SITE-SPECIFIC DRUG DELIVERY USING CHITOSAN MICROPARTICLES

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Abstract:

Chitosan (CS) is gaining increasing importance in the field of drug controlled release owing to its good biocompatibility, non-toxicity and biodegradability[1]. Recently, CS has also been shown to have mucoadhesive properties[2,3] and to enhance the penetration of macromolecules across the intestinal and nasal barriers[3,4]. These properties have encouraged greater prospects for its use in the oral and nasal administration of proteins and peptides. Furthermore, CS has been found to be a promising carrier for colon-specific drug delivery[5].

From a technological point of view, CS has unique properties which makes it an excellent material in microencapsulation technologies. In spite of this, only a few articles on this specific area have been published so far. Four main approaches have been proposed for the preparation of CS microparticles: (i) ionotropic gelation with an opposite charged polyelectrolyte, such as sodium tripolyphosphate or alginate[6], (ii) simple or complex coacervation[7,8]; (iii) spray-drying[9] and (iv) solvent evaporation[10]. The main limitation to all these procedures is that the microspheres obtained are unable to control the release of the microencapsulated compound after their oral administration.

Our objective was to develop two new types of microparticulate controlled release systems based on CS to achieve site-specific delivery of drugs following oral administration. With both systems, our aim was to overcome the problem due to the high solubility of CS in the acidic pH of the stomach while avoiding chemical crosslinking with aldehydes.

Key words:

Chitosan, Drug delivery, Eudragit®, Microencapsulation, Oral administration

Materials and methods:

Materials:

The following chemicals were obtained from commercial suppliers and used as received: chitosan glutamate (150 and 350 KD for Sea cure®G110 and Sea cure®G210), chitosan (50, 150 and 300 KD and degree of deacetylation was > 80%, for Sea cure®123, 223 and 320) (*Pronova Lab., Drammer, Norway*); Eudragit®S-100 and Eudragit®L-100 (*Rohm Pharma, Germany*); amoxycillin, sodium diclofenac, liquid paraffin, cottonseed oil (*Sigma Chimie, Madrid, Spain*); Span®85, Tween®80, Antifoam®A (*Fluka Química, Madrid, Spain*); analytical grade reagents and Milli-Q water (*Milli-Q Plus, Millipore Iberica, Madrid, Spain*) were used throughout.

Preparation of reacetylated chitosan/amoxycillin microspheres:

Amoxycillin (AM) loaded reacetylated CS microspheres were prepared by an emulsification followed by solvent evaporation/extraction method.The inner phase (10 ml of 1% w/v CS solution in acetic:acetone 1:1, containing 15% w/w of AM based on polymer) was emulsified into the continuous phase (150 g of cottonseed oil, containing 0.1% Span® 85) under mechanical stirring (500 rpm) (ratio of inner phase to continuous phase = 1:15). Afterwards, the solvent of the inner phase was first evaporated at room temperature; and then, 300 ml of ethylene acetate were added to extract the remaining solvent. Finally, 25 ml of 98% acetic anhydride were added and after a prefixed reacetylation time, the microspheres were isolated by vacuum filtration, washed and freeze-dried (-30°C, 24hr; *Labcomcon apparatus, Labcomco Corporation, Kansas City, MI*).

Preparation of pH-sensitive chitosan/Eudragit® microspheres:

CS core-microspheres were obtained by a spray-drying technique and then microencapsulated within an enteric polymer.

Polysaccharide solutions were prepared by dissolving CS glutamate in water and CS in diluted acidic solution (0.5 M acetic acid) at room temperature. Sodium diclofenac (SD) (5% w/w based on polymer) was dissolved in 2 ml of methanol and added to the CS solution. Solutions (100 g) were spray-dried (*Buchi Mini Spray Dryer, type 190, Switzerland*) with a 0.3 mm nozzle at a feed rate of 10 ml/min. The nozzle air pressure was 400-500 Nl/h and the inlet temperature 170°C. Finally, the product obtained was freeze-dried.

The CS core-microspheres were dispersed in 5 ml of acetone:methanol (ratio 2:1) in which Eudragit® (S-100 or L-100) was previously dissolved to give a 1:5 coat/core ratio. This organic phase was then poured into 70 ml of liquid paraffin containing Span®85 and Antifoam®A (1% and 0.1% w/w, respectively). The system was maintained under agitation (*Janke & Kanke model RW 20*; 100 rpm) at room temperature for 3hr to allow the evaporation of the solvent. Finally, the microparticles were collected, rinsed with n-hexane and freeze-dried (-30°C, 24hr).

Morphological characterization of microspheres:

The morphology, surface appearance and inner structure of the microspheres were examined by scanning electron microscopy (SEM). The particles were coated with gold palladium to achieve a film of 20 nm thickness (*Sputter coater, Balzers SCD 004, Liechtenstein*) and observed microscopically (*SEM, JSM-6400, Tokyo, Japan*). For the observation of the inner structure, particles were dispersed in water, frozen and sectioned using an ultramicrotome (*Cryocut 1800 Reichert-Jung*).

The particle size of CS microspheres was directly measured in an optical microscope. The size of CS/Eudragit® microspheres was determined using a Counter Coulter® Multisizer II system (*Coulter electronics, Luton, England*).

Evaluation of in vitro drug release:

The evaluation of in vitro drug release from CS uncoated microspheres was performed by the dialysis technique in either artificial gastric juice (pH=1,2) (AM loaded microspheres) or pH=7.4 phosphate buffer (SD loaded microspheres) (250 ml, 50 rpm, 37°C). At desired times, the samples were withdrawn, filtered (0.2 um NY filters) (*Lida Manufacturing Corp., kenosha, WI*) and assayed for drug release by measuring absorbance at 229 nm (AM) and 276 nm (SD).

Assessment of SD release from CS core-coated microspheres was performed by incubating the microspheres in 1ml isotonic phosphate buffer containing 0.02% w/v polysorbate 80, at 37°C in a horizontal shaker (*Heidolf promax 2020, Germany*) (n=3). The pH of the release medium was gradually increased from 5.8 to 7.4. At specific time intervals, the microspheres dispersions were centrifuged (*Sigma, model 2-15, Madrid, Spain*) at 2,500 g for 5 min, and the supernatants filtered and assayed for drug released by measuring its absorbance at 276 nm.

Results and discussion:

Reacetylated chitosan/amoxycillin microspheres:

The first system developed consisted of AM-containing reacetylated CS microspheres, which were obtained by an emulsification-and-solvent evaporation/ extraction method. Several studies have been focused on the utilization of solvent evaporation methods to obtain microspheres of CS containing teofiline, 5-fluouracil, oxantrazole or cisplatin[11-16]. Independent of the particularities of each technique, a common restriction to all is the limited capacity of the microspheres produced to control the release of the encapsulated compound following oral administration, and the necessity of a further cross-linking process in order to avoid their rapid dissolution in the stomach. This is due to the presence of free amino groups in the CS molecule which become ionized in the acidic media leading to the almost immediate dissolution of the polymer. Covalent crosslinking with aldehydes such as glutaraldehyde has been, so far, a way of overcoming this problem[12-14,16]. Nevertheless, the toxicity of aldehydes enormously limits the exploitation of the crosslinked microspheres. Furthermore, this crosslinking process is not totally effective in preventing the release of the encapsulated compound.

With respect to this, the main advantage of our method is that it allows the reacetylation "in situ" of microspheres to form a reacetylated CS layer on their surface which will control the water intake and gelification of CS at acidic pH; and, as a consequence, the adhesion to the gastric mucosa and the antibiotic release.

Several formulations were prepared which differed in the reacetylation period of time and in the CS type. All the microspheres obtained were spherical, had good flow properties and a particle size less than 100 μm (*figure 1*).

The effect of the molecular weight (Mw) and type of CS salt, as well as the reacetylation time, and hence[17] degree of CS reacetylation, on drug release was evaluated in experiments conducted at several pHs. Results showed that the release rates of the microspheres prepared by the different conditions were

slowed down at varying degrees. As an example, *figure 2* shows the release profiles of two unreacetylated formulations prepared using CS glutamate (Sea cure G110) and CS acetate (Sea cure 123 in acetic acid) in pH 1.2. On the other hand, *figure 3* depicts the effect of the reacetylation time (0, 2, 6 and 18 hr) on AM release from CS microspheres in pH=1.2.

The results of this study suggested that CS was an appropriate carrier for AM-microsheres preparation, and that it was possible to modify the drug release rate by very simple reacetylation surface treatment. This system is proposed for the treatment of gastric ulcers caused by "helycobacter pylori". Nowadays, the therapeutic treatment of this pathology implies the administration of very high doses of antibiotics several times per day. Therefore, the development of a dosage form, which can be administered by the oral route and adhere to the stomach mucosa, gradually releasing antibiotics at this level, might be meaningful from the standpoint of the quality of life of the patients. At this respect it is interesting to point out that the CS important antacid and antiulcer effects could act as coadjuvant, contributing to the dose of drug decrease[18,19].

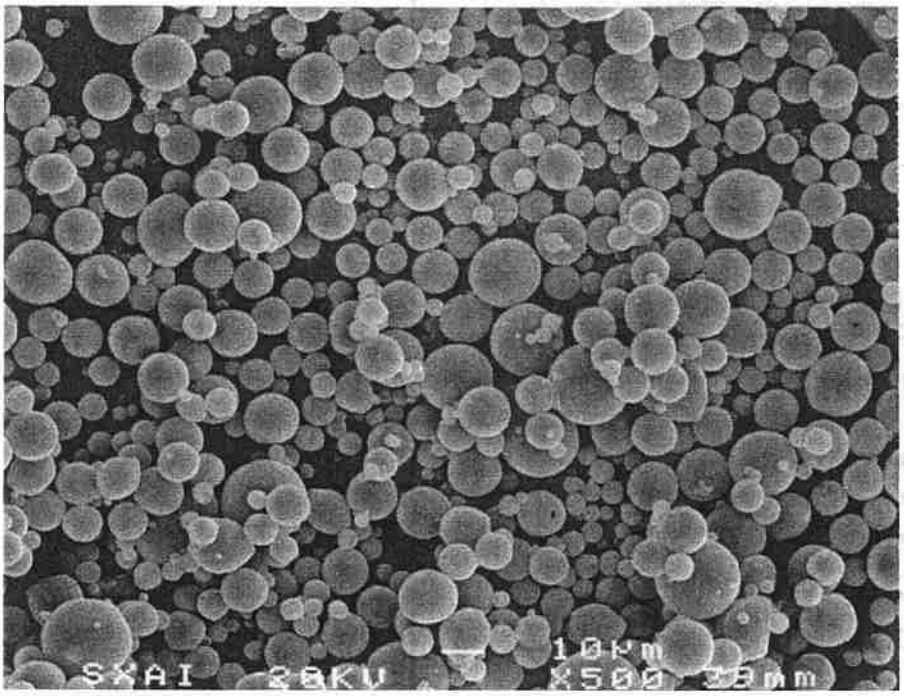

Fig.1. Microphotograph of chitosan-amoxycillin microspheres prepared by the emulsification-solvent evaporation/extraction method

pH-sensitive microspheres:

On the other hand, we have developed a new pH-sensitive colonic multicore micropaticulate system which combines biodegradability by the colonic bacteria and pH dependent release. It consist of mucoadhesive CS microcores entrapped

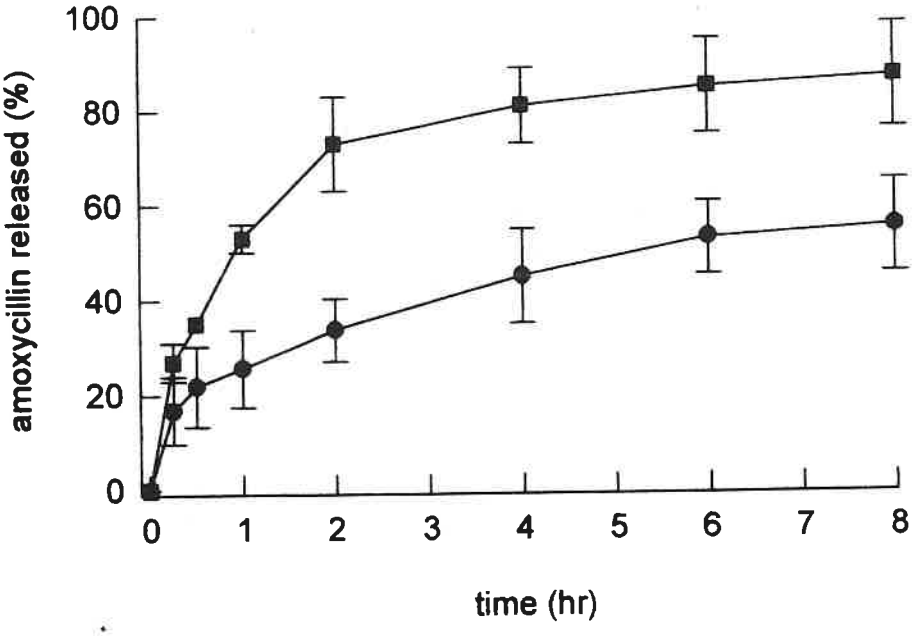

Fig. 2. Amoxycillin release from untreated chitosan microspheres. Key: (■) Sea cure G110 and (●) Sea cure 323.

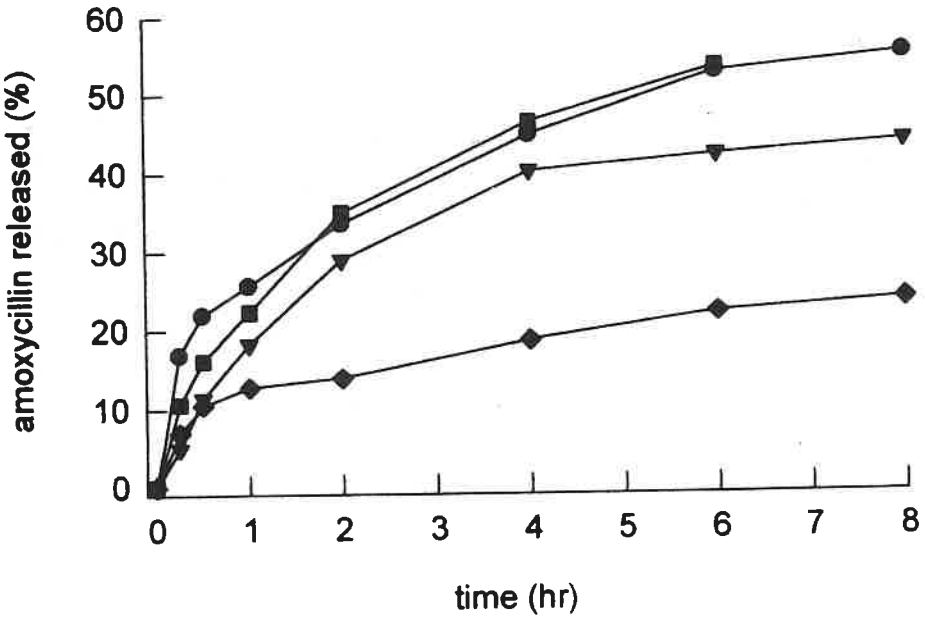

Fig.3. Effect of the reacetylation treatment on amoxycillin release in pH=1.2. Key: (■) 0 hr - untreated; (●) 2 hr; (▼) 6 hr and (◆) 18 hr (Sea cure 320).

within enteric microspheres made of acrylic polymers (Eudragit®). The CS microcores (size = 1.8 to 2.9 μm) were prepared by a spray-drying technique and then microencapsulated into Eudragit®L and Eudragit®S (size: 152-223 μm) using an oil-in-oil solvent evaporation method, obtaining a multireservoir system. SD was used as drug model compound. It was expected that this drug could benefit from the proposed system; since it was assumed that both the Eudragit® polymer coating and the CS would protect the compound entrapped in the CS cores as it passes through the acidic and enzymatic environment of the stomach, allowing release to the colon. On the other hand, the special advantage of the CS particles for anti-inflammatory drugs relies on their ability to weaken the gastro-intestinal side-effects caused by these drugs. This ability is not only related to the prolonged release but also to the well-known antacid and anti-ulcer effects of CS. Moreover, SD is particularly well absorbed in the colon. As it is shown in *figure 4*, SD release from CS microcores was adjustable by changing the CS molecular weight and type of CS salt.

Furthermore, by coating the CS microcores with Eudragit®, perfect pH-dependence release profiles were attained. No release was observed at acidic pHs. However when reaching the Eudragit® pH solubility, a continuous fast release for a variable time (8-12 hr) was achieved. This continuous release was found to be highly dependent upon the type of Eudragit® (*figure 5*). We propose a combined mechanism of release, which considers the dissolution of the acrylic coating, the swelling of the CS microcores and the dissolution of SD and its further diffusion through the CS gel cores, as well as Cs degradation in the colonic region.

Conclusions:

Two types of CS microparticulate systems were developed which are proposed to achieve site specific drug delivery in the gastric cavity and intestinal tract, respectively, after oral administration. With the first system, the reacetylation of the CS microspheres surface allows to control the drug release rate. With the second, by coating the CS-microcore microspheres with pH-sensitive acrylic polymers, it is possible the control of the region of the intestine where the coating polymer will be dissolved and the drug released.

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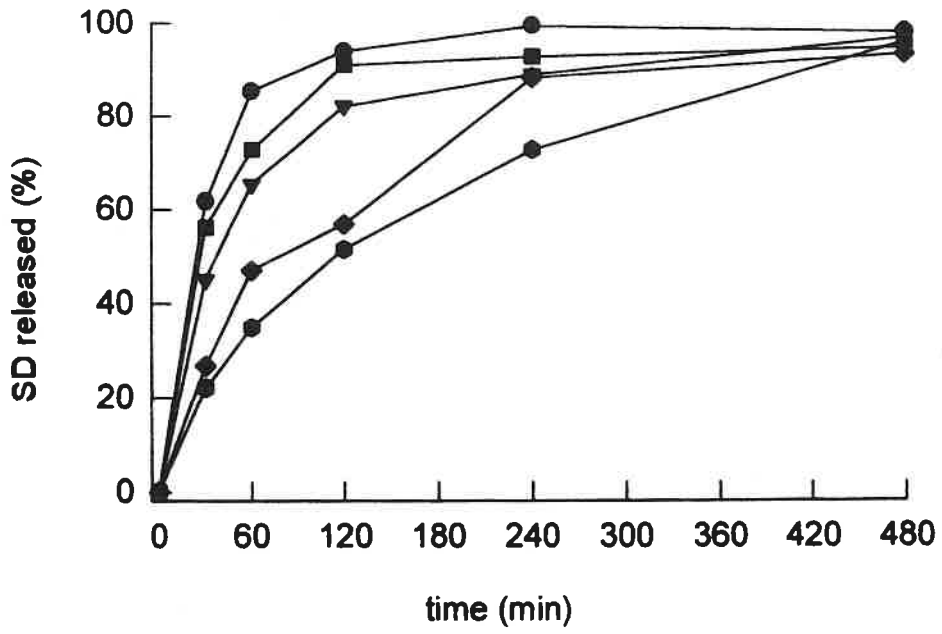

Fig. 4. Effect of chitosan molecular weight and type of salt on sodium diclofenac release. Key: (●) Sea cure 123; (■) Sea cure 223; (▼) Sea cure 320; (◆) Sea cure G1120 and (●) Sea cure G210.

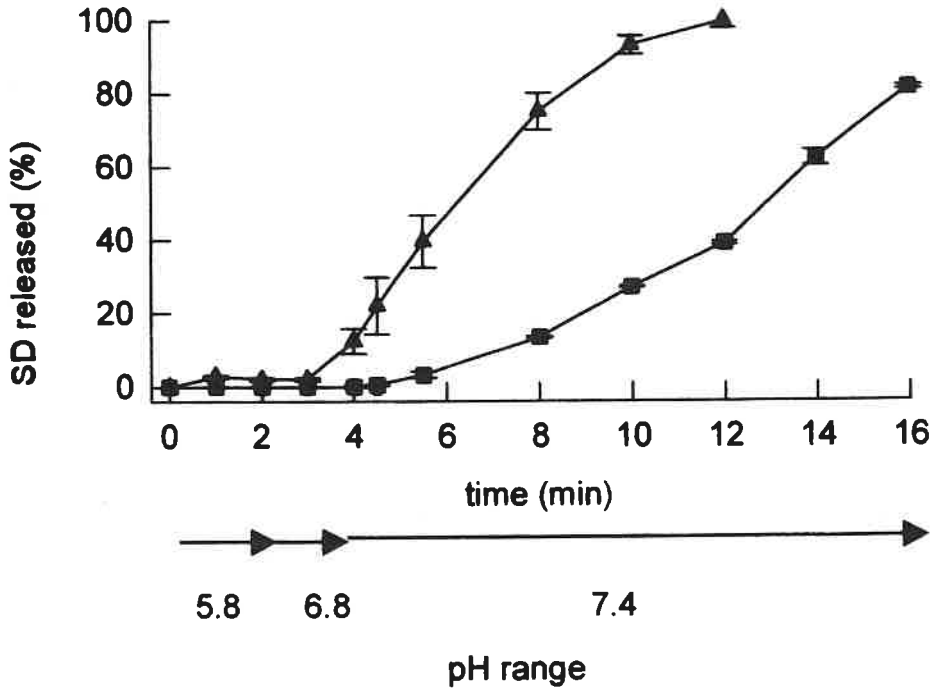

Fig.5. Effect of pH on sodium diclofenac release from chitosan/ Eudragit® microspheres as a function of core/coat ratio and type of Eudragit®. Key: (▲) Eudragit® L and (■) Eudragit® S.

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Chitin Foam and Chitosan Gel as a Carrier of Controlled Release of Drug

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Abstract

A chitin foam included various dyes was obtained by lyophilization of chitin gel which was prepared by decalcination of cast chitin dope, since a mild solvent, calcium chloride dihydrate saturated methanol, was found for chitin. Anionic dyes were adsorbed tightly to chitin foam in which little difference between carboxylic acid and sulfonic acid group. Nitro and hydroxyl groups became to contribute the tight inclusion of model drugs into polymeric micelle in addition to sulfonic group, but few influence by carboxyl group, when dicarboxylic PEG biscarboxylate was applied to N-acylation of chitosan. As both polymers are susceptible for lysozyme, the controlled release of model drug would be expected with use these polymers as drug carrier.

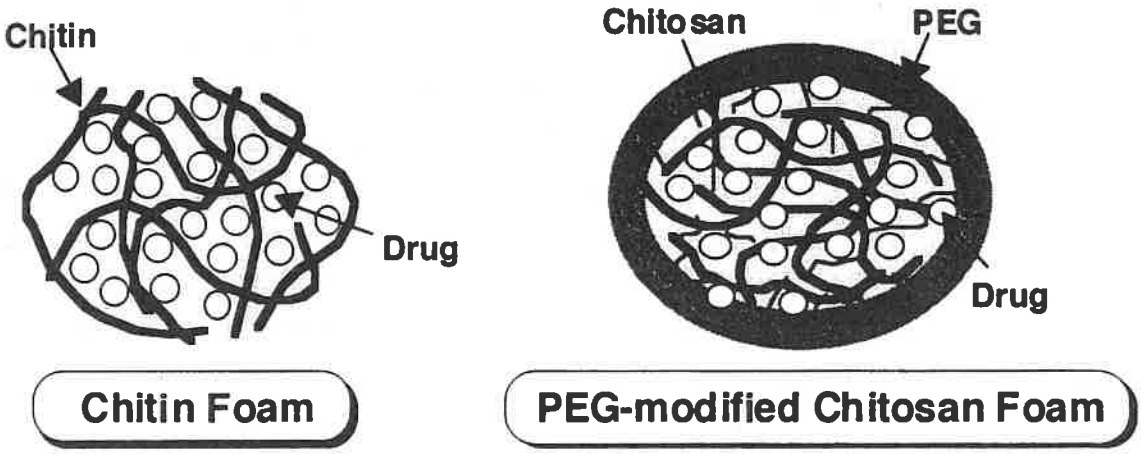
Keywords : controlled release of drug, PEG-acylated chitosan, Tight inclusion of anionic drug, Functional groups to attribute drug inclusion

Introduction

Though chemical modifications are expected to give high solubility, an immunogenicity might become serious problem on the application for biomedical materials.

In this report, new solvent system for chitin and the formation of polymeric micelle through the acylation of chitosan

by nontoxic polyethylene glycol biscarboxylate(PEG-BC) were investigated to apply for the carrier of drug delivery system. Chitin was firstly regenerated into porous foam included with model drug and the hydrogel of PEG acylated chitosan was prepared with inclusion of model drugs followed by lyophilization. The tight inclusions of model drugs were shown on chitin foam with some variety on functional group of drug, but narrow inclusion specificity was shown for N-acylated- PEG-chitosan probably due to a high concentration of chitin in the center of micelle structure.



Scheme 1. Models of chitin Foam and PEG-chitosan Foam

Materials and Methods

Materials: Chemicals and solvents were purchased from Wako Chemical Co. Ltd. and used without purification. Chitin was prepared from Queen crab shells according to the methods of Hackman(1). Chitosan of 98 % deacetylated was prepared twice autoclave treatments of chitin powder in 40% (w/v) sodium hydroxide aqueous solution each for 2 hrs.

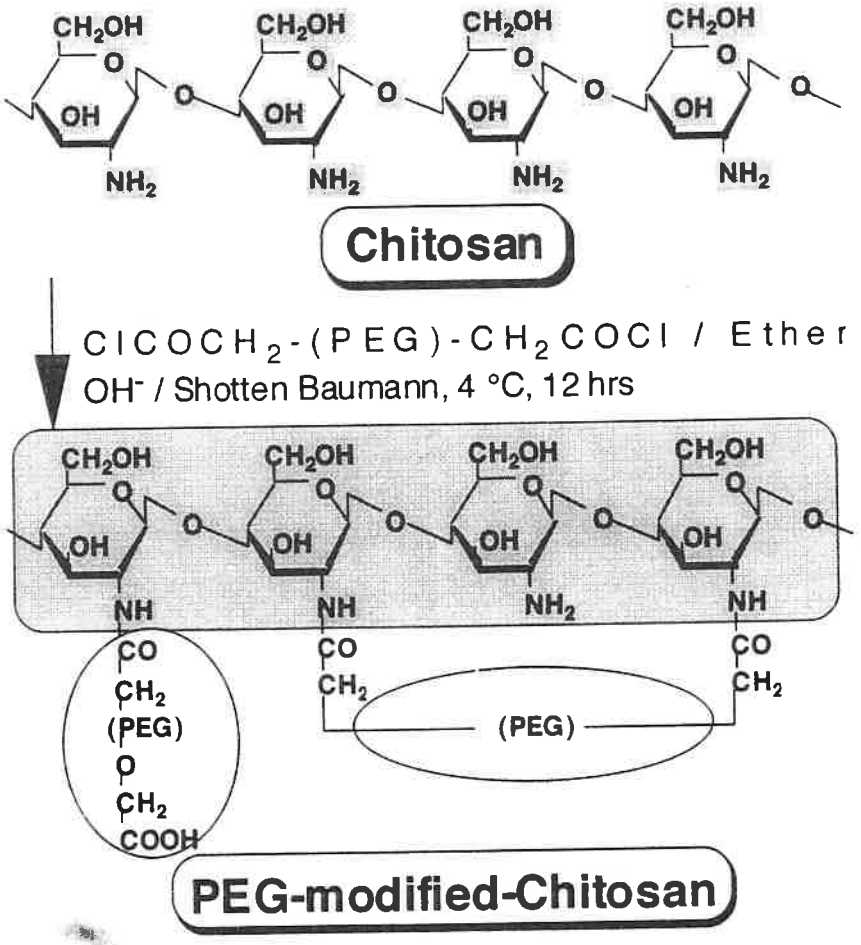
Solvent and preparation of chitin foam: Calcium chloride dihydrate (270 g) was suspended in 300 ml of methanol and refluxed for 30 min. Insoluble part was removed to prepare a calcium chloride saturated methanol at room temperature. Chitin fine powder (1 g) was suspended in 100 ml of solvent and then refluxed for 1 hr to dissolve(2). The insoluble material was removed by filtration through flannel. The chitin foam was cast on glass plate at room temperature following to mixing with model drug aqueous solution and then decalcinated by immersing into 1%

(w/v) sodium citrate aqueous solution with gentle stirring for 3 hrs at room temperature followed by rinse with deionized water. Decalcinated gels with various model drugs were lyophilized to prepare porous foam(3). The amount of model drug in chitin foam was estimated spectrometrically after equilibrate with constant volume of water.

Modification of chitosan by PEG biscarboxylate: PEG(Mw 600) biscarboxyl chlorides was prepared by reacting thionyl chloride with PEG biscarboxymethyl ether in chloroform (100 ml) under refluxing at 70 °C for 5 hrs. Then chloroform and unreacted materials were removed by distillation to give PEG biscarboxyl chloride(PEG-BC-Cl). Chitosan gel of fine particle was prepared by precipitating with dropwise addition of sodium bicarbonate (10 g) methanol solution (1 L) into chitosan (3 g) in 2% (w/v) acetic acid aqueous solution (100 ml). The volume of chitosan gel was adjusted to 600 ml by water after extensive rinse to remove methanol and salts. The aqueous chitosan gel was reacted with PEG-BC-Cl ether solution at 5 °C and pH 7 under vigorous stirring for 1.5 hrs and then for another 12 hrs at 5 °C under stirring(4). (Scheme 2.) The reaction product of gel was rinsed with deionized water and ethanol repeatedly to removed impurity. The product was lyophilized after several washes with deionized water (Yield 0.9 g). The structure of the product was confirmed by IR spectroscopy and estimated quantitatively by elemental analysis.

Inclusion of model drug: The inclusion of model drug was achieved by immersing the lyophilized water-insoluble fraction (20 mg) into 45 ml of aqueous solution of dye (0.1 mg/ml) under diminished pressure at room temperature for 30 min. The immersion procedure was repeated three times following to release of pressure and then the gel was rinsed with deionized water several times to remove free dye followed by lyophilization to give porous foam of chitin derivative for drug delivery system.

Controlled release of model drug from chitin gel: The rate of extraction was measured time dependently according to the procedures of Japan pharmacopoeia (5). The dye included foam (20 mg) was placed in 1 L of physiological salt solution under stirring at 100 rpm and room temperature. The concentration of the dye extracted was measured spectrophotometically.



Scheme 2. Preparation of PEG-modified-Chitosan

Results and Discussion

A new solvent system for chitin has been studied to regenerate the chitin porous foam under mild condition for the controlled release of model drugs. An acylated chitosan by PEG bis-carboxylate was also prepared for the carrier of controlled release of drug in which PEG biscarboxylate participated as a cross-linker and a hydrophilic branch. The PEG acylated chitosan was easy to become hydrogel with adsorption of water probably by the formation of polymeric micelle.

Cationic model drugs were little adsorbed both into chitin foam and chitosan hydrogel, when both polymers were included by model drug in aqueous solution. Monoanionic dyes were adsorbed slightly for chitin foam (Figure 1.), but almost completely not for chitosan hydrogel (Figure 2.).

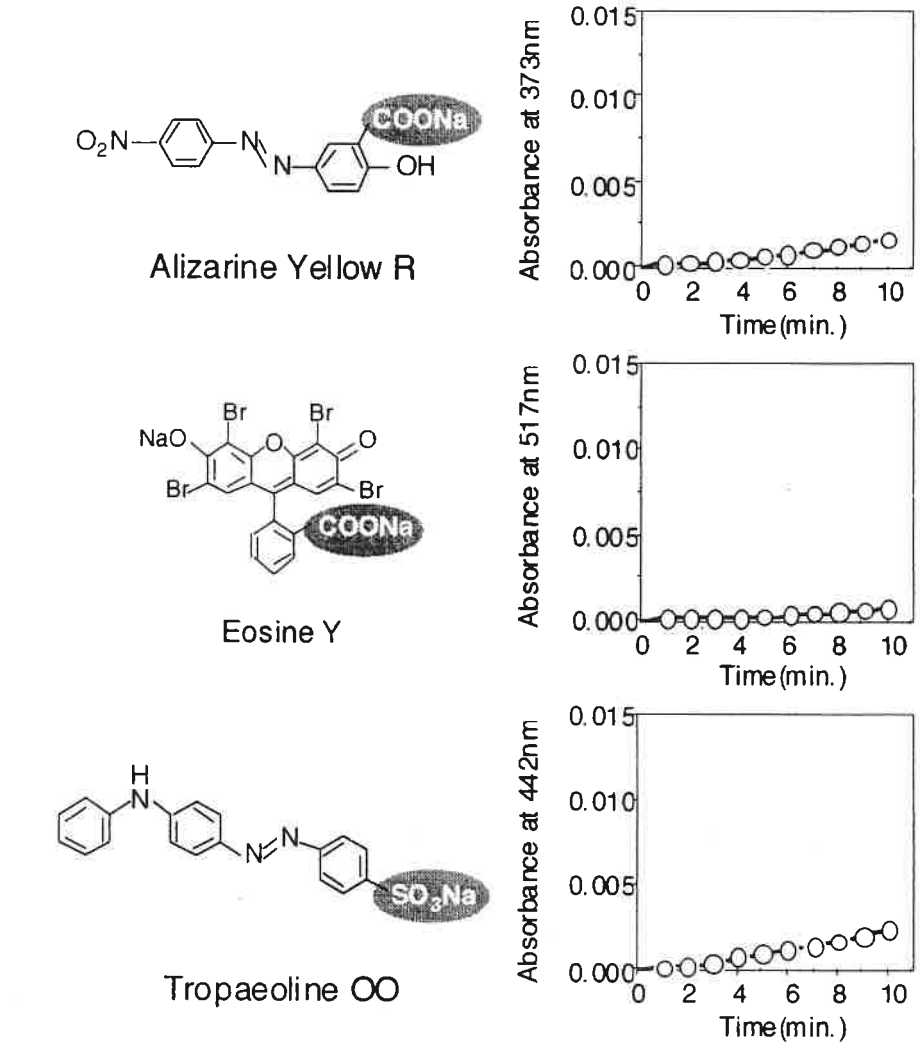


Figure 1. Effect of Acidity for Chin Foam

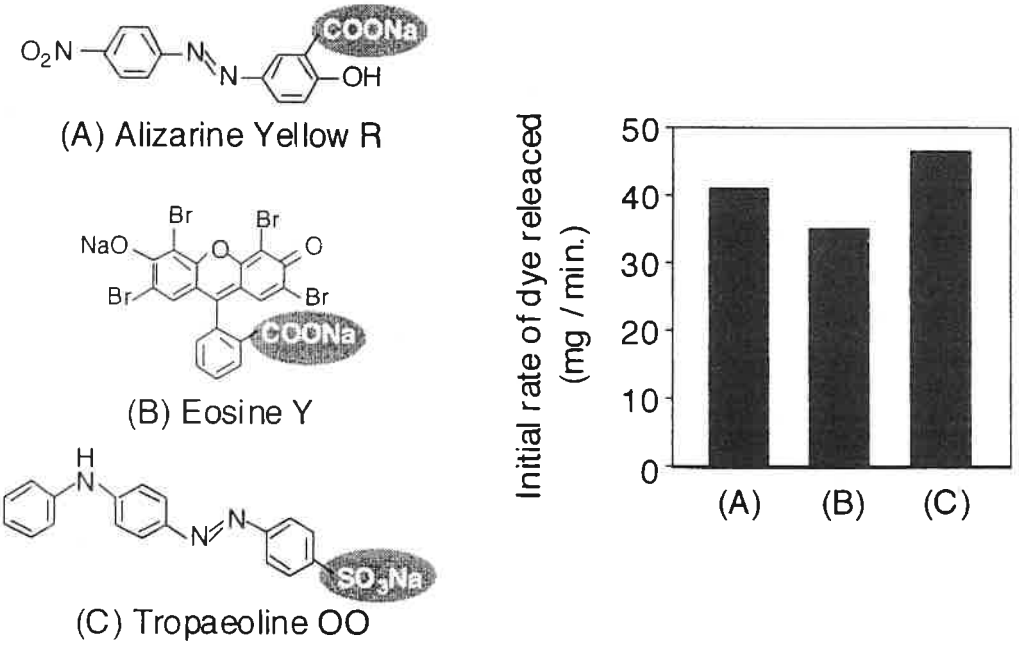


Figure 2. Effect of acidity for PEG-Chitosan Foam

Dianionic dyes, showed a tight adsorption ability to chitin foam when there was sulfonyl groups (Figure 3.).

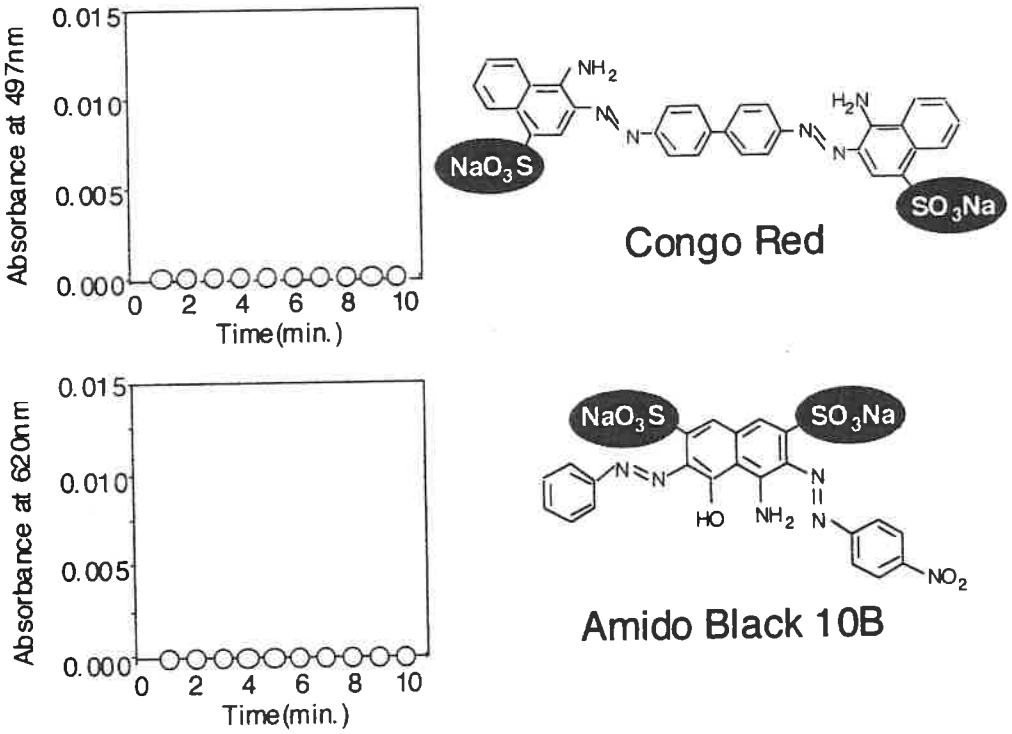


Figure 3. Dianionic dyes adsorption of Chitin Foam

There was little influence by the distance between sulfonyl groups and also by nitro group on the dye molecule. But a remarkable influence of distance between sulfonyl groups was shown by PEG-chitosan foam (Figure 4.).

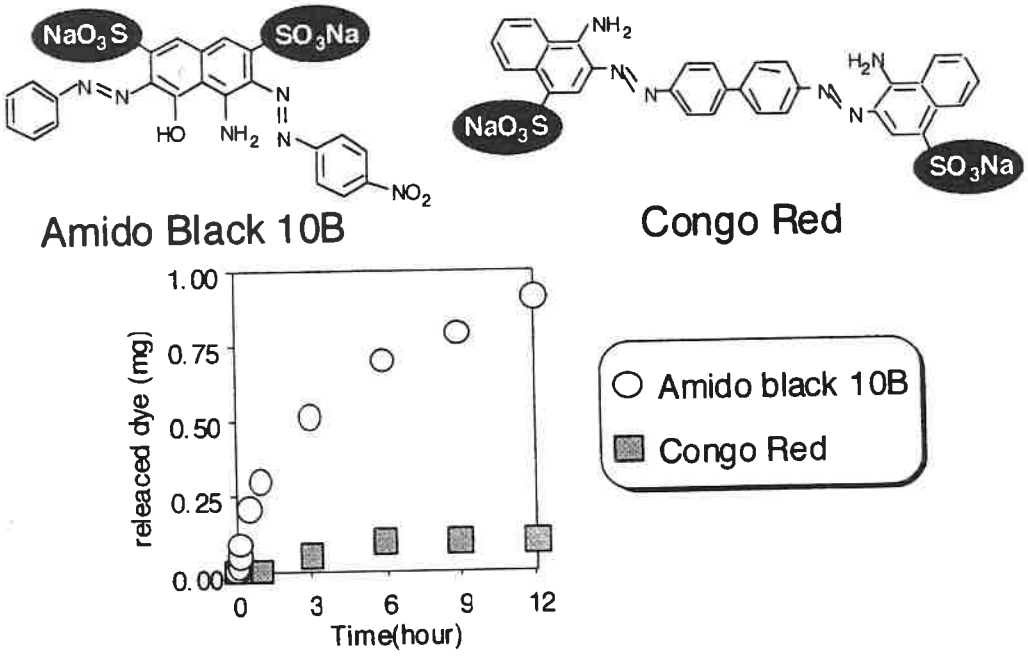


Figure 4. Distance effects of sulfonyl groups for PEG-Chitosan

The longer distance between sulfonyl groups induced the slower rate of drug release (become tighter adsorption).
Also there was serious influence was shown by nitro group on the adsorption of anionic dye to PEG-chitosan foam, in addition of a slight influence by hydroxyl groups (Figure 5.)

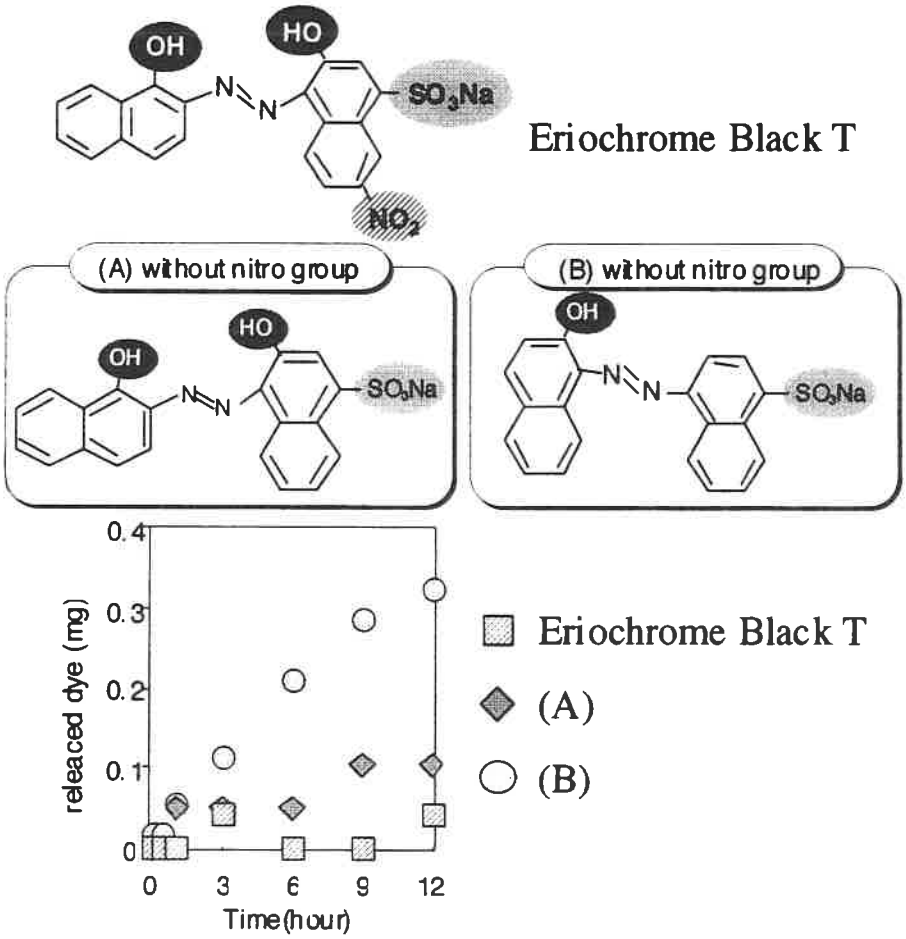


Figure 5. Effect of Hydroxyl and Nitro group

These phenomena are likely attributed to the grafting of hydrophilic PEG molecule to chitosan. As PEG was linked with amino group of chitosan through acetamide bond, the core part of chitosan gel is quite similar to that in chitin foam except packing effect of chitin molecules by the formation of polymeric micelle structure.

Conclusion

A chitin foam was prepared with various model drug applying new solvent system.

Only an anionic model drug was included tightly in chitin foam among various model drugs - little difference between carboxylic acid and sulfonic acid groups.Dianionic model drug (bis-sulfonyl groups) was adsorbed more tightly than those of monoanionic drug

The sulfonic group was the most effective factor to adsorb for PEG-chitosan foam.The nitro group was found to be additive factor to make tight adsorption for PEG chitosan in addition to the hydroxyl group.

The drug adsorption behavior of chitin was varied remarkably by the introduction of PEG-carboxylic group probably due to the formation of polymeric micelle structure.

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SOME ASPECTS ON THE CREATION OF CHITOSAN BIOACTIVITY

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ABSTRACT

A bioactivity of chitosan seems to be the most interesting behaviour of this natural polymer. The creation of chitosan bioactivity by formation of suitable controlled molecular, super - molecular and chemical structure defined by average molecular weight and its distribution, polydispersity, crystallinity or chemical substitutions for modified forms of chitosan in a paper is presented and discussed.

Keywords : chitosan forms, biodegradation, oligoaminosscharides, bioactivity creation, antimicrobial and antivirus activity, biostimulation.

INTRODUCTION

A bioactivity of chitosan seems to be the most interesting behaviour of this natural polymer. The important progress in research and development of this unique property for chitosan is observed during past years. Utilization of chitosan bioactivity, apart of medical applications mainly as the wound healing dressings (1 - 3), is going to be realized also on the ecological protection and biostimulation of plant growth as well as the modern medical uses (1 - 8).
The aim of this paper is to present some aspects on the creation of natural bioactivity of chitosan by modification of its molecular, super - molecular and chemical structure. Presented results are the part of research project realized by an author of this paper at the Institute of Chemical Fibres, Lodz, Poland.

MATERIALS AND METHODS

Two grades of initial chitosan produced by Vanson Co., USA with $\bar{M}v$ = 830.3 kD and DD= 85.6 % as well as by Sonat Co., Russia with

$\bar{M}v$ = 307.6 kD and DD= 89.2 % were used for preparation of microcrystalline chitosan. Microcrystalline chitosan with assumed molecular and super - molecular structure in a form of aqueous gel - like dispersion and powder according to the original method (9) was prepared. Sulfonated chitosan containing different amount of sulfonate groups on the base of liophilized microcrystalline chitosan with $\bar{M}v$= 332 kD, DD= 71% and WRV=170% was prepared according to modified recipe (10).
Degradation process of microcrystalline chitosan was carried out according to previously described method (11) . Sulfonated chitosan degradation process with precipitation of soluble derivative by ethyl alcohol saturated with sodium acetate was carried out in the similar conditions as for microcrystalline chitosan (12).
The bioactivity of tested chitosan forms for inhibition of plant virus infection, antibacterial and antifungal activity and biostimulation of lettuce seed germination was tested as previously described (4 - 7, 12).

RESULTS AND DISCUSSION

Microcrystalline chitosan used in these studies was prepared with assumed parameters of manufacture process in two forms as aqueous gel - like dispersion and powder (Table 1).

Table 1. Some properties of microcrystalline chitosan dispersion used

Symbol of MCCh	Form	Polymer content , wt%	$\bar{M}v$* ,kD	DD ,%	WRV ,%	CrI ,%
MKCh/2	gd	3.00	43.0	85.5	870	-
MKCh/SO3	gd	4.49	245.0	89.2	810	-
MKCh/V1	gd	3.14	480.0	85.6	920	-
MKCh/D3	p	91.8	143.0	90.0	990	44.7
MKCh/MK	p	89.2	208.0	70.0	210	-
MKCh/C	p	92.3	341.0	79.3	240	41.2

gd- gel - like dispersion, p - powder

A chemical modification of microcrystalline chitosan has resulted in preparation of sulfonated chitosan containing different amount of combined sulfur (Table 2).

Table 2. Some properties of sulfonated chitosan

Symbol of sample	Form and colour	Sulfur content ,wt%	Intrinsic viscosity ,g/100cm^3
S1	powder, white	6.05	0.41
S2	powder, white	8.10	0.44
S3	powder, white	11.00	0.42

All above microcrystalline chitosan and sulfonated chitosan were subjected for testing of their degradability for 28 days period, also in a presence of lysozyme with concentration of 400 μg/cm^3.

The results for determination of properties of residual polymeric materials as well as a content of oligoaminosaccharides, in calculation to aminoglucose, produced during degradation are presented in Tables 3 - 5.

The degradation process has affected on the changes of molecular characteristic of residual polymer. The polydispersity of residual polymeric material has been also related to a type of degradation process (Tabs. 1, 3-4).The statistical degradation seems to be a main process for hydrolitic system whereas the depolymerization as well as statistical destruction with a different ratio are existing parallely at the enzymatic system (Tabs. 3 - 4). A degradation of microcrystalline chitosan powder shown a similarity of mechanism with a different value of ratio for two existing processes of statistical destruction and depolymerization.

The degradation, both hydrolitic and enzymatic, of sulfonated chitosan has resulted in data shown in Table 5.

The degradation studies of sulfonated chitosan samples (Tab. 5) shown that the hydrolitic and enzymatic processes are probably carried out through the parallel statistical destruction and depolymerization with a relative similar rate.

The degradation studies of microcrystalline chitosan and sulfonated chitosan shown a relation of this process to their molecular, super - molecular and chemical structure (Tabs. 1 - 5).

Table 3. Degradation results[x] of microcrystalline chitosan dispersion

Symbol of sample	Lysozyme concentration ,μg/cm^3	Molecular parameters of residual polymer			Oligoaminosaccharide content ,μg/mg of polymer
		$\bar{M}n$,kD	$\bar{M}w$,kD	Pd	
MKCh/2	0	3.42	11.41	3.34	0.01
	400	2.96	6.78	2.29	11.20
MKCh/SO3	0	7.59	60.39	7.95	0.01
	400	5.89	44.68	7.58	13.90
MKCh/VI	0	7.84	64.61	8.24	0.50
	400	6.11	52.64	8.61	9.80

x - 28 days degradation period

Table 4. Degradation results[x] of microcrystalline chitosan powder

Symbol of sample	Lysozyme concentration ,μg/cm^3	Molecular parameters of residual polymer			Oligoaminosaccharide content ,μg/mg of polymer
		$\bar{M}n$,kD	$\bar{M}w$,kD	Pd	
MKCh/D3	0	7.68	27.96	3.64	1.70
	400	6.58	37.99	5.77	20.50
MKCh/MK	0	5.00	13.49	2.70	2.80
	400	4.64	16.60	3.59	34.00
MKCh/C	0	6.69	21.30	3.18	0.40
	400	6.27	33.08	5.27	29.50

x - 28 days degradation period

A germination process of lettuce seeds carried out in an environment of used chitosan forms was carried out for testing their biostimulation activity. The average length of seed shoots after 2 days of germination, in comparison to control, was determined in a case of different chitosan. The results of above tests are presented in Table 6. The microcrystalline chitosan gel - like dispersion shown a highest germination biostimulating activity in a case of

low and medium average molecular weight (Tabs. 1, 6). The sulfonated chitosan with combined sulfur content of 7.10 wt % was also characterized by a highest germination bioactivity (Tab.6).

Table 5. Degradation results[x] of sulfonated chitosan

Symbol of sample	Lysozyme concentration ,μg/cm^3	Sulfur content in residual polymer ,wt%	Intrinsic viscosity ,g/100cm^3	Oligoaminosaccharide content , μg/g of polymer
S1	0	6.0	0.053	17.3
	400	6.6	0.042	19.5
S2	0	6.9	0.028	16.7
	400	6.4	0.035	19.9
S3	0	8.2	0.046	11.8
	400	6.1	0.041	17.4

x - 28 days degradation period

Table 6. Average lengths of shoots for lettuce seed germinated in tested chitosan forms environment.

Symbol of sample used	Form of initial sample	Concentration used ,wt%	Average length of shoots, % in comparison to control
MKCh/2	gd	0.01	103
		0.02	104
MKCh/SO_3	gd	0.01	109
		0.02	103
MKCh/V1	gd	0.01	103
		0.02	101
S1	p	0.01	108
		0.02	97
S2	p	0.01	136
		0.02	108
S3	p	0.01	107
		0.02	42

gd - gel-like dispersion, p - powder

The bioactivity of selected chitosan forms for plant protection using different pathogens was also tested. The antibacterial activity for microcrystalline chitosan in a form of gel - like dispersion distinquished by different average molecular weight and sulfonated chitosan were used for testing of their antibacterial activity. The results of this test described as the minimum chitosan concentration inhibiting a growth of bacteria (MIC) are presented in Table 7.

Table 7. The antibacterial activity of microcrystalline chitosan dispersion and sulfonated chitosan.

Type of sample	MIC, % for	
	Cmm	Ec
oligomers	(1.0)*	(1.0)
MCCh (L)	0.1 - 0.2	0.2 - 0.3
MCCh (M)	0.3 - 0.5	0.5
MCCh (H)	(1.0)	(1.0)
S-1	(1.0)	(1.0)
S-2	(1.0)	(1.0)
S-3	(1.0)	(1.0)

* - no inhibition of bacteria growth with this concentration., Cmm - Clavibacter michiganese subsp. michiganese, Ec - Escherichia coli.
L - low Mv, M - medium Mv, H - high Mv.

The antibacterial activity of microcrystalline chitosan dispersion relates to its average molecular weight level, being also a parameter acting on its susceptibility for degradation, as well as to a type of bacteria (Tab.7). The oligoaminosaccharides and microcrystalline chitosan with high average molecular weight are not inhibited the bacteria growth.At the same time the sulfonated chitosan was expressed by no inhibition of tested bacteria growth.
The antifungal activity of tested chitosan forms in a case of Botrytis cinerea in vitro is shown in Table 8.
Microcrystalline chitosan with low and medium average molecular weight was inhibited a growth of tested bacteria in 100 % with concentration of 5mg/cm^3, whereas with concentration of 2.5 mg/cm^3 - in 95 - 98%. However, a sulfonated chitosan was characterized by much lower (25 -35%) effectivity (Tab.8).
The antiviral effectivity of tested chitosan forms is shown in Table 9.

Table 8. Influence of selected chitosan forms on the growth of Botrytis cinerea fungi in vitro.

Type of chitosan form	Specification	Concentration used, mg/cm^3	Inhibition of fungi growth, % in comparison to control
MCCh	dispersion $\bar{M}v = L$	0.5	48
		1.0	80
		2.5	95
		5.0	100
MCCh	dispersion $\bar{M}v = M$	0.5	38
		1.0	79
		2.5	98
		5.0	100
sulfonated chitosan	S = 6.05 wt%	0.5	0
		1.0	0
		2.5	25
		5.0	35

Table 9. Influence of tested chitosan forms on inhibition of ALMV virus infection for bean plants.

Type of chitosan form	Specification	Inhibition, % in comparison to control, for concentration of, wt%	
		0.1	0.01
MCCh	dispersion, $\bar{M}v = VL$	<90	<30
MCCh	dispersion, $\bar{M}v = L$	<95	<40
MCCh	dispersion, $\bar{M}v = M$	95 - 100	45 - 50
MCCh	dispersion, $\bar{M}v = H$	100	50 - 60
MCCh	powder, $\bar{M}v = M$	<100	90
MCCh	powder, $\bar{M}v = H$	100	<80
S-1	S = 6.05 wt%	91	48
S-2	S = 8.10 wt%	86	33
S-3	S = 11.00 wt%	73	25

Microcrystalline chitosan, both as a gel - like dispersion and powder, is characterized by excellent antivirus activity in a case of ALMV viruses on the

bean plants (Table 9). A chemical modification of chitosan by sulfonated groups has drasticly reduced its antiviral activity (Tab. 9).

CONCLUSIONS

1. Microcrystalline chitosan with polycationic character is distinquished by a high antiviral activity in a range of medium and high average molecular weight, related also to a virus -plant type system. At the same time this chitosan form is characterized by optimum antifungal activity with low and medium average molecular weight as well as antibacterial activity with low average molecular weight.
2. Biostimulation of the plant growth is especially visuable for microcrystalline chitosan with low and medium average molecular weight.
3. Modification of microcrystalline chitosan structure during its preparation allows to create its optimum bioactivity.
4. Chemical modification of chitosan by introduction of sulfonate groups has changed its charge character for polyanionic with elimination or reduction its antimicriobial activity and leaving of high ability for biostimulation.
5. A most probably mechanism of bioactivity for microcrystalline chitosan with polycationic character seems to be a multifunctional with different ratio and rate of processes such as:
   - biodegradation
   - induction of plant natural resistance
   - membrane effect
   - direct action.
6. A chemical modification of chitosan with introduction of anionic type of groups seeems to be affected mainly on the membrane process in connection with biodegradation and sligh induction of plant natural resistance.The anionic charge modification of chitosan has mainly affected for biostimulation of plant growth.

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Fundamental Study on Oral Administration of Chitin and Chitosan in Dogs.

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Abstract

The changes of chitin and chitosan in the gastrointestinal tract and the systemic effects of oral administration were evaluated in dogs. Chitin and chitosan were packed in nylon bags (45 μm mesh) and were administered orally. The weight of the chitosan in the bags recovered from the feces was decreased to <10% ot the original weight and it formed a film, while chitin did not change in shape or weight. When chitosan bags were inserted surgically into the small and large intestines, about 40 % weight loss of chitosan was observed. When the chitosan bags were placed in large intestinal loops for 24 hr, about 26 % weight loss was observed in the presence of feces. An *in vitro* study showed that chitosan became a gel in artificial gastric juice and its weight decreased by 15 %, but there were no changes in artificial intestinal juice. The plasma total cholesterol level decreased significantly when chitosan was administered orally every day for 2 weeks to dogs (3 g daily in week 1 and 6 g daily in week 2), while chitin and cellulose caused no changes.

Keywords: Chitin, chitosan, cholesterol, dogs, gastrointestinal tract, oral administration,

Recently, chitin and chitosan have attracted major interest as functional foods. The cholesterol-lowering action of chitosan administered orally was first reported by Sugano *et al.* in 1978, and has since been confirmed by several investigators to be associated with an increase in the fecal excretion of cholic acid[2-8]. On the other hand, chitin do not display a cholesterol-lowering action[9]. The extent of digestion of chitosan in the gastrointestinal tract has been unclear. To evaluate chitin and chitosan as functional foods, it is important to investigate their changes in the gastrointestinal tract.

In this study, we evaluated the changes of chitin and chitosan in the gastrointestinal tract and their systemic effects after oral

administration in dogs.

Materials and methods

Animal

Seventy-eight mongrel dogs aged 3 to 5 years old and weighing 9-12 kg were used in this study. All of the dogs were healthy according to clinical and hematological tests.

Reagents

Chitin and chitosan: Chitin and chitosan were supplied by Sunfive Inc. (Tottori, Japan). Chitin was prepared from squid pen as minute particles with an average diameters of 0.5 or 3 mm. The molecular weight was approximately (2-3) x 10^5. Chitosan was prepared from chitin which was purified from crab shell by chemical deacetylation. Approximately 80 % of the deacetylated particles (DAC80) had an average diameter of 0.5 and 3 mm and the molecular weight was approximately 8 x 10^4. Cellulose (average diameter: 0.12 mm) was purchased from Wako (Osaka, Japan)

Artificial gastric and intestinal juice: Artificial gastric juice was made from of 2.0 g/L of NaCl (Wako, Osaka, Japan), 3.2 g/L of pepsin (Wako, Osaka, Japan), and 24 ml/L of 1 N-HCl (Wako, Osaka, Japan). Artificial intestinal juice consisted of 15 g/L of $NaHCO_3$ (Wako, Osaka, Japan) and 2.8 g/L of pancreatin (Wako, Osaka, Japan).

Experimental design

Experiment 1: Sixty dogs were divided into three groups for oral administration (n=24), insertion into the small intestine (SI group, n=18), and insertion into the large intestine (LI group, n=18). The oral group was divided into two subgroups given chitin (ϕ 0.5 and 3 mm : n=3 each) or chitosan (ϕ 0.5 and 3 mm: n=9 each). A nylon bag (3 x 1 cm) was made of 300 mesh (pore size 45 μm) nylon fabric (Nytal, No282, Swiss) and 50 mg of chitin or chitosan was placed inside each bag. Three bags were administered to the dogs treated orally. The SI and LI groups were divided into 2 subgroups given 0.5 and 3 mm chitosan (n=9 each). Three bags were surgically inserted under general anesthesia in each dog. Bags were recovered from the feces within 2 days after administration or insertion and were washed in water for 10 min. Then the bags were dried at 38 ℃ for 7 hr and weighed.

Experiment 2: Six mongrel dogs were divided into 2 groups of 3 with and without removal of fece. The large intestine was exposed at laparotomy under general anesthesia and was ligated with sutures at

proximal, middle, and distal sites to make two loops 10 cm long. In the group without fecal removal, three nylon bags that measured 3 x 1 cm and contained chitosan particle with an average diameter of 0.5 mm, were inserted surgically into the proximal loop without removal of intestinal contents, and three similar bags without chitosan particles were placed in the distal loop. In the fecal removal group, three of the same bags with chitosan particles were placed the proximal loop after removal of the intestinal contents, and three bags without chitosan particles were similarly placed in the distal loop. The incisions in the intestinal wall were closed with sutures, and then the abdominal wall, subcutaneous tissue, and skin were closed with sutures in a routine manner. The dogs were sacrified at 24 hr postoperatively and the bags were recovered. The bags were then weighed following washing and drying in the manner described above.

Experiment 3: Nylon bags measureing 5 x 5 cm were made with 300 mesh (pore size 45 μm) nylon fabric and 300 mg of chitosan particles with an average diameter of 3 mm were placed in each bag. Then each bag was immersed in 300 ml of artificial gastric or intestinal juice. The bags were recovered after 1, 2, 4, 8, and 12 hr and were weighed following washing and drying.

Experiment 4: Twelve dogs were divided into 3 groups treated with chitin (n=3), chitosan (n=6), and cellulose (n=3). The chitosan group was divided into 2 subgroups: chitosan-L and chitosan-H subgroups (n=3 each). In the chitin, chitosan-H, and cellulose groups, 3 g/day of the test agent was given to each dog during the first week, and subsequently 6 g/day was given in the second week. In the chitosan-L group, a dose of 1 g was given daily in the first week, and subsequently 2 g was given daily in the second week. Body weight (BW) and general condition including appetite, vigor and hair condition were evaluated before administration of each test agent (Day 0) and on the 7th, 14th, 21st, and 28th days after administration (Day 7, 14, 21, and 28, respectively). On these days, blood was collected from the jugular vein using a heparinized syringe with an 18G needle. The red blood cell count (RBC) and the white blood cell count (WBC) were measured by a hemocytometer (Celltack, Nippon Koden, Tokyo), and the packed cell volume (PCV) was measured by the micro-hematocrit method. Plasma total protein (TP) was measured by refractometry. The differential leukocyte count (DLC) was determined by light microscopy of a Giemsa-stained blood smear. Plasma was separated from the residue by centrifugation at 2500 rpm for 10 min for biochemical analysis. The levels of Plasma transaminases (AST and ALT), glucose, total cholesterol (T-Chol), blood urea nitrogen (BUN), and total bililbin

(T-Bil) were measured by autoanalyzer (Cobas reday, Japan Rosh, Japan).

Statistical analysis

Statistical analysis was performed using Student's *t* - test.

Results and discussion

Experiment 1

Changes in the weight of chitin and chitosan in the nylon bags after oral administration are shown in Table 1. The weight loss of chitosan with an average particle diameter of 0.5 or 3 mm was 93.8 +/- 3.9 and 82.4 +/- 6.4% of the original weight, respectively, and the particles formed a film, while chitin did not change in weight or shape. Table 2 shows the relationship between the change in the weight of chitosan and the route of administration. When chitosan was surgically inserted into the small and large intestines, about 40 % weight loss was observed.

TABLE 1

Changes in the weight of chitin and chitosan after oral administration.

Sample	Shape	Weight loss (%)
Chitin	powder	2.0 ± 2.2[a)]
	flake	2.4 ± 1.9
Chitosan	powder	93.8 ± 3.9
	flake	82.4 ± 6.4

a) Mean ± S.D.

Experiment 2

Changes in the weight of chitosan in the large intestinal loop are shown in Table 3. When bags containing chitosan were placed in the large intestinal loop for 24 hr, about 26 % weight loss was observed in the animals without removal of feces, while no weight loss was observed in the animals with removal feces. There was also no change in the weight of the bags without chitosan particles.

Experiment 3

Changes in the weight of chitosan in artificial gastric and intestinal juice are shown in Table 4. Chitosan became a gel in artificial gastric juice and its weight decreased by 15 %, but there was no change in artificial intestinal juice.

TABLE 2
Relationship between the change in the weight of chitosan and the route of administration.

Route	Shape	Weight loss (%)
Oral	powder	93.8 ± 3.9[a]
	flake	82.4 ± 6.3
Small intestine	powder	34.2 ± 7.5
	flake	36.9 ± 5.6
Large intestine	powder	33.8 ± 3.5
	flake	34.4 ± 6.8

a) Mean ± S.D.

TABLE 3
Effect of feces on change in the weight of chitosan.

Group	Weight loss (%)
Feces treated	2.7 ± 3.9[a]
Feces untreated	26.0 ± 11.6

a) Mean ± S.D.

TABLE 4
Changes in the weight of chitosan in artificial gastric juice.

Time (hr)	Weight loss (%)
1	0.6 ± 1.2[a]
2	0.3 ± 2.2
4	11.6 ± 20.9
8	14.6 ± 4.8
12	14.4 ± 3.5

a) Mean ±S.D.

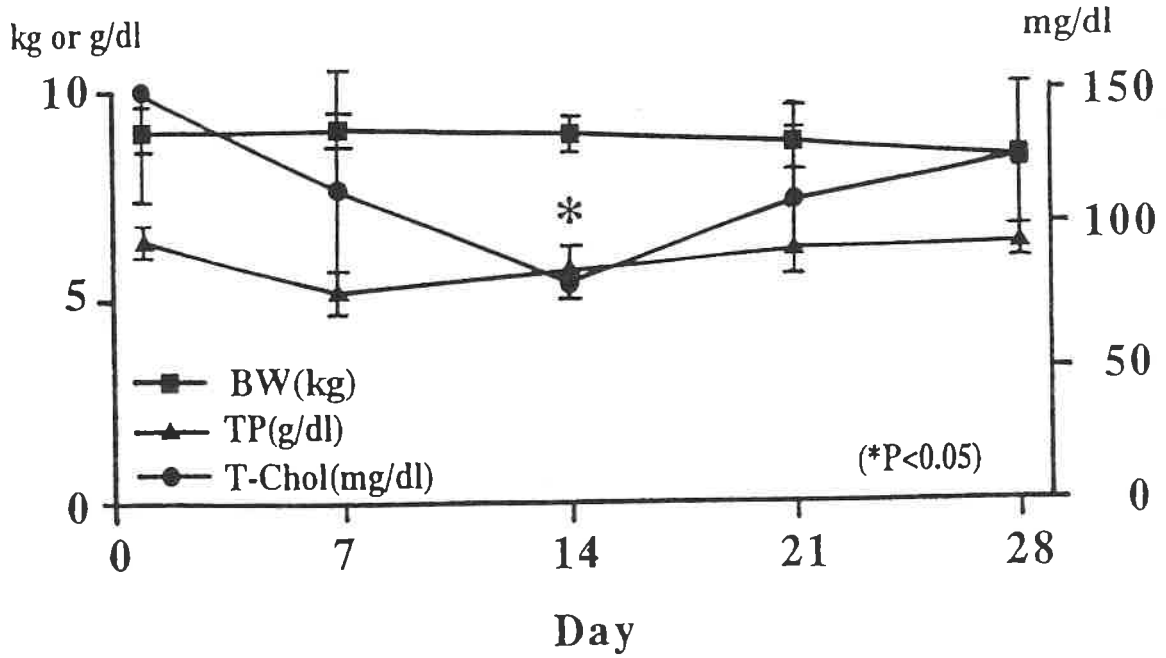

Figure 1 Changes of total cholesterol (T-chol), total protein (TP), and body weight (BW) in the chitosan-H group. Each dog in the chitosan-H group was given 3 g/day of chitosan during the first week, and subsequently 6 g/day was given in the second week.

Experiment 4

The general condition showed no change in all groups. Figure 1 shows the changes of T-Chol, TP, and BW in the chitosan-H group. The plasma T-Chol level decreased significantly and BW decreased slightly on Day 14, but there was no change in other parameters. The plasma T-Chol level decreased to 77% on Day 7 and subsequently to 54 % on Day 14, and then recovered at the initial level on Day 28. The other groups showed no changes in any of the parameters.

The present study showed that chitin did not undergo changes in weight and shape, but chitosan was degraded in the gastrointestinal tract. In addition, chitosan was found to be affected in the stomach and large intestine, but not in the small intestine. About 90 % weight loss of chitosan was observed when it was administered orally, while it became a gel in artificial gastric juice and only showed 15 % weight loss. This suggests that chitosan was lost from the bag into the gastrointestinal tract following the change to a gel in the stomach. From the fact that 26 % weight loss of chitosan was observed in the presence of feces in the large intestine, it appears that bacterial flora influenced the weight of chitosan. Hirano *et al.* (1990) reported that chitosanase was found in bacteria flora and in dietary fiber. The

bacterum producing chitosanase is Bacillus spp[11]. It is known that main type of bacterial flora in canine feces is Bacteroidaceae[12], there are few data regarding other bacterial species. In the future, further investigation will be necessary regarding this point.

In the clinical study, the cholesterol level was reduced by feeding chitosan in dogs, but chitin did not have this effect. This result is consistent with data on other animals including humans, mice, rats, chickens, and rabbits. Hirano *et al.* (1990) reported that oral administrations of 0.7-0.8 and 1.2-1.4 g/kg of chitosan affected the plasma cholesterol level in the rabbit and chicken, respectively. In addition, Maezaki reported that oral administration of 0.05-0.1 g/kg of chitosan affected the plasma cholesterol level in humans. The present study showed no change of the cholesterol level when 0.1- 0.2 g/kg of chitosan was administered orally. These data suggest that the influence of chitosan on cholesterol differs between species.

The mechanism of the hypocholestemia of chitosan may be as follows. After chitosan is dissolved by gastric juice, it mixes with food containing fat in the stomach. This mixture of chitosan and food becomes a gel in the intestine. As a result, absorption of fat is reduced and the cholesterol level in the serum and liver is decreased[14]. However, this theory is based on the premise that most chitosan administered orally is not absorbed from the gastrointestinal tract. In contract, the present study showed loss of chitosan in the large intestine, making it likely that chitosan was absorbed from the large bowel following degradation by bacteria or was utilized by the bacteria in the feces.

Deuchi *et al.* (1995a, 1995b) have reported that the side effects of chitosan include disturbance of the absorption of minerals and fat soluble vitamins. In this study, however, no side effects of chitosan were seen.

Conclusion

Chitin did not undergo changes in weight and shape, but chitosan was degraded in the gastrointestinal tract. In addition, chitosan was found to be affected in the stomach and large intestine, but not in the small intestine. From the fact that 26 % weight loss of chitosan was observed in the presence of feces in the large intestine, it appears that bacterial flora influenced the weight of chitosan. In the clinical study, the cholesterol level was reduced by feeding chitosan (3 g daily in week 1 and 6 g daily in week 2in dogs), but chitin did not have this effect.

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Mechanism of Wound Healing Acceleration by Chitin and Chitosan

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Abstract

Chitin and chitosan activate complement C3 and C5, but not C4. Chitin and chitosan also stimulate fibroblast to produce interleukin-8 (IL-8). Fibroblasts produced much IL-8 by the stimulation of chitosan (50 ml/ml) than by it of TNF-a (100 ng/ml). The complement activation and IL-8 production would be a very important role in the wound healing acceleration by chitin and chitosan.

Key words: Chitin, chitosan, complement, IL-8, wound healing

We already reported that 200 mg/kg subcutaneous administration of chitosan induced severe hemorrhagic pneumonia in dogs[1] and the 10 mg/kg administration induced systemic activation of chemiluminescence response (CL) in canine circulating peripheral polymorphonuclear cells (PMN)[2]. The increasing of CL response was observed in the PMN recovered from the chitosan treated dog after incubation with normal plasma and in the PMN of chitosan non-treated dog after incubation with the plasma recovered from the chitosan treated dog. These reactions had recognized that both activation of the PMN and the plasma had induced by the administration of chitosan to dogs. The effect of the plasma PMN activation easily decreased by heating in 56℃ for 30 min, therefore, it is easily supposed that these effects would be brought about by complement activation especially in activation of thermolabile factors[2]. Furthermore, in vitro experiments, when chitin and chitosan were used for a stimulator of PMN CL response with normal plasma, chitin and chitosan induced 30% and 50% intensity of the zymosan induced CL intensity (100%), respectively. Without plasma or with heat treated plasma, chitin and chitosan did not induce CL response in canine PMN[3]. On the other hand, a supernatant of plasma incubated with chitin or chitosan had an effect on increasing of CL response to zymosan in the PMN recovered from non-treated dogs and was decreased this effect by heat treatment[4]. This plasma treated with chitin or chitosan also increased the number of migrated PMN in an experiment of Boyden chamber method[5]. On the other hand, chitin and chitosan have been observed to accelerate wound healing properties and the attainment of a skin good-healing without scar[6-7]. Histological findings are suggestive of the fact that these materials constitute a stimulant for the migration of PMN and mononuclear cells, and accelerate the regeneration of vascular granulating tissue[3, 8]. In the wound healing process, fibroblasts are recognized to be critical. It is currently accepted that they follow inflammatory cells into site of tissue injury and contribute to wound healing through the

synthesis of structural proteins. They also facilitate the wound contraction and the reorganization of the extracellular matrix. Fibroblasts have also been generally regarded as target cells of cytokines and growth factors. Recent investigations have focused on the role of fibroblast-secreted cytokines. Furthermore, fibroblasts are capable of secreting a broad range of cytokines[9-11]. However, there are no reports on the effect of chitin and chitosan on cytokine production of fibroblast.

Materials and methods

Six normal adult beagle dogs, 1-3 old, weighing 8-15 kg, and 39 ddy mice, 6 weeks old were used for determination of systemic complement activation by the subcutaneous administration of chitosan. To determine direct effects of chitin and chitosan, fresh normal human plasmas collected from three healthy male volunteers were used.

DRUG

Chitin and chitosan suspensions:Commercial squid pen chitin purified from *Ommastrephes bartrami* (Nippon Suisan Co., Ltd., Tokyo, Japan) which was b-chitin of 9% deacetylation and an average molecular weight of over 100,000 and chitosan flake (Flonac C, Kyowa Tecnos Co., Ltd., Japan), which was comprised of 82% deacetylated a-chitin purified from crab shell and had an average molecular weight of 80,000, ash of maximum 1.2%, and heavy metals as Pb, Cd and As of maximum 5 ppm, were pulverized into 3 mm with a mill (Ube Industries, Ltd., Japan, CF-400). These fine powders were sterilized by ethylene oxide gas, and were suspended into physical saline in the concentration of 30 mg/ml for dog and 5 mg/ml for mouse (the original suspension). Zymosan suspension: After Zymosan (Sigma, USA) of 0.1 g was suspended into 10 ml of physical saline, its suspension was centrifuged at 2,000 rpm for 10 min and the supernatant was diluted by HEPES added Hank's balanced salt solution (HEPES-HBSS, phenol red free, Nihon-suisan Co. Ltd., Tokyo, Japan) up to 10 ml after decantation. Luminol solution: Luminol was diluted into 2 mg/ml by HEPES-HBSS and then 50 ml of triethylamine (Wako, Tokyo, Japan) was added into this solution. After 45 min ultrasonication, this solution was filtrated by 0.45 microns Millipore filter in order to remove insoluble Luminol particle. Anti-complement serum: One mg of anti-dog C3 goat serum (Bethyl Laboratories, Inc., USA), anti-mouse C3 goat serum (Bethyl Laboratories, Inc., USA), anti-human C3 goat serum (Bethyl Laboratories, Inc., USA), anti-human C4 goat serum (Bethyl Laboratories, Inc., USA), and anti-human C5 goat serum (Bethyl Laboratories, Inc., USA) were dissolved in 2 ml of physical saline and stored in -80^{0}C. Agarose gel: After 1.5 g of purified agar, powder (Nacalai Tesque, Inc., Kyoto, Japan) was dissolved in 100 ml of phosphate buffer solution, 30 mg of sodium azide (Nacalai Tesque, Inc., Kyoto, Japan) was added. Fibroblast cell culture medium: Eagle's minimum essential medium (E-MEM, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with 10% fetal calf serum (FCS, ICN Biomedicals Japan Co. Ltd., Tokyo, Japan), 60 mg/ml of antibiotic (Kanamycin, Meiji Pharmaceutical Co., Ltd., Tokyo, Japan) and 1 mg/ml of anti-fungal agent (Fungizone, BMS, Tokyo, Japan) in a plastic culture dish (Corning, New York, USA) was used. Cytokine ELISA kit: IL-8 ELISA kit was purchased from Panafarm Laboratories (Kumamoto, Japan). The sensitivity of this assay is 80 pg/ml.

Methods

Effect of subcutaneous administration of chitosan on complement activation: Six dogs, they were divided into two groups, 10 mg/kg chitosan group (3 dogs) and control group (3 dogs). Each dog in the chitosan group was administered 10 mg/kg chitosan in two parts of neck subcutaneous tissue, using a syringe with 20 gage needle. The injection volume was adjusted to 1 ml/kg by a dilution of the original suspension with physiological saline. In control group, 1 ml/kg of physiological saline was injected subcutaneously as the same manner in the chitosan group. Mice were divided into three groups, 10 mg/kg chitosan group (12 mice), 50 mg/kg chitosan group (12 mice), and control group (15 mice). In the chitosan groups, 350 ml of chitosan suspension was prepared in a syringe with 24 gage needle by a dilution of the original suspension with physiological saline and was administered in dorsal subcutaneous tissue. In control group, 350 ml of physiological saline was administered as same manner of the chitosan groups

Blood collection, separation and blood examination:In dogs, 5 ml of blood was collected from jugular vein with heparin (10 IU/ml) before chitosan or physiological saline administration (Pre), and at 3 days (day 3) and 6 days (day 6) after administration of chitosan or physiological saline. The blood examination described as below was performed by 1 ml of the collected blood, and another 1 ml was used for CL examination. Red blood cell count (RBC), white blood cell count (WBC), each differentiation of white blood cells, and hemoglobin concentration (Hb) were measured in a routine manner. The concentration of complement 3 (C3) in the plasma was measured, which was separated from another 3 ml of the collected blood by centrifugation at 3,000 rpm for 15 min at 4 ^{0}C. The aliquots of ca. 1 ml blood from 9 mice which were divided to 3 groups were collected by direct heart puncture with heparin (10 IU/ml) under anesthesia by ether, before administration of physiological saline (Pre), and at 3 days (day 3) and 6 days (day 6) after administration of physiological saline from 3 mice each. In the chitosan groups, blood collection was performed on 3 days and 6 days after administration of chitosan from 5 mice each. The aliquots of collected blood were pooled at -80 ^{0}C and the blood analyses of the half of them were done as same as described in dogs. C3 estimation was performed after separation of plasma by centrifugation at 3,000 rpm for 15 min at 4 ^{0}C by using the blood remained. In human, blood was collected from cephalic vein with heparin (10 IU/ml) in the Tottori University Health Care Center, and was separated by centrifugation at 3,000 rpm for 15 min at 4 ^{0}C as soon as possible. Each plasma for the complement estimation were stored at -80 ^{0}C before use.

Chemiluminescence (CL):The CL measurement according to Makimura and Sawaki (1992) [12] was taken on Lumat LB-9501 (Berthold Co., Germany).

Complement estimation:The amounts of C3, C4, and C5 in blood were estimated by the single radial immunodiffusion method. Each optimum dilution of anti-serum was decided by preliminary test. Anti-dog C3 goat serum, anti-mouse C3 goat serum, anti-human C3 goat serum, anti-human C4 goat serum and anti-human C5 serum was diluted 20, 40, 40, 80, and 30 times, respectively, by agarose gel previously warmed at 50 ^{0}C and then was coagulated in an ice water bath. A center hole was created in the coagulated agar by a small corkscrew. The test serum was used 2.5 ml and placed in the center hole of the agar. The incubation time was 48 hr under 4 ^{0}C.

Table 1. Complement activation by chitin, chitosan, and zymosan

complement	agent	incubation time 10	20	30	40
C3	chitosan	63±02**	60±05**	59±04**	58±06**
	chitin	83±11*	77±10**	73±13**	63±21**
	zymosan	66±03**	64±04**	64±04**	64±04**
C4	chitosan	100±02	100±02	100±02	100±02
	chitin	100±02	100±02	100±02	100±02
	zymosan	100±02	100±02	100±02	100±02
C5	chitosan	56±19**	23±32**	23±32**	23±32**
	chitin	73±04**	45±04**	45±04**	45±04**
	zymosan	56±19**	23±32**	23±32**	23±32**

The concentration of each agent is 10 mg/ml. Data were expressed as the mean % and standard deviation in each agent. Individual data were converted to % relative to the initial complement level (pre-incubetion).Statistical analysis was performed between each initial level and after incubation level (*: $p<0.05$; **: $p<0.01$).

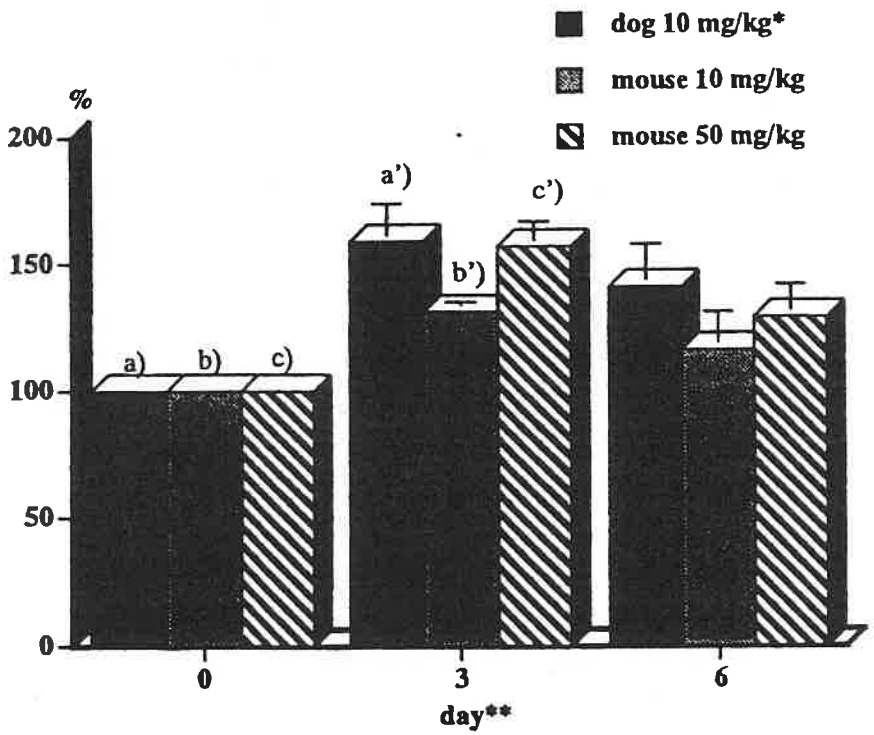

Fig. 1. Effect on C3 concentration in peripheral plasma after subcutaneous injection of chitosan.

*10 mg/kg of chitosan was administered subcutaneously.
**day 0 means before injection (pre.), days 3 and 6 mean days after injection.
a') and c') are significatly increased compare to a) and c)($p<0.01$).
b') is significantly increased compare to b)($p<0.05$).

We had demonstrated that inflammatory cells migrated to subcutaneously administered chitin or chitosan[3], and chemotactic substances were created in plasma by incubation with chitin or chitosan[4]. These effect would suggest humoral agents in a body fluid such as plasma and cellular interstitial fluids played very important role to chitin and chitosan. In general, it has been well known that three anaphylatoxins, such as C3a, and C5a, are created by complement activation through the complement alternative pathway[14]. C3a stimulates subcutaneous mast cells producing histamine[15-17] and leukotriene B4[18, 19], which cause intensive dilation of peripheral vessels and edema. C5a stimulates phagocytes to upregulate adhesive receptor for endothelium[20]. Furthermore, C5a also induces upregulation of FC receptor for antibodies, and complement receptors, CR1 and CR3 which make regands to C3b and iC3b, respectively[14]. When chitin and chitosan are administered to subcutaneous tissue, it is easy to recognize from the present *in vitro* study, that subcutaneous complement will waste rapidly by contact to these materials, since the plasma C3 increase in chitosan administered dogs and mice will explain by these mechanisms. On the other hand, when chitosan and chitin were administered in a human plasma, C3 and C5 were decreased while C4 did not changed. It has been known that a polyglucan such as zymosan induced the complement alternative pathway, and the present result of zymosan also well agree with the previous report[21, 22]. There are no doubt that chitosan and chitin also induces the activation of complement alternative pathway. In the alternative pathway, C3b is created by a C3i enzymatic degradation of C3[14, 23], but a creation of C3i by chitosan is still unknown. Further investigation on the mechanism of complement activation by chitin and chitosan will be required.

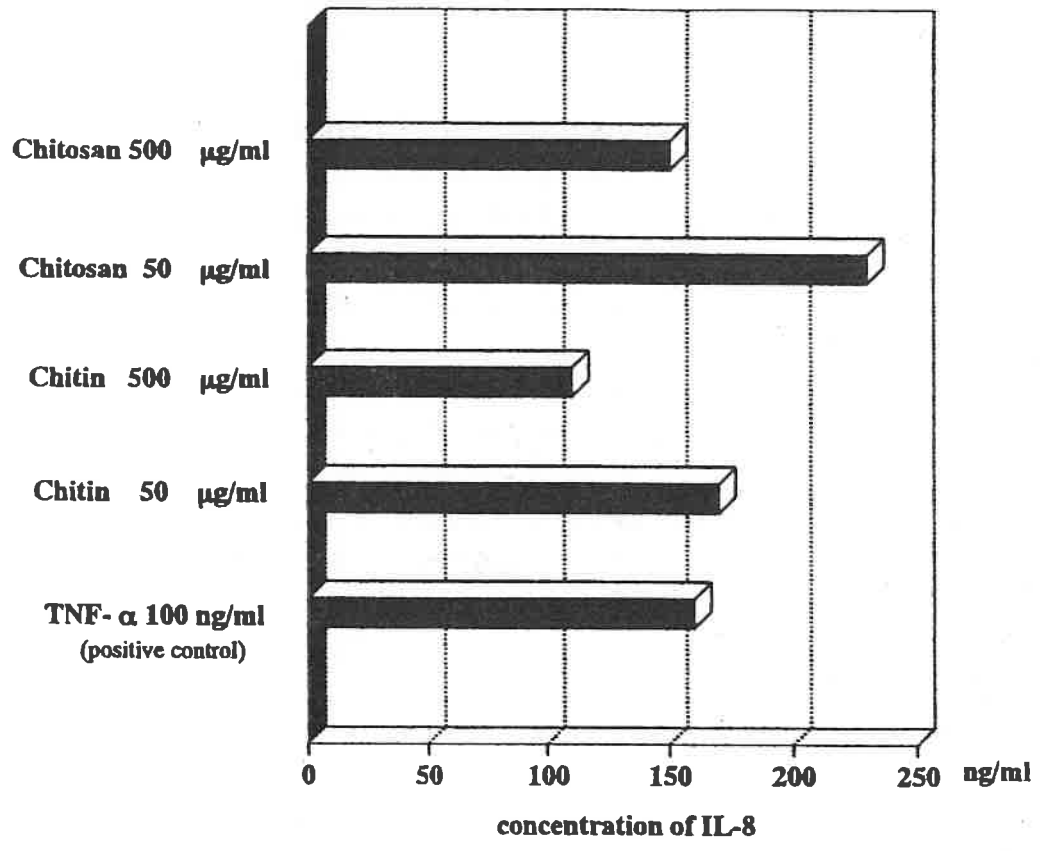

Fig.2. Interleukin-8 (IL-8) relaeased from rat dermal fibroblasts stimulated with chitin and chitosan.

IL-8 production from rat dermal fibroblasts::Results were shown in Fig.2. IL-8 released from rat dermal fibroblasts stimulated with chitin and chitosan was higher in the 50 mg/ml of chitosan than the 100 ng/ml of TNF-a positive control, and almost same level of positive control in the 500 mg/ml of chitosan and the chitins. IL-8 is a potent activator and chemoattractant of neutrophils. Fibroblasts, endothelial cells, and monocytes are well known as producers of IL-8, when they are stimulated by agents such as IL-1 or TNF-α [24]. Human IL-8 is reported to be angiogenic in the rat cornea. It also induces the migration of human umbilical vein endothelial cells *in vitro*[25]. Chemotaxis of epidermal cells in response to IL-8 has also been observed[26]. In the present results support the clinical findings of angiogenesis and migration of neutrophils by chitin and chitosan are a result of persistent release of IL-8 from fibroblasts caused by direct contact of chitin and chitosan.

Conclusion

Chitin and chitosan will induce effective complement activation and IL-8 production from fibroblasts in the applied wound. There are no doubt one of the initial biological activation will start by the alternative pathway complement activation. C5a produced by this activation and IL-8 from fibroblast will act effective PMN migration to the wound.

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CHITIN-GLUCAN COMPLEX OF *Aspergillus niger* AND ITS DERIVATIVES: ANTIMUTAGENIC, ANTIINFECTIVE AND ANTIVIRAL ACTIVITY

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Abstract

Insoluble chitin-glucan complex (CG) isolated from the cell walls of the filamentous fungi *Aspergillus niger* was solubilized in water by means of carboxymethylation and two lower-molecular weight fractions were prepared by ultrasonication of the high-molecular weight carboxymethyl chitin-glucan (CM-CG). Non-degraded CM-CG showed protective antimutagenic activity against cyclophosphamide when injected intraperitoneally or intravenously to mice prior to the mutagen application. CM-CG also inhibited adherence of the pathogenic *Candida albicans* cells to human buccal and vaginal epithelia and prevented experimental candidosis in rabbits. Superficial pretreatment of the leaves of bean or cucumber plants with the solutions of the low-molecular weight fractions of CM-CG prior to inoculation of tobacco necrosis virus (TNV) led to a significant decrease in number of viral lesions caused by TNV on the plant leaves.

Keywords: Chitin-glucan, *Aspergillus niger*, carboxymethylation, ultrasonication, antimutagenic effect, adherence inhibition, plant protection

Materials and methods

Isolation of the crude chitin-glucan complex

The crude, water-insoluble chitin-glucan complex was isolated from the cell walls of the industrial strain *Aspergillus niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovak Republic). The mycelium was subjected to a hot alkaline (1 M NaOH) digestion for 1 h. The alkali-insoluble sample was subsequently five times washed with distilled water, acetone, and finally with diethylether. The dry sample contained 2.24 % nitrogen which corresponded to a content of ca. 30 % chitin.

Preparation of the carboxymethylated chitin-glucan complex (CM-CG complex)

Derivatization of the chitin-glucan complex was performed using the modified procedure described by Sasaki *et al.*[1]. Briefly, 10 g of the chitin-glucan complex was suspended in a mixture of 12.4 mL of aqueous NaOH (300 $g.L^{-1}$) and 125 mL of isopropanol. The suspension was vigorously stirred at 10 °C for 1 h. Subsequently, sodium salt of monochloroacetic acid was added (7.9 g for achievement of the substitution degree 0.5) in 14 mL of water, and the mixture was stirred at 70 °C for 2 h. Excess NaOH was neutralized with 6 N HCl and the salts were removed by dialysis.

The non-dialyzable portion was dried, dissolved in water, centrifuged and freeze-dried. The degree of substitution of the obtained carboxymethylated chitin-glucan complex was 0.43 as determined by the potentiometric titration with a KOH solution[2]. The content of carbon in this sample was 38.3 %, hydrogen 5.74 %, and nitrogen 1.61 %.

Ultrasonication of the CM-CG complex

The obtained CM-CG complex (250 mg of the lyophilized sample dissolved in 25 mL distilled water) was treated by ultrasonication (20 kHz, 100 W) in the ice bath using a horn type (ϕ = 1.5 cm) ultrasound generator UZD 300 (PERSON-Ultragen, Nitra, Slovak Republic). Aliquots (1 mL) of the biopolymer solution were withdrawn from the ultrasound vessel at the defined time intervals of 5, 10, 20, 30, and 50 min. These aliquots, as well as the final 60-min ultrasonicated sample were further analyzed by the combined methods of high-performance liquid chromatography (HPLC) and the elemental analysis. The final sample of the 60-min ultrasonicated CM-CG complex was further fractionated by gel filtration, and the two fractions obtained were subsequently investigated by ^{13}C NMR spectroscopy.

High-performance liquid chromatography

All HPLC experiments were performed at ambient temperature with a system that included a high-pressure pump (LCP 3001; Laboratorní přístroje, Prague, Czech Republic), an eight-port switching valve equipped with two 100-μL loops (Model PK 1; Vývojové dílny, Czechoslovak Academy of Sciences, Prague), and two in series connected stainless-steel HPLC columns (250 x 8 mm) packed with SEPARON HEMA-BIO 1000 sorbent (mean particle size = 10 μm; Tessek Ltd., Prague). The separation process was monitored with a differential refractometric detector (RIDK 102; Laboratorní přístroje, Prague). The mobile phase used was 0.1 M aqueous $NaNO_3$ solution. The flow rate was 0.4 mL/min. A set of pullulans P-5, P-100, P-200, P-400, and P-800 (Shodex Standard P-82; Macherey-Nagel GmbH+CoKG, Düren, Germany) was used for the calibration of the HPLC system. Samples containing 0.1 mg of the pullulan standard dissolved in 100 μL of the mobile phase were loaded onto the HPLC column. The elution volumes corresponding to the the maxima of the chromatographic curves were assigned by the values of $M_{peak} = (M_w \times M_n)^{1/2}$ of the pullulan calibrants, where M_w and M_n are the weight- and the number-average molecular weights, respectively.

The original and the ultrasonicated CM-CG samples were analyzed by HPLC applying both analytical and semipreparative separation modes. The biopolymer concentrations used while operating HPLC in the analytical separation mode were 3-4 mg/mL, whereas for the scaled-up procedure they were increased of up to 70 mg/mL. During the HPLC run under the semipreparative separation mode, defined fractions were collected for the subsequent elemental analysis. All analyzed samples were characterized by the corresponding values of M_{peak}. The M_w and M_n molecular-weight averages of the samples were calculated using the computer program described by Šoltés *et al.*[3]. Taking into account that the HPLC system was calibrated using pullulan standards as the reference materials, the molecular-weight characteristics of M_{peak}, M_w, and M_n of all CM-CG samples should be regarded to as the relative values.

Elemental analysis

The solid biopolymers, obtained by dialysis and subsequent freeze-drying of the CM-CG samples collected upon the semipreparative HPLC, were analyzed for their carbon, hydrogen, and nitrogen content using the EA 1108 device (FISONS Instruments, UK).

Gel filtration

Gel filtration of the final 60-min ultrasonicated CM-CG complex was performed on the

glass column (150 x 1.5 cm) packed with Sepharose CL-6B (Pharmacia, Uppsala, Sweden). Sodium-phosphate buffer (0.1 M, pH 7.5) was applied as the mobile phase at a flow rate of 0.2 $mL.min^{-1}$. The 250 mg sample, dissolved in 6 mL of the mobile phase, was loaded onto the column. The separation process was monitored with a differential refractometric detector RIDK-102 (Laboratorní přístroje, Prague). Fractions (4 mL) were collected using a sample collector (SF62; Mikrotechna, Prague). The two pooled fractions - tubes 16-22 and tubes 34-65 - were further dialyzed (Dialysis Tubing- Art. No. 44146; Servapor, SERVA, Germany) against distilled water for 48 h. During the first hours of the dialysis, a significant amount of mono- and oligosaccharides was detected in the waste water using the phenol-sulfuric acid assay[4]. The dialyzed samples were freeze-dried. The yields of the two lyophilizates, designated as Samples I and II, were 3.3 and 49.5 %, respectively, relative to the initial amount of the CM-CG sample prior to ultrasonication.

^{13}C NMR spectroscopy

For the quantitative determination of the glucan:chitin ratio in Samples I and II ^{13}C NMR spectroscopy was applied. The ^{13}C NMR spectra were recorded at 298 K in D_2O solutions (30 mg/mL) using the Bruker AM-300 instrument.

Antimutagenic assay

Cyclophosphamide (Cyclostin® 200) was purchased from Pharmitalia, Carlo Erba GmbH (Italy). Cyclophosphamide (CP) administration to mice consisted of two intraperitoneal (i.p.) injections of 80 mg/kg body weight (b.w.) separated by 24 hours. Original, high-molecular weight CM-CG was applied in doses of 50 or 100 mg/kg b.w. either i.p. 24 h or intravenously (i.v.) 1 h prior to each CP injection. CP and CM-CG were dissolved in saline (Imuna, Šarišské Michaľany, Slovakia) and the applied volume was 0.1 mL/10 g b.w. 5 mL blood samples were collected from the tail vein 24 h after the second injection of CP. Acridine orange-coated slides were prepared and peripheral blood cell preparations were made according to the method of Hayashi *et al.*[5]. The slides were packed in plastic bags and stored in refrigerator overnight at 4 °C and evaluated within the next two days. Reticulocytes (RETs) and micronuclei (MN) were identified with the fluorescent microscope Fluoval (Zeiss, Germany). For each animal, 1000 RETs were analyzed for the presence of MN. The mice were randomly divided into eight groups consisting of eight animals each. The control groups consisted of 50% of animals treated i.p. and 50% treated i.v. with saline or CM-CG prior to i.p. saline administration in the same time intervals as animals exposed to CP. Five groups of mice were injected with cyclophosphamide. One of these groups was treated with saline (four mice i.v. and four mice i.p.) prior to CP application. The other four groups were treated i.p. (-24 h) or i.v. (-1 h) with CM-CG in two concentrations 50 or 100 mg/kg prior to CP administration. Statistical evaluation of the results was performed using the Mann-Whitney-Wilcoxon test[6].

Inhibition of Candida albicans adhesion.

The experiment was carried out in two variants:

var. 1: epithelium + *C. albicans* + CM-CG (inhibition assay)

var. 2: epithelium + *C. albicans* + buffer solution (control)

The experiments were performed three times. For the performance of the experiments, three suspensions were used:

suspension A: 25 mg CM-CG or its fractions in 1 mL phosphate buffer, pH 7.0

suspension B: 24 h *C. albicans* culture cultivated on Sabourad agar suspended in phosphate buffer pH 7.0 up to the density 5 according to MacFarland's scale[7]

suspension C: buccal or vaginal epithelium obtained by scraping, in the phosphate buffer, pH 7.0 and 3-times washed with the same buffer solution.

1 mL of suspension A was mixed with 1 mL of suspension B and the mixture was incubated 24 h at

4 °C. Subsequently, 0.2 mL of this mixture is added to 0.2 mL of suspension C and incubated at constant shaking 90 min. The mixture was filtered through Synpor 1 membrane filter and rinsed with 100 mL phosphate buffer, pH 7.0. The preparation on the microscope slide glass was dried, fixed with an open flame and stained according to Gram. Number of the Gram-positive yeast cells touching or lying on the 100 Gram-negative epithelial cells of irregular shape was evaluated microscopically. In vivo assay of the CM-CG ability to prevent experimentally induced candidosis was tested on the female rabbits. 21 animals were used in the test. The suspension of the virulent *C. albicans* cells in distilled water corresponding to density 10 on MacFarland's scale was mixed with CM-CG solution (25 mg/mL in physiological buffer) 1:1 (v:v). This mixture was applied intravaginally to the group of 7 rabbits once daily for 7 days (group A). Another group of 7 animals was infected with *C. albicans* according to the same time schedule (group B). The third group of 7 animals were treated with CM-CG alone (group C). After the 7th day, the vaginal swab was taken from all animals, that was later cultivated for 7 days in Sabouraud's medium with glucose (the control swab was taken from all 21 animals before the test).

Plant antiviral assay.

Leaves of bean (*Phaseolus vulgaris* L. cv. Haricot Garonel) and cucumber cotyledons (*Cucumis sativus* L. cv. Laura) were treated with CM-CG solutions (0.0005-0.5%) 24 h prior to inoculation with Tobacco Necrosis Virus (TNV) in 500 μg/ml concentration. The protective effect of CM-CG preparation was evaluated on the basis of comparison of the number of the local viral lesions that appeared on the cotyledons or leaves of the pretreated and untreated control plants (leaves treated with distilled water). The percentage of inhibition was calculated according to the formula suggested by Kopp *et al.*[8].

Results and discussion

The HPLC analysis of the original CM-CG complex and of the high-intensity sonicated CM-CG samples revealed the bimodal elution profiles of all samples containing higher and lower molecular weight components. As evident from Fig. 1, already after a short-time ultrasonication (≈ 20 min), the M_{peak} value of the earlier eluted high-molecular-weight component, equaling in the initial CM-CG to 650 kDa, has increased up to 680 kDa and did not change significantly upon further treatment (Fig. 1, curve A). On the other hand, the prolonged ultrasonication led to a continual decrease of the M_{peak} value of the later eluted component (Fig. 1, curve B). The molecular-weight averages (M_w and M_n) as well as the carbon, hydrogen, and nitrogen contents of the ultrasonicated CM-CG samples are listed in Table I. It should be pointed out that while the nitrogen content of the original CM-CG complex was 1.61 %, in the high-molecular-weight component of the final 60-min ultrasonicated sample that was equal to 3.89 %. On the other hand, in the low-molecular-weight component the content of nitrogen (1.67 %) was comparable to the value found in the original CM-CG complex.

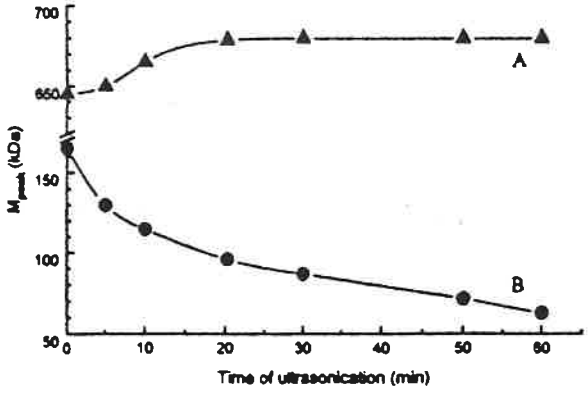

Fig. 1. Molecular weight (M_{peak}) dependence on the duration of ultrasonication. Curve A relates to an earlier eluted component of CM-CG, curve B corresponds to the later eluted one

Table I. The carbon, hydrogen, and nitrogen contents and the molecular-weight averages of the ultrasonicated CM-CG samples

	High-molecular-weight component					Low-molecular-weight component				
Time of sonication (min)	C(%)	H(%)	N(%)	M_w (kDa)	M_n (kDa)	C(%)	H(%)	N(%)	M_w (kDa)	M_n (kDa)
5	42.9	6.21	2.84	650	640	37.9	6.31	1.75	138	95
10	39.8	5.78	3.02	662	654	37.1	6.24	1.69	120	85
20	39.8	5.79	3.46	680	670	36.8	6.09	1.66	101	72
30	39.9	5.78	3.56	680	671	36.1	6.10	1.50	95	69
60	39.8	5.69	3.89	680	670	36.9	6.12	1.67	75	57

The gel filtration record of the final 60-min ultrasonicated CM-CG sample is depicted in Fig. 2. As can be seen from this record, Sample I is represented by a narrower elution curve than the one corresponding to Sample II. This finding correlates well with the polydispersity values (M_w /M_n) of ca. 1.01 and ca. 1.32 (*cf.* data in Table I) calculated for the high- and low-molecular weight components of the final 60-min ultrasonicated CM-CG complex, respectively.

The quantitative estimation of the glucan:chitin ratio in the Samples I and II was carried out on the basis of their ^{13}C NMR spectra (Fig. 2). The glucan component in the spectrum is represented by its characteristic signal of C-3 at ca. 86 ppm, while the chitin component is represented by the signal of N-linked C-2 at ca. 56 ppm. The C-2 signal of the chitin component has a shape of a doublet due to the partial N-acetylation of the aminogroup. The integration of the specified signals revealed that the content of chitin in Sample I was 27.1 %, while in Sample II its content was only 13.8 %.

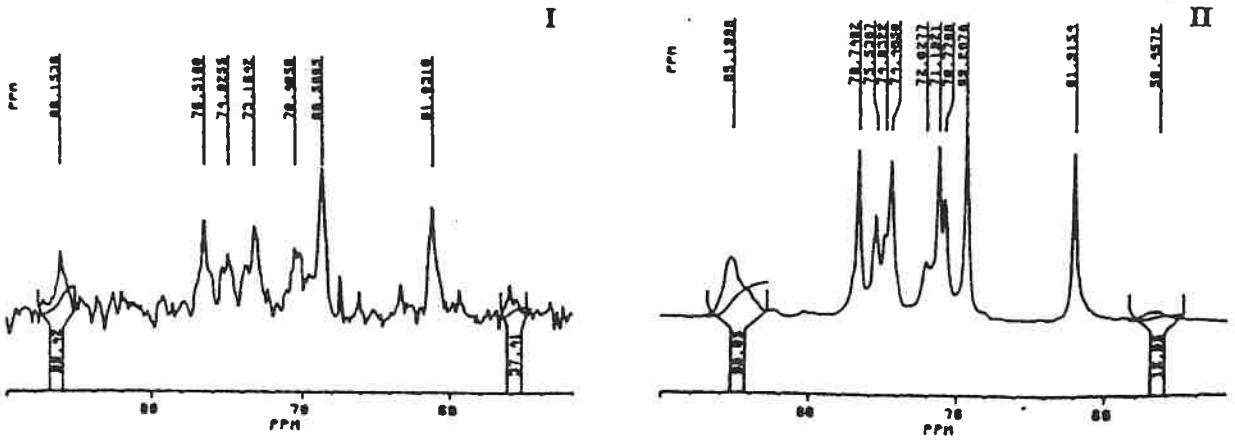

Fig. 2. ^{13}C NMR spectra of the Sample I (I) and Sample II (II)

Our results demonstrate that ultrasonication was efficient for depolymerization of the CM-CG complex prepared from the cell walls of *Aspergillus niger*. The original CM-CG complex was shown to contain two components . Although the bicomponental character of CM-CG complex was preserved through the 60 min of ultrasonication (Fig. 1), changes in the certain parameters (*e.g.* nitrogen content, M_w and M_{peak} values) were observed in the higher and lower molecular weight components (Samples I and II, respectively) (Table I). The rise of the M_{peak} value, *i.e.* the increase of the hydrodynamic parameter of the hydrated macromolecule within the first 20 min of sonication (Fig. 1., curve A) might have resulted from the unwinding of the biopolymer chains due to the action of ultrasonication[9]. The M_{peak} value of the low-molecular-weight component of the CM-CG complex decreased significantly (Fig. 1, curve B), while its content of nitrogen remained almost unchanged with the extended ultrasonication (Table I).

The observed consistent increase of nitrogen content in the high-molecular-weight component with the prolongation of the ultrasonic treatment (Table I) implies that the ultrasonication caused a preferential depolymerization of the glucan component of the CM-CG complex. Degradation of the polymeric glucan to the oligomeric molecules and their subsequent elution in the Sample II resulted in accumulation of chitin component in Sample I, which was observed as a relative increase of the nitrogen content. It is very plausible that in the chitin-glucan complex the linear rod-like chitin chain segments form a more crystalline structure[10], while the helical chains of the branched (1→3, 1→6)-β-D-glucan constitute a more amorphous moiety[11] which is more susceptible to the depolymerizing action of the ultrasonication. The glucan chains are most probably initially deprived of the most exposed (1→6)-linked side chains which are degraded to the mono- and oligosaccharides that were subsequently removed by dialysis, while the larger glucan fragments remained in the dialysate and were eluted at HPLC or gel-filtration together with the degraded chitin fragments in the lower-molecular-weight component (Sample II).

The comparison of the ^{13}C NMR spectra of the Samples I and II (Fig. 2) indicates that Sample II has lower molecular weight which is evident from the narrower shape of the signals in its spectrum. The spectra also provide information on the heterogeneity in N-acetylation of the amino group in chitin component (which results in doublet appearance of the signal of N-linked C-2 atom at ca. 56 ppm) as well as on the relative content of chitin in both samples. Since the signal at ca. 86 ppm is unambigously ascribable to C-3 of (1→3)-β-D-glucan[12] and the signal at ca. 56 ppm is known to correspond to C-2 of N-acetyl-D-glucosamine[13,14], relative intensities of these two signals can be used for evaluation of the chitin content in the samples. Integration of these signals indicated that chitin content in Sample I was 27.1 %, while in Sample II it was 13.8 %. In other words, Sample I contained approximately twice as much chitin as Sample II. Comparison of the data on the nitrogen content obtained by means of the elemental analysis (Table I) shows that the ratio of the chitin contents in Samples I and II is 2.3, which is in quite reasonable agreement with the spectrometric data. These results indicate that ^{13}C NMR spectroscopy may be used for the off-line monitoring of the chromatographic separation of different polysaccharides.

The CM-CG preparation was tested for inhibition of the clastogenic effect of cyclophosphamide.[6] The effects of i.v. and i.p. pretreatment with CM-CG on the frequency of micronuclei in peripheral blood reticulocytes induced by CP are demonstrated in Table I. The frequencies of micronucleated reticulocytes (MNRETs) in CM-CG treated animals were close to those observed in the saline-treated control group. Both ways of CM-CG administration, i.p. 24 h or i.v. 1 h prior to CP injection significantly reduced its clastogenic effect. The effect of CM-CG was concentration-dependent, since the higher dose (100 mg/kg b.w.) had a more pronounced effect than the lower dose (50 mg/kg b.w.).

Table II. Frequencies of MNRETs in peripheral blood of CP-treated female ICR mice injected i.p. or i.v. with non-degraded high-molecular weight CM-CG[6]

Group	MNRETs/1000	
	Individual animal data	Group mean (±SD)
Control saline	1,2,3,1,1,0,2,1	1.38 ± 0.92
CM-CG 50 mg/kg	1,1,0,3,0,1,3,3	1.50 ± 1.31
CM-CG 100 mg/kg	0,0,1,3,2,1,1,2	1.25 ± 1.04
CP	63,68,60,71,68,62,71,66	66.13 ± 4.12[a]
CP + CM-CG 50 mg/kg i.v.	64,59,43,77,51,45,57,51	55.88 ± 11.05[a,b]
CP + CM-CG 50 mg/kg i.p.	51,60,61,50,52,39,48,60	52.63 ± 7.52[a,c]
CP + CM-CG 100 mg/kg i.v.	40,38,45,40,43,43,43,36	41.00 ± 3.02[a,d,e]
CP + CM-CG 100 mg/kg i.p.	39,43,38,39,41,43,41,42	40.75 ± 1.91[a,d,e]

[a] Significantly higher than control groups (saline and CM-CG)
[b] Significantly lower than CP ($P < 0.05$)
[c] Significantly lower than CP ($P < 0.01$)
[d] Significantly lower than CP ($P < 0.0002$)
[e] Significantly lower than CP + CM-CG 50 mg/kg i.p. and i.v. ($P < 0.01$)

The results of the inhibition tests of adherence of the virulent *Candida albicans* cells to human buccal and vaginal epithelia are summarized in Table II.

Table III. Inhibition of the adherence of *C. albicans* cells to the human epithelial cells by non-degraded high-molecular weight CM-CG

Epithelium	CM-CG concentration (mg/mL)	% inhibition
Buccal	6.25	84.14
Buccal	12.50	85.46
Buccal	25.00	86.44
Vaginal	25.00	84.07

The results indicate that CM-CG is a potent inhibitor of the adhesion of the virulent candidal cells to the human buccal and vaginal epithelial cells. The inhibition effect was of a comparable magnitude for the buccal and vaginal human epithelium and increased slightly with the concentration of CM-CG in the case of the human buccal epithelium cells. These data imply that the preparations

based on CM-CG might be used in the prevention or treatment of the mycotic infections.
This suggestion was experimentally proven by *in vivo* tests of prevention of *C. albicans* vaginal infection in rabbits. In the control non-treated group of rabbits (group B, see section Materials and Methods) all animals showed positive mycotic infection and suffered from vaginal discharge, while in the group of animals in which the infective agent was applied together with CM-CG (group A) and in the animals treated with CM-CG only (group C) the mycological medical finding was negative.

Figures 3 and 4 demonstrate the inhibition effect of the CM-CG fractions on the development of the viral lesions on the leaves of bean and cucumber, respectively. As can be seen, both tested fractions revealed high concentration-dependent inhibition activity, while the highest values of inhibition (97 %) were obtained using either of the Samples I or II in 1.5 mg/mL concentration for the pretreatment of the bean leaves. Although the precise mechanism of the protective action of the polysaccharides in plants is not known yet and may involve increased production of some enzymes, such as peroxidase and chitinase, induction of phytoalexins or other metabolites[15], it is obvious that water solutions of CM-CG could be used for antiviral plant protection.

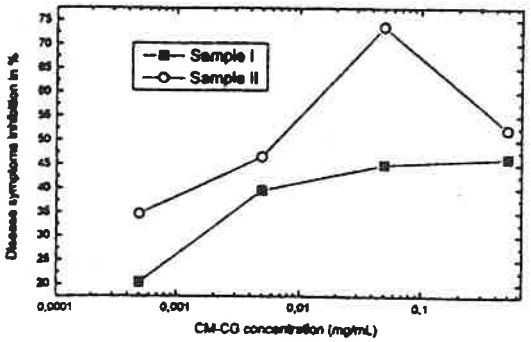

Fig. 3. Influence of CM-CG on development of disease symptoms induced by TNV on bean leaves

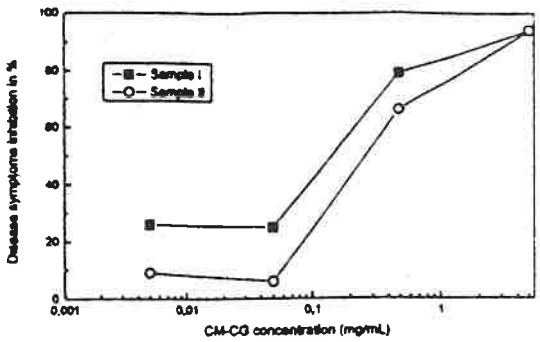

Fig. 4. Influence of CM-CG on development of disease symptoms induced by TNVon cucumber cotyledons

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CHITIN HEALTH PRODUCT «MYCOTON» PRODUCED FROM FUNGI

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Abstract

A new chitin-containing material named MYCOTON is produced from *Higher Basidiomycetes*. The material consists of chitin, glucans and melanins. Tests on laboratory animals revealed absence of any toxicity. The clinical tests were carried out on the base of the "1st Municipal Hospital" and the "Center of Liver Surgery" in Kiev. MYCOTON is used as an universally sanitation and preventive remedy - *Natural Health Product*. None negative case was observed at 7 year the use. MYCOTON has demonstrated almost all wonderful therapeutic properties described for chitin and chitosan. In certain cases these properties are explicitly intensified by the presence of β-glucans and melanins in it. MYCOTON is able to remove various food poisonings as well as negative impacts of intensive medicinal chemotherapy. It improves considerably the health of people who received high radiation doses. Application of MYCOTON in clinic as a hepatoprotector reduces general intoxication. It is very effective in the case of gastrointestinal pains and disturbances, pains in the liver and kidneys. MYCOTON also removes the allergic reactions, pimples and abscesses on a face and a body.

Keywords: Chitin, fungi, wound dressing, hepatoprotector, enterosorbent, immunomodulator, health product.

Materials and methods

The chitin-containing fiber material MYCOTON is produced from the mycelial mass of *Higher Basidiomycetes* (mainly species of *Aphyllophorales*) (1). The diameter of fibers is 3 - 5 μ, the length - up to 20 mm. The material consists of chitin - 60 - 95%, glucans - 5 - 35% and melanins - up to 10%. The content of ash elements is less than 0.1% (2). The fibers possess good elasticity and strength both in the humid and dry states. It is possible to obtain the cotton-like material, to produce the nonwoven dressings both in a pure form and with other fibers.

The cotton-like material and the nonwoven sheets of 0.1, 0.2 and 0.5 mm thick were used in the clinic of the "1st Municipal Hospital" for treatment of wounds and ulcers of patients suffering from diabetes mellitus. Patients with hepar insufficiency were treated in the "Center of Liver Surgery" by the powder MYCOTON *per os* 10 days in a dose of 0.5 g 3 - 6 times daily. Patients of the control group were treated by a method with the use of *Hemodez* (*Neocompensan*) (3). Clinical researches and laboratory tests of the blood biochemical parameters, percentage content of various bile acids, parity of bile acids among themselves were criteria of a conducted detoxication therapy (4).

A study of a radionuclids extraction by MYCOTON was carried out on 3 age groups of people, 10 persons in each, residing in the radioactive contamination regions. The measurements were performed by a human radiation spectrometer, simultaneously analyses of urine was made.

Results

MYCOTON as a wound dressings. The application of chitin for wound healing as powder (5-7) or its introduction into the ointments (8, 9) are the simplest procedures. Sheet bandages are manufactured as films, sponges, artificial skin, nonwoven and woven dressings. The process for obtaining such materials involves the stages of dissolution of chitin or chitosan, stretches of fibers, their precipitation, cutting into pieces and sheet casting by paper-making machine (10-13). In most of patents either chitin or chitosan are proposed to be used in compositions with other fibers. There are patents authors of which propose a fabric from chitin or chitosan threads and woven sheets for wound healing (14,15).

Fungi are known to be the other source of chitin for the production of dressings. The main advantage of fungi consists in the fact that their biomass has natural fiber (hyphal) structure. Chitin is present in cell walls of hyphae. After treatment cell walls may preserve the fiber structure. It is possible to make films of them (16) or to produce nonwoven dressings (17). The main disadvantage of the latter is the absence of elasticity in the dry state. This fact prohibits to use such fungal chitin materials in a pure form and other elastic fibers are added or humid hyphal mass is coated on to an elastic support (18-20) or fungi are growing on the nutrient medium in which the matrix of synthetic fibers is inserted (21).

Our studies are focused on the elaboration and trials of chitin dressings from fungal biomass. The obtained material has passed the full test programm on laboratory

animals and has received a permission for clinical trials. MYCOTON can be used for healing wounds in different forms. Cotton-like material and the nonwouven napkins to be the most perspective forms from a practical point of view. They have good hygroscopicity (20 ml/g) and high drainage ability (100 mm). MYCOTON has a high bacteriostatic effect. Its major advantage is the ability to biodegrade completely in a body. MYCOTON is an atraumatic remedy.

The treatment of fresh wounds and burns by MYCOTON differs in simplicity and efficiency. For treatment it is necessary to apply to the damaged surface a layer of the cotton-like material of 2 - 3 mm thick, to cover with a napkin and to fix with a bandage. MYCOTON easily stops the bleeding, removes pain, prevents inflammatory processes and suppuration. The complete healing usually occurs for 5 - 7 days. In the case of skin-deep damages it is possible to use efficiency the nonwoven sheet dressings. The most significant advantage of MYCOTON consists in the fact that repeated dressings are not needed. Wounds and burns heal quickly and without traces of scars.

Of great interest is the use of MYCOTON for treatment of unhealing trophic ulcers which usually appear in patients suffering from diabetes. Such purulent wounds are not often treatable with antibiotics. The clinical trials were conducted on 31 patients with the severe manifestation of ulcers. In most cases the cotton-like material is convenient for healing the purulent ulcers. Originally wound was filled in densely with cotton-like MYCOTON, closed by bandage and then left for a day. Then the derivated fuse of the material and purulent contents were taken out from a wound. The cleared surface of a wound was covered by a lamina of MYCOTON for a day and then the material was substituted by a fresh portion. Epithelisation of a wound surface occurs during 5 - 7 days and an ulcer is healed without leaving noticeable traces on a body. The microbiological tests have shown that the surface of ulcers after the contact with MYCOTON is sterile. It inhibits the development of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and some other bacteria usually stable to antibiotics. Traditional methods of treatment of such unhealing trophic ulcers in control group allow to rescue from partial amputation of lower limbs only 30% of the patients suffering from diabetes. The application of MYCOTON increases a percentage of recovery up to 68%.

MYCOTON as a hepatoprotector. MYCOTON was used by the "Center of Liver Surgery" for clinic treatment of such diseases as: hepatic cirrhosis, cancer intoxication, liver-kidneys

insufficiency tumour and non-tumour genesis. The clinical effect of MYCOTON was tested on 28 patients. Stabilization of hemodynamics, reduce hypodynamia, and obvious increase of appetite were observed in all patients on the 4th day.

Blood biochemical parameters are: the content of total bilirubin (TB), direct bilirubin (DB), indirect bilirubin (IB), alanine transaminase (AlT), asparagin transaminase (AsT), alkaline phosphatase (AP), lactate dehydrogenase (LD), serum urea (SU), serum urea nitrogen (SUN), creatinin, total protein, serum albumins and globulins. They are indexes of the hepatic intoxication level. The outcomes of clinical trials are shown in Tab. 1. These data testify about a significant fall of the intoxication level in the patients.

Table 1

Dynamics of biochemical blood indexes after 4 day-treatment by MYCOTON

Blood index	Control group		MYCOTON treatment group	
	initial level	4 days later	initial level	4 days later
TB, *Mkmol/l*	105.4 ± 3.8	141.2 ± 2.9	112.2 ± 3.4	97.3 ± 3.1
DB, *Mkmol/l*	85.3 ± 3.7	98.2 ± 3.2	88.4 ± 2.9	76.3 ± 1.6
IB, *Mkmol/l*	20.1 ± 1.4	43.3 ± 1.3	24.3 ± 1.8	22.1 ± 2.1
AlT, *Mmol/h/l*	2.8 ± 0.06	3.2 ± 0.09	3.6 ± 0.08	3.1 ± 0.06
AsT, *Mmol/h/l*	2.0 ± 0.09	2.9 ± 0.06	2.2 ± 0.08	1.5 ± 0.09
AP, *Mmol/h/l*	3.4 ± 0.2	3.5 ± 0.3	3.5 ± 0.4	2.5 ± 0.5
LD, *Mmol/h/l*	3.9 ± 0.4	4.2 ± 0.3	4.1 ± 0.5	3.2 ± 0.4
SU, *Mmol/l*	9.1 ± 0.7	9.7 ± 0.5	8.9 ± 0.6	7.7 ± 0.6
SUN, *Mmol/l*	4.8 ± 0.4	4.7 ± 0.2	5.2 ± 0.3	4.3 ± 0.3
Creatinine, *Mmol/l*	0.25 ± 0.06	0.27 ± 0.03	0.31 ± 0.04	0.26 ± 0.05
Total protein,	62.9 ± 6.3	58.7 ± 3.1	61.8 ± 4.4	64.2 ± 2.8
albumins, g/l	55.1 ± 3.4	54.2 ± 3.1	54.8 ± 3.5	56.3 ± 3.8
globulins, g/l	45.0 ± 2.8	45.2 ± 2.4	45.4 ± 3.5	45.6 ± 2.6

Content of various bile acids are presented in Tab. 2.

Table 2

Dynamics of bile acids after 4 day-treatment by MYCOTON

Bile acids	Initial level, Mmol/l	4 days after, Mmol/l	Statistic, P
Taurocholic	5.83	6.40	0.1
Taurodeoxycholic	7.21	9.68	0.01
Glycocholic	20.67	16.76	0.01
Glycodeoxycholic	9.98	8.70	0.01
Cholic	5.50	11.04	0.01
Deoxycholic	16.79	20.14	0.05
Litocholic	36.55	44.93	0.01

MYCOTON as a Natural Health Product. In addition to the described cases of clinical tests, MYCOTON is used by orally for many years as an universally sanitation and preventive remedy, that is to say in the sense which is put into the Natural Health Product conception.

None negative case was observed for the 7 year period. MYCOTON has demonstrated almost all wonderful therapeutic properties described for chitin and their derivatives. In certain cases these properties are explicitly intensified by the presence of β-glucans and melanins in it.

First and foremost it ought to note that MYCOTON is able to remove various food and alcoholic poisonings as well as negative impacts of intensive medicinal chemotherapy.

MYCOTON improves considerably the health of people who received high radiation doses during their work at the Chernobyl Nuclear Power Plant and life in the contaminated zone. The oral application of MYCOTON for 10 days by 0,5 r 3 times daily allows reducing the content of radionuclids by 4 - 5 times (Fig. 1).

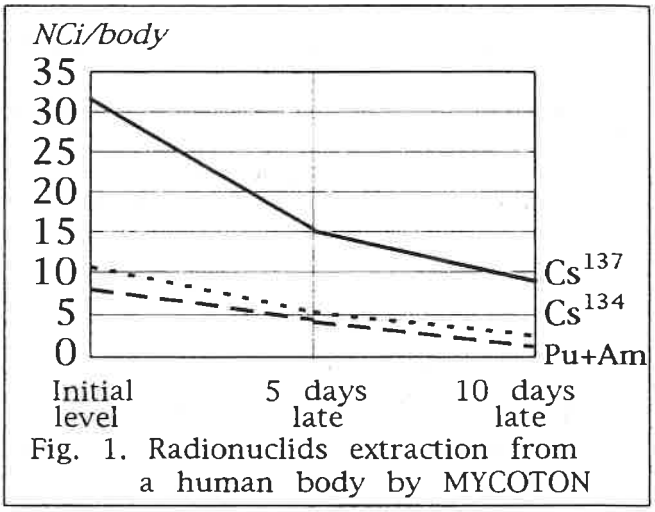

Fig. 1. Radionuclids extraction from a human body by MYCOTON

MYCOTON also removes the allergic reactions. The oral application within 4 - 5 days makes it possible to shake off pimples and abscesses on a face and a body. Of great interest is the influence of MYCOTON on elderly people. They mark a noticeable improvement of general condition, increase in cheerfulness, working capacity and decrease in cases of sick rate.

Discussion

A wide range of favorable influence of chitin and its derivatives, including our remedy MYCOTON, on the health forces us to think about the nature of its action, what is the health and how does chitin keep an organism in a good state? Undoubtedly, this is a very complex problem, but we can consider it in a very simplified scheme which is acceptable to our theme.

In our opinion the major factor of health is the level of toxins in a body. Many organic and inorganic compounds are delivered into a human organism. They are continuously transformed and during this process unhealthy and toxic substances are formed. This is the natural metabolic-waste

product source. Toxins may appear in an organism due to the development of pathogenic bacteria, viruses and fungi. Food-stuffs and water of low quality may also be the source of toxins. Also, many drugs may slag an organism under the chemotherapy. Other sources of toxins may exist as well. A metabolism between different parts of a body is realized by blood. Toxins from any source are also scattered by blood. If toxins are removed poorly, that most often leads to the disturbance of a health and to one or another disease.

In a body the functions for removal of toxins are performed by a gastrointestinal tract, liver and kidneys as well as by a skin and lungs which purify blood similar to filters. During evolution these organs were adapted to the definite natural toxin loading in a normal healthy people. In the last century an intensive technogenic pollution of the environment has begun. Many toxic chemical compounds, heavy metals and radionuclides are accumulating in the increasing quantity in a biological cycle of substances. So, a toxin loading increased significantly. The natural protective organs are not able to cope with all situations even in a healthy man and if the functions of the liver, kidneys or gastrointestinal tract are weakened or disturbed, then the situation may become more severe.

How can we help to our organism in the situation when it is impossible to return to the natural way of life? It is necessary to add a new filter to the natural organs. The blood-vascular system in a body is closed in all organs. It is open only in the gastrointestinal tract. From blood through the organs of endocrine secretion about 10 l of liquid (saliva, gastric juice, biliary juice, pancreas and intestine secretions) are secreted daily into the gastrointestinal tract and then almost the same quantity of liquid is sucked into blood. Thus, the permanent drainage of the blood liquid fraction leaks through the gastrointestinal tract. We believe the additional filter for purification of a body should be interposed in the path of this natural drainage. Chitin is the best material for such filter. First, it is nontoxic even in high doses. Second, chitin is a good sorbent of heavy metals, radionuclides, cholesterol and other toxins. Third, low-molecular-weight forms of chitin possess proper medical, immunomodulating, oncostatic and other properties. The known results on application of chitin as a component of dietary supplement testify to this fact (22).

MYCOTON may be considered as a more advanced material for a toxin filter as compared to chitin from crabs. MYCOTON is also nontoxic, but it possesses stronger sorption capacities. The advantage of the fungal chitin is established

by many researchers (23). We have studied sorption capacities of MYCOTON in detail on many chemical elements including radionuclides (2). Contrary to other enterosorbents MYCOTON does not absorb most of biogenic microelements (K, Na, Ca, Mg, etc.) and does not disturb the mineral metabolism. The unique high sorption properties of MYCOTON are achieved due to the natural fiber structure of fungal hyphae and the spatial microfibrillar chitin net which have preserved in it. By our calculations the active sorption surface of chitin microfibrils in 1 g of MYCOTON exceeds 1000 m^2. Glucan matrix assists to preserve the fiber structure of hyphae and the spatial arrangement of microfibrils. β-1,3 and β-1,6-glucans of cell walls of fungi are close to chitin by their chemical nature and manifest many analogous properties which are valuable for medicine (24). Due to the presence of glucans the immunomodulating, bactericidal and oncostatic properties are strengthened in MYCOTON. Melanins impart it essential advantages. Melanins are the best bioprotectors against active chemical radicals, UV- and penetrating radiation (25). Due to it MYCOTON possesses a higher bacteriostatic activity relative to many pathogenic bacteria.

Conclusion

The action of MYCOTON on health status may be explained for simplicity as follows:

- it sorbs various toxins from digestive juices inside of the gastrointestinal tract and removes them from a body;
- under the effect of digestive juices a part of chitin and glucans splits up to oligosaccharides and is imbibed into blood, thus stimulating the immune system;
- melanins are partially dissolved together with glucans and block the action of chemical radicals and inhibit the development of pathogenic bacteria.

Thus, MYCOTON fulfils three main tasks on preservation and restoration of the health: it removes toxins, inhibits a development of infections and stimulates the immune system. By its complex action MYCOTON completely corresponds to the Natural Health Product conception.

At present only basic prerequisites of MYCOTON are studied and it is necessary to carry out a great complex of biomedical investigations, first, to understand rather well its influence on a human organism and, second, to know well its disadvantages and to find ways for a further perfection.

Chitin-containing material MYCOTON has great perspectives for wide application in medicine both for a preventive maintenance and for treatment of many diseases.

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