUS ION IN FOOD

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Abstract: The removal of nitrous acid in stomach was tested by natural amino compounds such as sodium glutamate, glucosamine, chitosan, and chito-oligosaccharides. The consumption of nitrous acid was evaluated under the artificial gastric condition (0.15 M HCl). The consumption of nitrous acid by sodium glutamate was almost similar to that by glucosamine. Chitosan showed the highest consumption among natural compounds used in this study and the concentration of nitrous acid was decreased to 12.8% within 6 hours, thus demonstrates that chitosan is useful for the effective removal of nitrous acid in foods.

Keywords: chitosan, scavenger, nitrous acid, food, nitrosamine, diazoquinone, carcinogenesis, mutagenesis

INTRODUCTION

Chitosan is a natural amino-polysaccharide and glucosamine is a monomer of chitosan. Since these amino-sugars are effective for the improvement of some diseases such as hypercholesterolemedia by chitosan and osteoarthritis by glucosamine, these are suitable for the supplement of food. On the other hand, nitrous acid in general foods is caused to produce dangerous materials such as carcinogenic or mutagenic compounds in the human stomach. Nitrous acid is easily produced from nitric ions in foods by the action of oral bacteria or reducible ingredients. Nevertheless, various nitrates or nitrites, which is a source of nitric ions, have been artificially added to general foods such as meets and fishes on the purpose of the meat color cured agent. Furthermore, plants or vegetables absorb and store nitrates from soil. In order to avoid carcinogenic/mutagenic compounds in foods, the removal of nitrous acid under gastric stage is important for the human health care. It is well known that the chemicals bearing primary amino group is a good scavenger of nitrous acid under acidic condition. Therefore, natural compounds such as amino acids or sugars would be good candidate for the removal of nitrous acid. In this study, we report the effective removal of nitrous acid by chitosan under artificial gastric condition.

EXPERIMENTAL

Materials

The 100% deacetylated chitosan (DAc100) was purchased from Koyo chemicals Co., Japan.

Chito-oligosaccharides (Olig. 7. Olig. 91) Olig. 7(DP. 7), Olig. 91(DP 91) were prepared by modified Muraki's method [1]. In brief, oligosaccharides were roughly separated from enzymatically hydrolyzate of chitosan by using cellulose column, and then these were purified in each degree of polymerization (DP) by gel permeation column chromatography (GPC). The DP of oligosaccharides was estimated by GPC with Pullulan as standards. Other reagents were purchased Wako Pure Chemical Industries and Aldrich Co., Ltd and used without further purification.

Analysis of the nitrous ion

The amount of nitrous ion was estimated by modified Shinn's method [2]. Briefly, 0.5 ml of 1% solution of sulfanilamide in 10% aqueous HCL and 0.5 ml of 0.12% aqueous solution of N-1-naphthyl-ethylenedianine dihydrochloride were added into 5 ml of sample solution including 0.1–0.4 ppm of nitrogen. At this time, the color of sample solution turned to rose, and the absorbance at 540 nm of the solution was measured.

Analysis of nitrosamine

The amount of resulting nitrosamine in sample was estimated by modified Howard's method [3]. The nitrosamine was prepared from the reaction of sample solution and dimethylamine in 0.15 M HCl at room temperature for 1h. After the acidic solution was immediately converted to basic by addition of KOH, the nitrosamine was collected by steam distillation. The distillate including nitrosamine was turned to nitrous acid by addition of excess HCl. Finally the amount of nitrous acid (corresponding nitrosamine) was analyzed by the above procedure [2].

Removal of nitrous acid by amine compounds

For the artificial gastric condition, 0.15M HCl was used. Chitosan, oligo. DP 7, oligo. DP 91, glucosamine, and sodium glutamate were chosen as the removal agent (scavenger) of nitrous acid. A prescribed amount of scavenger and sodium nitrite were mixed in 5 ml of 0.15 M HCl. The amount of consumed nitrous acid was measured with time-courses. The concentration of scavenger was settled to 5–100 mg/5 ml (0.36–300 ppm of nitrogen).

RESULTS AND DISCUSSION

Table 1 shows the concentration of nitrogen measured in each condition and residual ratio of nitrogen. In all cases, the residual ratio of nitrogen (RRN) by glucosamine as scavenger was slightly more effective than that of sodium glutamate. The RRN by glucosamine, oligomer, and chitosan was much depended on their degree of polymerization (DP). The higher DP of amino-sugar showed more effective decrease of nitrogen. This dependence on DP would be caused by

the difference of reaction rate against nitrous acid. After 6 hours, the RRN value by chitosan showed 5-folds more than that by sodium glutamate. In Table 1-b, especially, the concentration of nitrogen was decreased to 2.69 ppm by chitosan within 6 hours. These results suggest that chitosan is most effective scavenger of nitrogen among the natural amino compounds used in this study.

Table 1-a. Change of nitrogen concentration with time-courses removal agent: 100 mg/5 ml *concentration of nitrogen initial concentration of nitrogen: 302 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig.91		Chitosan		Glutamate	
Reaction time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	127	42.1	24.4	8.1	21.7	7.2	24.8	8.2	143	47.4
3.0hr	49.2	16.3	3.55	1.2	2.66	0.9	2.58	0.9	44.4	14.7
6hr	10.2	3.4	0.0647	0.0	0.0754	0.0	0.16	0.1	17.2	5.7

Table 1-b. Change of nitrogen concentration with time-courses removal agent: 50 mg/5 ml *concentration of nitrogen initial concentration of nitrogen: 302 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig. 91		Chitosan		Glutamate	
Reaction time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	140.8	46.6	76.2	25.2	71.2	23.6	64.1	21.2	148	49.0
3.0hr	93.5	31.0	29.2	9.7	24.6	8.1	19.1	6.3	66.9	22.2
6hr	33.3	11.0	5.17	1.7	4.2	1.4	2.69	0.9	24.9	8.2

Table 1-c. Change of nitrogen concentration with time-courses removal agent: 20 mg/5 ml *concentration of nitrogen initial concentration of nitrogen: 16.9 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig. 91		Chitosan		Glutamate	
Reaction time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	12.4	73.4	8.93	52.8	8.09	47.8	8.58	50.8	11.6	68.6
3.0hr	8.86	52.4	4.76	28.2	4.32	25.6	4.0	23.7	8.41	49.8
6hr	4.73	28.0	1.56	9.2	1.18	7.0	0.925	5.5	4.35	25.7

Table 2-a. Change of nitrogen concentration with time-courses removal agent: 5 mg/5 ml *concentration of nitrogen initial concentration of nitrogen: 3.15 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig. 91		Chitosan		Glutamate	
Reaction Time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	3.19	101.3	2.86	90.8	2.73	86.7	2.78	88.3	3.12	99.0
3.0hr	2.64	83.8	2.08	66.0	1.97	62.5	1.87	59.4	2.58	81.9
6hr	2.12	67.3	1.23	39.0	1.11	35.2	0.929	29.5	1.72	54.6

Table 2-b. Change of nitrogen concentration with time-courses removal agent: 10 mg/5 ml *concentration of nitrogen initial concentration of nitrogen: 3.15 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig. 91		Chitosan		Glutamate	
Reaction Time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	3.09	98.1	2.44	77.5	2.26	71.7	2.13	67.6	2.91	92.4
3.0hr	2.57	81.6	1.61	51.1	1.41	44.8	1.18	37.5	2.46	78.1
6hr	1.69	53.7	0.739	23.5	0.594	18.9	0.403	12.8	1.56	49.5

Table 2-c. Change of nitrogen concentration with time-courses removal agent: 20 mg/5ml *concentration of nitrogen initial concentration of nitrogen: 3.78 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig. 91		Chitosan		Glutamate	
Reaction time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	2.81	74.3	1.86	42.9	1.8	47.6	1.88	49.7	2.98	78.8
3.0hr	2.19	57.9	1.14	30.2	0.871	23.0	0.818	21.6	2.32	61.4
6hr	1.49	39.4	0.445	11.8	0.253	6.7	0.176	4.7	1.44	38.1

Table 2-d. Change of nitrogen concentration with time-courses. removal agent: 50 mg/5ml *concentration of nitrogen initial concentration of nitrogen: 3.78 ppm **residual ratio of nitrogen

	Glucosamine		Olig. 7		Olig.91		Chitosan		Glutamate	
Reaction time	* C ppm	**.ratio %	* .C ppm	**.ratio %	* C ppm	**ratio %	* C ppm	**ratio %	* C ppm	**ratio %
1.5hr	2.73	72.2	0.978	25.9	0.638	0.6	0.28	7.4	2.63	69.6
3.0hr	1.87	49.5	0.279	7.4	0.14	0.1	0.122	3.2	1.85	48.9
6hr	0.982	26.0	0.336	8.9	0.24	0.2	0.0973	2.6	0.943	24.9

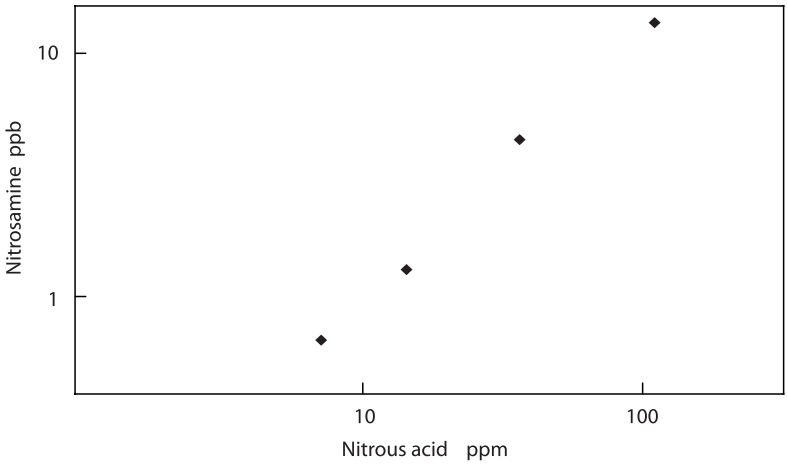

Figure 1. The relationship between the concentration of nitrosamine and nitrous acid in reaction medium

Table 2 shows the change of nitrogen concentration with time-courses under lower concentration of nitrogen (3–4 ppm: which is almost similar level in human stomach). In these cases, the RRNs decreased by natural amino compounds. Among them, chitosan, olig. DP 7, and DP 91 were excellent for removal agents of nitrogen., When the volume in stomach are settled to ca. 1,000 mL, the amount of the removal agent (10 mg/5mL) corresponds to 2 g under the conditions in Table 2-b. After 6 hours, chitosan consumed the amount of nitrogen 3.9 folds more than sodium glutamate. In the absence of scavenger, the concentration of nitrogen decreased to 12.8%. Fig. 1 shows the correlation between the concentration of nitrosamine and that of nitrous acid in reaction medium. Each value indicates the initial nitrogen concentration of the nitrous acid. From the results in Fig. 1, the concentration of nitrous acid was proportional to that of nitrosamine. Therefore, the amount of carcinogenesis, which is a dangerous material for human body, is also proportional to that of nitrous acid. Since chitosan is most effective scavenger of nitrous acid, it would be useful for the inhibitor of cancer promoting factor in foods.

It has been reported that the cancer disease in vivo was much associated with amino acid [4], since amino acid is guessed on the consumption of nitrous acid in vivo. Fig. 2 shows the reaction mechanism of chitosan, glucosamine, and sodium glutamate with nitrous acid under acidic condition. The primary amino group in both chitosan and glucosamine reacts with nitrous acid and produce 2,5-anhydro-D-mannose (reaction 1and 2) [5]. In the case of sodium glutamate, the reaction with nitrous acid would proceed as reaction 3. The consumption rate of nitrous acid would be much associated with the rate of each reaction 1–3. From the results described here, the reaction 1 by chitosan was fastest among all amino compounds used in this study. Although the reason for the fast reaction in chitosan is not clear at the present stage, the simultaneous hydrolysis of glycoside bonds will be an effective factor on the acceleration of the reaction by nitrous acid.

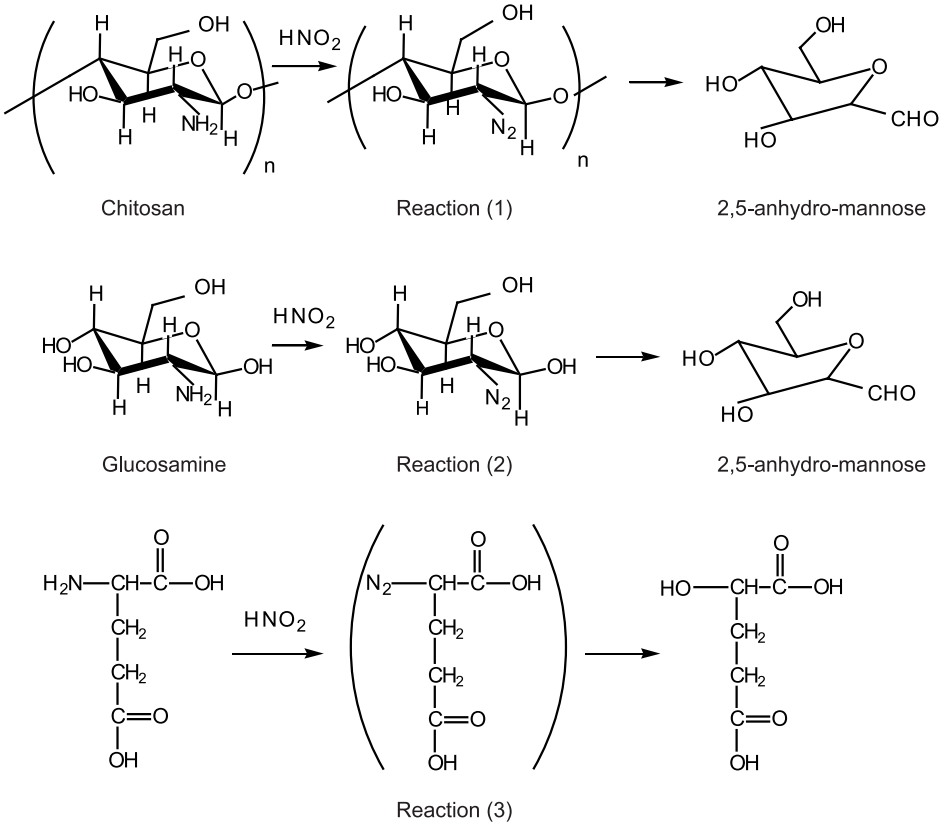

Figure 2. The reaction of amino compounds with nitrous acid

In general, most of plants and vegetables include nitrates, which is a source of nitrous acid. On the other hand, dimethylamine is included in most of fishes. In addition, several kinds of dialkylamines and monophenols are contained in other foods. By the action of nitrous acid, dialkylamines and monophenols in food are converted to nitrosamines and diazoquinones, which are caused by carcinogenesis or mutagenesis, respectively [6–8]. To maintain the human health care, the effective scavenger of nitrous acid in food is required. In this study, chitosan was most suitable scavenger of nitrous acid under artificial gastric conditions. As a future subject, the effect of chitosan on the prevention of carcinogenesis/mutagenesis preventive effect will be studied in vitro or in vivo.

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HEXOSAMINIDASE ACTIVITY IN THE PLASMA OF KOREANS

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INTRODUCTION

Increased activity of plasma *β-N*-hexosaminidases (Hex) is found in hyperthyroid individuals [1], infants aged less than 12 months [2, 3] and cancer patients [4, 5], although there is a general lack of fluctuation in total Hex activity relative to sex and age in normal human serum and plasma. In this experiment we measured the Hex activity of 1,835 plasma samples using 4-methyl-umbelliferyl-*N*-acetylglucosamine as substrate. The mean value and standard deviation (sd) of the samples was 1,914 ± 1040 μU/ml. The value of 50 female samples and 38 male samples was above 5,034 μU/ml, which exceeded the mean + 3sd value. Although their etiology is under investigation, about 40% of 50 female samples are linked with pregnancy.

EXPERIMENTALS

Samples

Plasma samples of in- or outpatients collected in clinical pathology department, Ajou University Hospital were kept at 4°C until use.

Hex activity assay

The fluorogenic substrate, 4-methylumbelliferyl-*N*-acetylglucosamine(MU-GlcNAc) was used as substrate. Assays were performed using black 96-well microtitre plates [6]. Fluorescence was monitored (Excitation 355 nm, Emission 460 nm) using a microtitre plate fluorometer (Fluoroskan II). The sample was added individually to the wells of a microtitre plate, containing 80 μl McIlvaine buffer (pH 4.0, 0.1 M) and 5 μl of the appropriate fluorogenic substrate at a concentration of 0.8 mM. The plate was placed in a shaking incubator at 40°C and reading was recorded over a 3-hour period. The reaction was terminated by addition of 100 μl of 1 M glycine/NaOH (pH 10.6) and fluorescence was read 10 min later. 1 μunit(μU) is defined as 0.1nmoles of released 4-MU per min/ml plasma.

RESULTS AND DISCUSSION

Optimization of Hex activity assay

As seen Fig. 1a, the optimum pH of plasma Hex was 4.0. This optimum was in good agreement with others [1–4]. Fig. 1b showed that samples of higher Hex activity already reached maximum value only after one- hour reaction time and that those of middle and lower activity were linear for at least 2-hour incubation. As there was a high variation of Hex activity among the samples, it was needed to optimize and standardize the assay protocol (Fig. 1b).

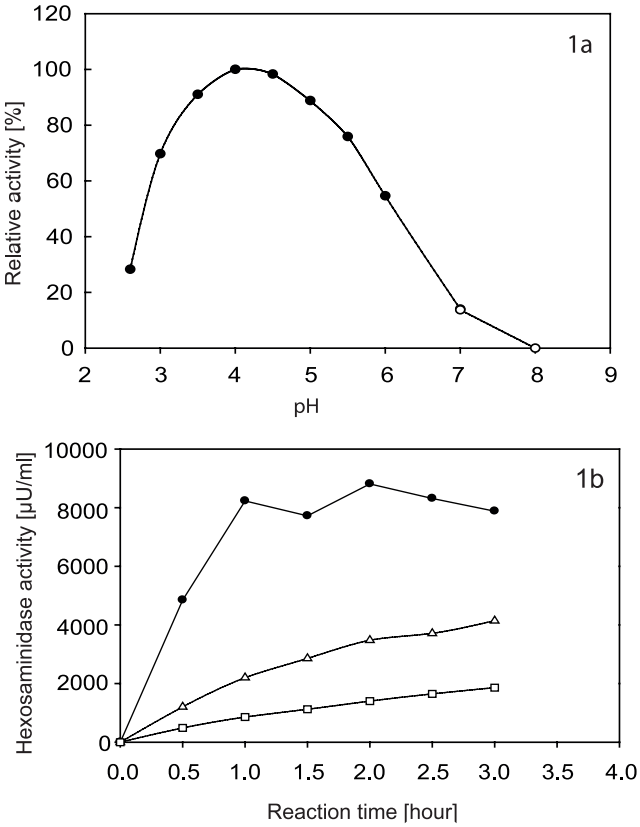

Figure 1. Effects of pH, incubation time and samples on Hex activity
1a: Effect of pH. pH 2.6–7.0: Citrate-phosphate buffer; pH 7.0–8.0: Phosphate buffer
1b: Effect of incubation time with different Hex activity samples.
— ● —: Higher Hex activity sample, — △ —: Middle Hex activity sample,
— □ —: Lower Hex activity sample

The incubation time of 1 hour was chosen for all samples, regardless of the fluctuation of Hex activity.

Distribution of Hex activity

As seen Fig. 2, the mean value and standard deviation of 1850 plasma samples was 1,914 ± 1,040 µU/ml. This means that there is a high variation of Hex activity among the samples.

50 female samples and 38 male samples showed a value higher than 5,034 µU/

ml, the mean + 3sd value. 40% of 50 female samples are linked with pregnancy and 12% are related to diabetes. In male samples, 21% are suffering from liver problems and 13% are diagnosed to be diabetes and to have kidney problems, respectively. The higher values both in diabetes and renal problems are very interesting, because the renal excretion of HEX decreased in diabetes and normal renal function [7].

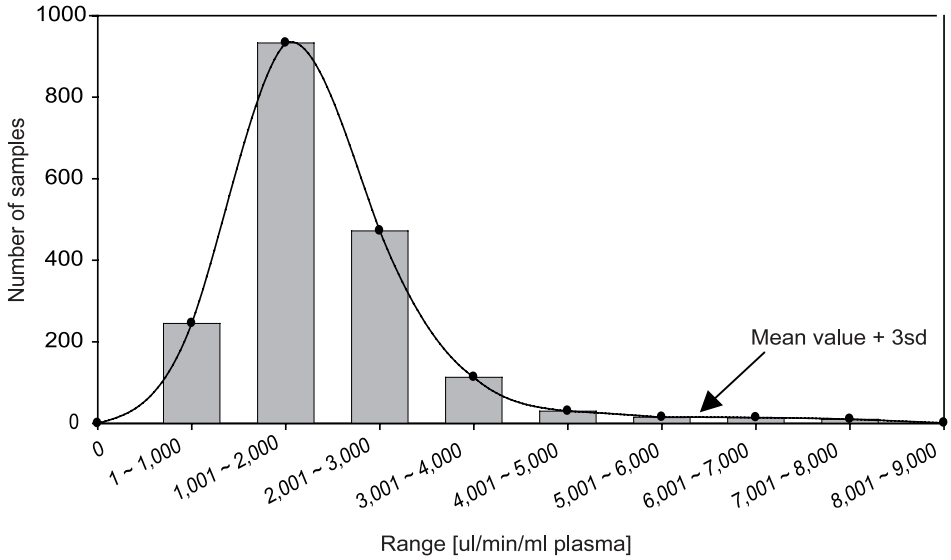

Figure 2. Distribution of Hex activity Total number of samples was 1,835. The arrow indicated Mean value + 3 sd

CONCLUSIONS

We measured the activity of *β-N*-acetylhexosaminidase in 1835 samples of human plasma. The mean value and standard deviation (sd) of Hex activity was 1,914 ± 1040 μU/ml. The activity of 50 female samples and 38 male samples was above 5,034 μU/ml, which exceeded the mean +3sd value. Although their etiology is under investigation, about 40% of 50 female samples are linked with pregnancy. In male samples, 21% are suffering from liver problems and 13% are diagnosed to be diabetes and to have kidney problems, respectively.

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