Aspects of protein breakdown during the lactic acid fermentation of prawn waste

Keiko SHIRAI (1), Isabel GUERRERO-LEGARRETA(1), Gabriela RODRIGUEZ-SERRANO (1), Sergio HUERTA-OCHOA(1), Gerardo SAUCEDO-CASTANEDA(1) and George, M. HALL (2)

(1) *Universidad Autonoma Metropolitana, Department of Biotechnology, Av. Michoacan y Purisima s/n Col. Vicentina. C.P. 09340 Mexico, D.F. Fax (5) 7244712*
E-mail: smk@xanum.uam.mx
(2) *Loughborough University, Department of Chemical Engineering, Loughborough Leics. LE11 3TU. (01509) 223923. United Kingdom.*

Abstract
Approximately 30 to 50 % of prawn is considered as waste, such as heads, tails and skeleton. We are currently looking at the recovery of high added-value products from prawn waste which has been stabilised by lactic acid fermentation. The aim of this particular study is to investigate the recovery of proteases from fermented prawn waste using ultrafiltration and gel permeation chromatography (GPC). And the deproteinisation of prawn shell using these enzymes for chitin purification.

The prawn waste (*Penaeus sp.*) was fermented by adding glucose and inoculating with *Lactobacillus sp.* isolated from tropical prawns. Lactic acid fermentation was carried out to pH 4.5 at 48 hours. Following fermentation, the liquid fraction was centrifuged to remove insoluble matter (mainly chitin) and the liquid was freeze dried for storage.

The latter was resuspended in distilled water and subjected to ultrafiltration. The concentration and the fractionation of this liquid was performed using membrane with a nominal molecular weight cut off (NMWC) of 1×10^4 Daltons.

Proteolytic activity and protein concentration were established in permeate and retentate at each ultrafiltrafiltration step using haemoglobin and casein as substrates, at pH 2 and 7 respectively, at 30^0 C. The protein fractions showed higher proteolytic activity in the neutral and alkaline range.

In addition, protease detection on electrophoresis gels was performed. Clear zones were detected after destaining showing protease activity bands. The optimum pH for proteolytic activity was in the region of pH 7-8

The peaks with proteolytic activity on casein (pH 7) were produced by GPC showing molecular weight of 56100, 28300 and 14300 Daltons. Those fractions were obtained from the retentate of NMWC of 10,000 Da.

The fractions obtained from ultrafiltration process were applied during the deproteinisation step for chitin purification, commercial proteases and chemical treatment were also tried to compare the effectiveness of the methods.

Keywords: Chitin, Chitosan, Lactic Acid Fermentation, Proteases, Ultrafiltration, Protein Purification..

Introduction
The catching and farming of crustacea in the recent past has shown a constant growth, due to the important international market for these species. As a result, the production of waste, mostly shells, viscera and small amounts of left-over meat, has

increased. Currently, the destination of the waste produced is mainly landfills and dumping, causing environmental problems.

The prawn wastes can be fermented with lactic acid bacteria and glucose, as the carbon source. The main advantage of the fermentation is to extend the shelf life of the waste. Another advantage is the possibility of recovering added-value products, such as lipids, pigments, chitin and endogenous proteases.

The proteases play an important role in Biotechnology, Food, Clinical and Physiology. The traditional origin of proteases has been from plants, micro-organisms and animals (terrestrial). Marine enzymes have not been studied and applied as the others, although they are highly efficient at low temperatures, have poor thermal stability and are both active and stable at neutral to alkaline pH. Many applications have been proposed, for example the production of crustacean and fish hydrolysates, flavourings, production of peptones for bacteriological media and extraction of chitin.

Purified trypsin, chymotrypsin, collagenases, carboxypeptidases and leucin-aminopeptidases from prawn (*Penaeus monodon* and *Penaeus californiensis*), showed optimal conditions of pH and temperature, 6-10 and 50-65°C, respectively (Jiang *et al.*, 1991; Vega-Villasante *et al.*, 1993, 1995: Le Gal *et al.*, 1995).
The present study focused on the partial purification and application of endogenous proteases for chitin recovery from prawn wastes.

Materials and Methods

Prawn wastes
The prawn wastes were purchased from the main seafood market in Mexico City. The waste was a mixture of several species of *Penaeus,* such as *aztecus, californiensis, vanamei.* The wastes were heads, that were minced and stored at -20° C.

Ensilation.
The prawn wastes were mixed with glucose, 10% w/w (wet basis) (Baker, Mexico), and 5% v/w (wet basis) of a culture of *Lactobacillus sp.* grown into APT broth (Difco, USA), 1×10^8 cfu/ml, as starter. The fermentation was carried out at 30° C over 48 hours at pH 4.5. After the fermentation the liquor obtained was separated by filtration through a sieve. This liquid fraction was centrifuged at 12,000 rpm at 4°C, 15 minutes (Beckman USA), the supernatant was freeze dried and stored at 10°C.

Partial purification of proteases from prawn silage.
Ultrafiltration.
The membrane used had nominal molecular weight cut off of 1×10^4. The total membrane area was 33.18 cm², the pressure was 1.0 bar and the feed rate of 4.5 mlmin⁻¹.

Proteolytic Activity Assay
Haemoglobin (Sigma, USA) and casein (Merck, USA) were used as substrates at concentration of 2% (w/v) at 30° C for 20 minutes. The reaction was stopped with

trichloroacetic acid 5% (w/v). The proteolytic activity unit was defined according to that of Kunitz (1947), *"the amount of enzyme that produces a change in the absorbance at 280 nm of 0.001 per minute at the conditions described"*.

Protein determination

The concentration of protein was determined by the following two methods:

i)Bio-Rad protein assay (Bio-Rad USA), this method was used in fractions collected from GPC.

ii) Lowry-Peterson method (Peterson, 1977),

In both methods bovine serum albumin (Sigma, USA) was used as the protein standard.

Gel Permeation Chromatography (GPC)

Sephacryl High Resolution 300 with a molecular weight range of 10-1000 kDaltons was used as packing into a column of 2 cm (inner diameter) and 28 cm (height) with a programmable and controller LCC-500, fraction collector Frac-100, peristaltic pump P-500, detector UV-1 and twin channel recorder 482 (Pharmacia Sweden). The mobile phase was phosphate buffer 0.05M and 0.15M sodium chloride at pH 7. For the molecular weight determination using GPC, the following known molecular weight compounds were used as standards: Dextran blue, Lactate dehydrogenase, bovine serum albumin, egg albumin, cytochrome C, tryptophan and tyrosine (Sigma USA).

Electrophoresis

Proteolytic Activity on to gels (Garcia-Carreño *et al.*, 1993).

On completion of the electrophoresis, the gel was immersed into casein 2% (w/v) 50mM of tris/HCl pH 7.0, during 30 minutes at 5°C , and incubated at 30°C 1 hour. The gels were fixed and stained with Coomassie Blue R250.

Chitin extraction

Demineralisation

The wastes of *Penaeus monodon* from Sri Lanka were minced and dried at 105°C. Later it was ground and sieved through a mesh (Tyler no. 100mm). This powder was demineralised with hydrochloric acid 1.2 M and washed until pH 7.0.

Enzymatic deproteinisation

After demineralisation, the waste was treated with enzymes: prawn proteases fractionated by ultrafiltration (membrane NMWCO 10,000 Daltons), trypsin from beef pancreas (BDH UK). The conditions carried out were for the former at 30°C and the latter at 50° C in a phosphate buffer 0.01M pH 7. The sampling was done at 24 hours and 48 hours.

The waste treated with the enzymes was filtered and total nitrogen determined by Kjeldahl, according to the technique performed by Black and Schwartz (1950).

The soluble protein concentration in the liquid was determined by Peterson (1977)

Results and Discussions

The lactic acid fermentation for extending the shelf life of Mexican prawn wastes was previously presented (Shirai et al., 1996). During this step, the enzymatic activity and acidification produced a liquor with a pH of 5.5.

The protein concentration and proteolytic activity of that liquor, as well as, the fractions from ultrafiltration, are shown at the Table 1. The specific activity obtained in the initial and fractions showed that the majority of the enzymes were in the retentate fraction, indicating a concentration of those active molecules.

Fraction	Protein Total (mg)	Total Activity (Units)		Specific Activity (Units/mg)	
		Casein	Haemoglobin	Casein	Haemoglobin
Initial	672	9317	2340	13.9	3.5
Retentate	544	969	136	46.1	6.5
Permeate	21	4240	1760	7.8	3.2

Table 1. Purification of endogenous proteases from prawn fermented heads. Activity on casein at pH 7 and haemoglobin at pH 2 of fractions from ultrafiltration performed at NMWC of 10,000 (YM10).

Comparing the activity at both pH, at pH 7 the activity was 2-7 fold higher than pH 2. According to of Vega-Villasante et al. (1995), the pH properties of digestive proteases from prawn of genus Penaeus, are around 8 or, depending on the species, slightly less alkaline. However, it is worth considering, those enzymes reported active at acid pH in another crustaceans: land crab (O'Brien and Skinner 1988) and squid (Hameed and Haard, 1985), which could explain the activity at acid pH.

Molecular weight determination of ultrafiltration fractions.

The molecular weight spectrum was determined for the initial liquor, retentate and permeate, as it is shown in the Figure 1. The aim of the use of the ultrafiltration was to clear the liquor of other compounds that do not show proteolytic activity.

Many of the compounds with a molecular weight higher or equal to 10 KD were retained, nevertheless in the permeate some compounds detected were larger than 10 KD, which explains the proteolytic activity found in the permeate (Table 1).

The shape of the molecule passing through the membrane, can affect the efficiency of the membrane for separation of proteins according to the molecular weight. Additionally, the pore size of the membranes will vary in the manufacture influencing the separation (Harris and Angal, 1990).
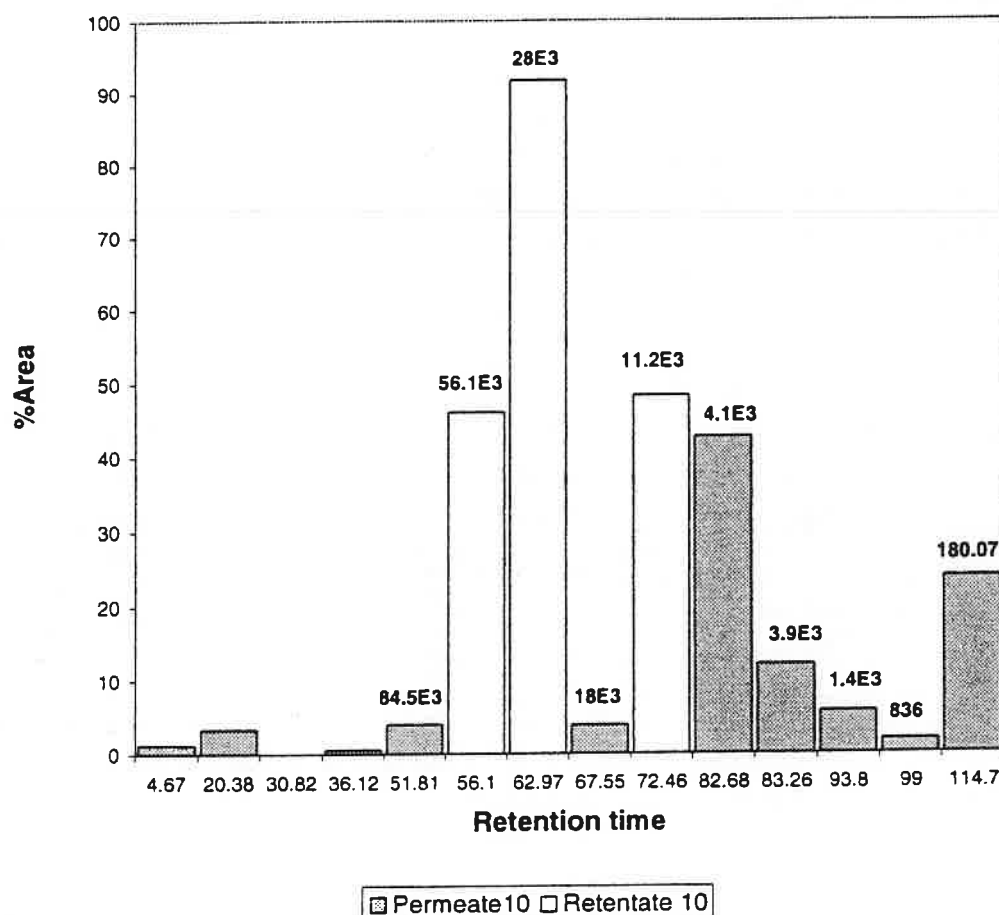
Figure 1. Molecular weight distribution of proteins from ultrafiltration fractions, permeate and retentate at NMWC 10,000 (YM10).

In Figure 2 is shown the results obtained from GPC for the retentate with protein amount recorded as absorbance at 280 nm. The specific activity was shown in the fractions corresponding to 56 KD, 26 KD and 13 KD. The specific activity was again higher at neutral pH in the fractions collected from GPC, (5-fold higher than pH 2). The proteolytic activity determination on electrophoresis gels showed that more clear zones were detected for the retentate than for the permeate, but light clear zones were also found at the band corresponding to 14KD for both.

There are in the literature, some works that reported several enzymes in this range of molecular weight. In *Pleuroncodes planipes* proteases with molecular weight from 16 to 65 KD were identified as serine proteinases and metal proteases (Garcia Carreno and Hernandez Cortes, 1995); Trypsin-like enzymes were found in *Euphasia superba*, the molecular weight determined was around 24 - 28 kD; *Penaeus monodon* has shown digestive proteases in the range of 18.5 kD to 50 kD (Jian *et al.*, 1991). The peak corresponding to 26 KD could be trypsin-like, on the basis of molecular weight, pH of activity but it is necessary to determine other characteristics, such as effect of inhibitors or activators.
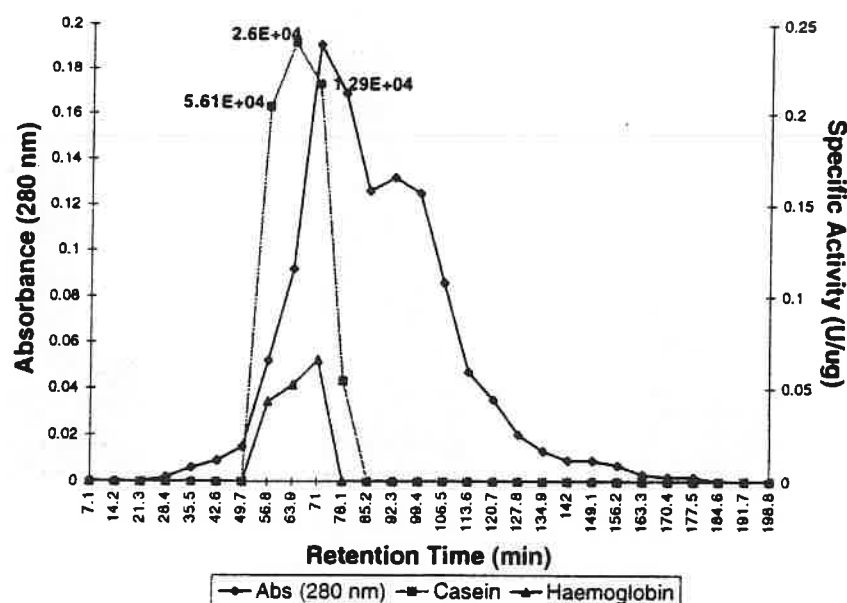
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Figure 2. Gel Permeation Chromatography of ultrafiltration fraction, retentate of NMWC 10,000 membrane. Specific activity on casein at pH 7 and haemoglobin at pH 2.

Later, the application of the retentate was done on prawn waste as a substrate. The percentage of soluble protein released after 48 hours of reaction, for the waste mixed with retentate, trypsin and without enzyme, were 63.2, 57, and 6.3 %, respectively (Figure 3). The time after 24 hours was not significantly different and the addition of enzyme improved considerably the deproteinisation. There were a decrease in the total nitrogen content in the sediment, that was related to the loss of protein during the deproteinisation (Figure 4). The advantage in the use of enzymes for deproteinisation is the specificity, which avoids depolymerisation and other adverse effects on the chitin quality.
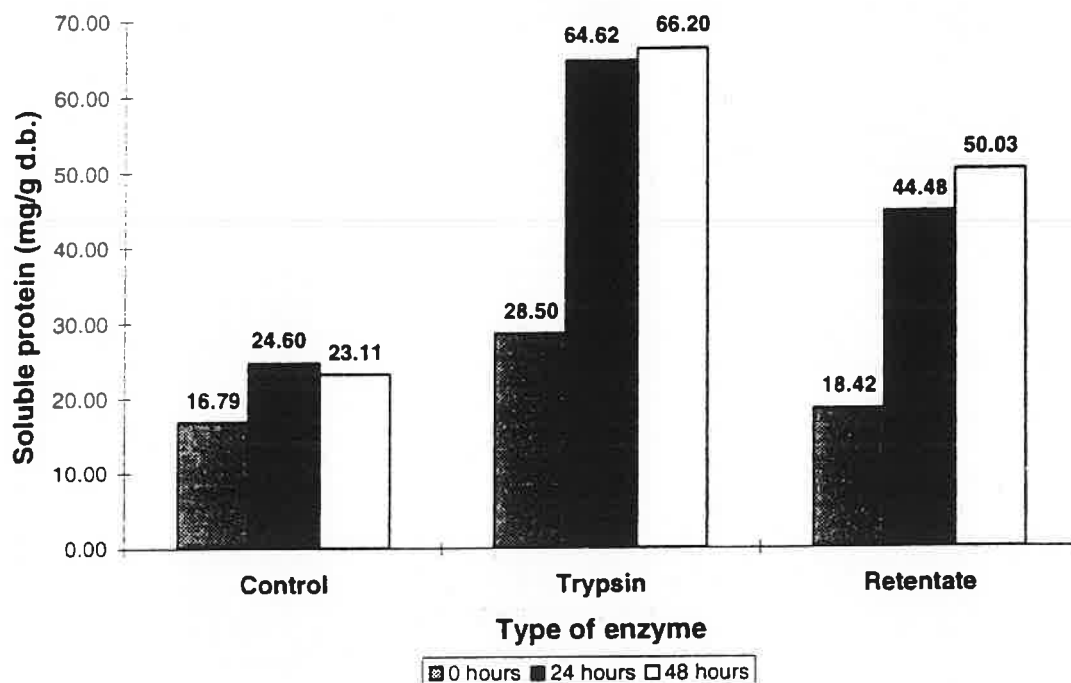
Figure 3. Enzymatic deproteinisation using trypsin from beef pancreas and retentate. Soluble protein determined by method of Peterson (1977).
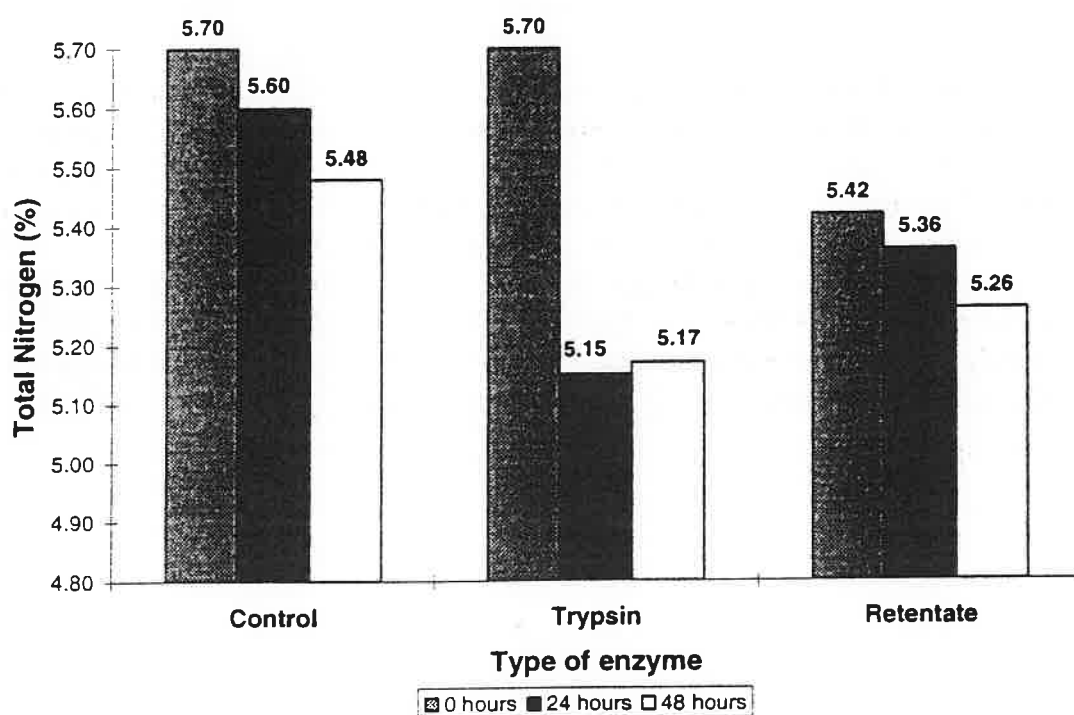

Figure 4. Enzymatic deproteinisation using trypsin from beef pancreas and retentate. Total nitrogen in sediment determined by method of Black and Schwartz (1950).

Conclusions

The present work has shown the feasibility of the recovery of proteases from lactic acid fermentation prawn wastes, these enzymes can be applied for the extraction of chitin.

Acknowledgements

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CORRELATION BETWEEN CHARACTERISTIC PROPERTIES OF CHITOSAN

Anna WOJTASZ-PAJĄK and Maciej M. BRZESKI

Sea Fisheries Institute, Kołłątaja 1, 81-332, Gdynia, (POLAND). Fax:(058) 20 28 31
E. Mail: biopak@mir.gdynia.pl

Abstract

A series of chitosans was obtained from the shells of common shrimp (*Crangon crangon* [L.]). Deacetylation of raw chitin was conducted by means of 50% water solution of sodium hydroxide. The time of reaction varied from 30 to 180 minutes and temperature from 100 to 140°C. Qualitative analysis of chitosans makes it possible to find mathematical correlation between the average viscometric molecular weight, viscosity of standard solution and deacetylation degree of the polymer:

$$\overline{M}_{\eta 2} = a + \left[b \cdot \exp\left(\frac{c}{\eta^d} \right) \right] \cdot \left(e \cdot DD^f \right)$$

where $\overline{M}_{\eta 2}$ is the average viscometric molecular weight, η is the viscosity of the standard solution [mPa·s], DD is the deacetylation degree [%] and a, b, c, d, e, f are coefficients.
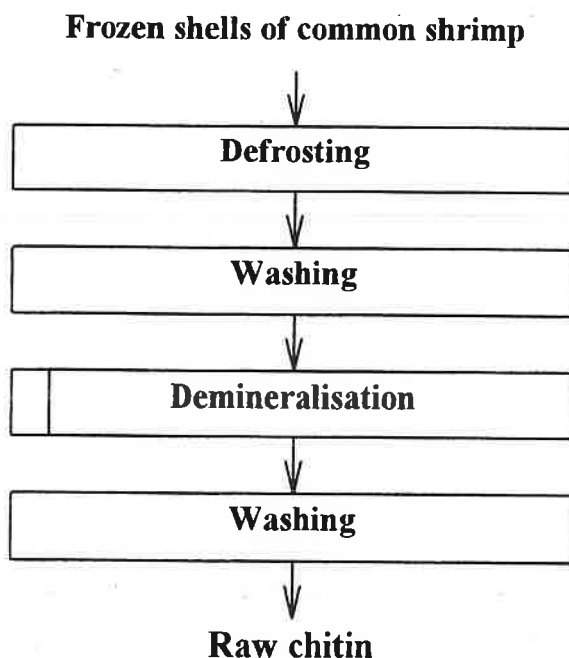
Keywords: chitosan, deacetylation degree, average viscometric molecular weight, viscosity, correlation

Materials and methods

Tests were carried out using the shells of common shrimp (*Crangon crangon* [L.]) as raw material. Dry weight of raw material was 35±1.2%, in which carapace constituted 40±2,3%; ash 28±1,5% and lipids 5±0,5%. Raw material was processed according to the flow diagram in *Figure 1* to obtain raw chitin.
Raw chitin was deacetylated by means of 50% water solution of sodium hydroxide in a single or double operation. Each step of repeated deacetylation was separated by washing. The ratio of the raw material to the NaOH solution was 1:10. The temperature of the reaction of deacetylation was changed from 100 to 140°C, and the time from 30 to 180 minutes..
Determination of water and ash content, deacetylation degree and viscosity of standard chitosan solution was conducted according to Polish Standard PN-89/A-86850[1]; the carapace content was determined according to Polish Standard PN-89/A-86790[2]. Lipids were determined by Bligh-Dyer[3] method. The average viscometric molecular weight was determined by Roberts and Domszy method[4].

Frozen shells of common shrimp

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        ↓
┌─────────────────────────────┐
│        Defrosting           │
└─────────────────────────────┘
        ↓
┌─────────────────────────────┐
│         Washing             │
└─────────────────────────────┘
        ↓
┌─────────────────────────────┐
│      Demineralisation       │
└─────────────────────────────┘
        ↓
┌─────────────────────────────┐
│         Washing             │
└─────────────────────────────┘
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Raw chitin

Figure 1 Flow diagram of raw chitin production from shells
of common shrimp (*Crangon crangon* [L.])

Results and discussion

As a result of the application of changing parameters of deacetylation, series of chitosans of the physical and chemical properties presented in Table 1 were produced.

Table 1. Physical and chemical properties of chitosans

Dry weight [%][a]	98.4 ± 0.61
Ash [% of dry weight][a]	0.18 ± 0.099
Deacetylation degree [%][b]	62 ÷ 90
Viscosity of standard solution of chitosan [mPa·s]	10 ÷ 1550
Average viscometric molecular weight[b]	337,000 ÷ 1,719,000

[a] average from n=57 ± standard deviation
[b] range for n=57

Based on experimental data, the relationship linking the average viscometric molecular weight to the deacetylation degree and the viscosity of standard solution of chitosan was determined. The derived equation is as follows:

$$\overline{M}_{\eta 2} = a + \left[b \cdot \exp\left(\frac{c}{\eta^d} \right) \right] \cdot \left(e \cdot DD^f \right)$$
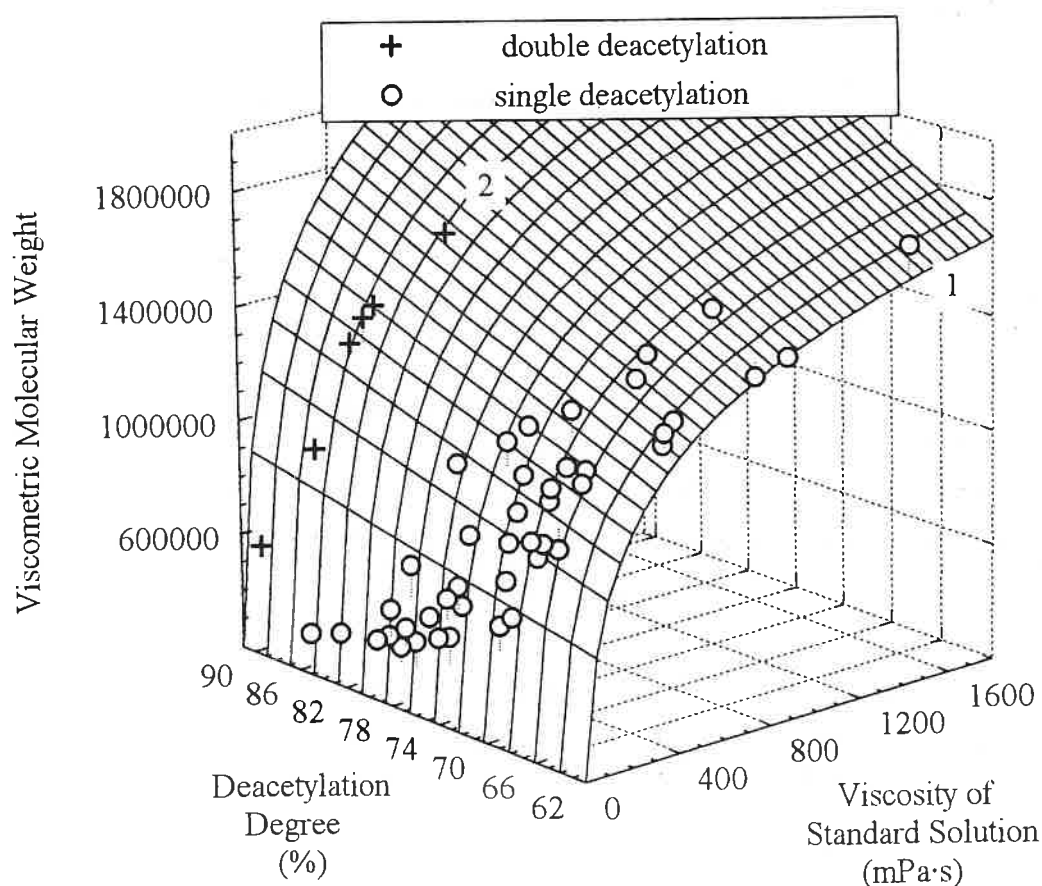
where $\overline{M}_{\eta 2}$ is the average viscometric molecular weight, η is the viscosity of the standard solution [mPa·s], DD is the deacetylation degree [%] and a, b, c, d, e, f are coefficients[5].

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The coefficients of the equation are presented in Table 2.

Table 2. Coefficients occurring in the formulated equation

| Coefficient	
a	7,47E+04
b	2,35E+02
c	-4,91E+00
d	2,52E-01
e	2,44E+02
f	9,80E-01

Diagram of the relationship between the average viscometric molecular weight and the viscosity of the standard solution and the deacetylation degree $\overline{M}_{\eta 2} = f(\eta, DD)$ is shown in *Figure 2*. Correlation between predicted and observed values and distribution of remainders of variable average viscometric molecular weight for the above relationship (*Figure 2*) is shown in *Figure 3* and *Figure 4*, respectively.

Figure 2   Relationship between the average viscometric molecular weight and the deacetylation degree and viscosity of standard solution of common shrimp (*Crangon crangon* [L.]) chitosan
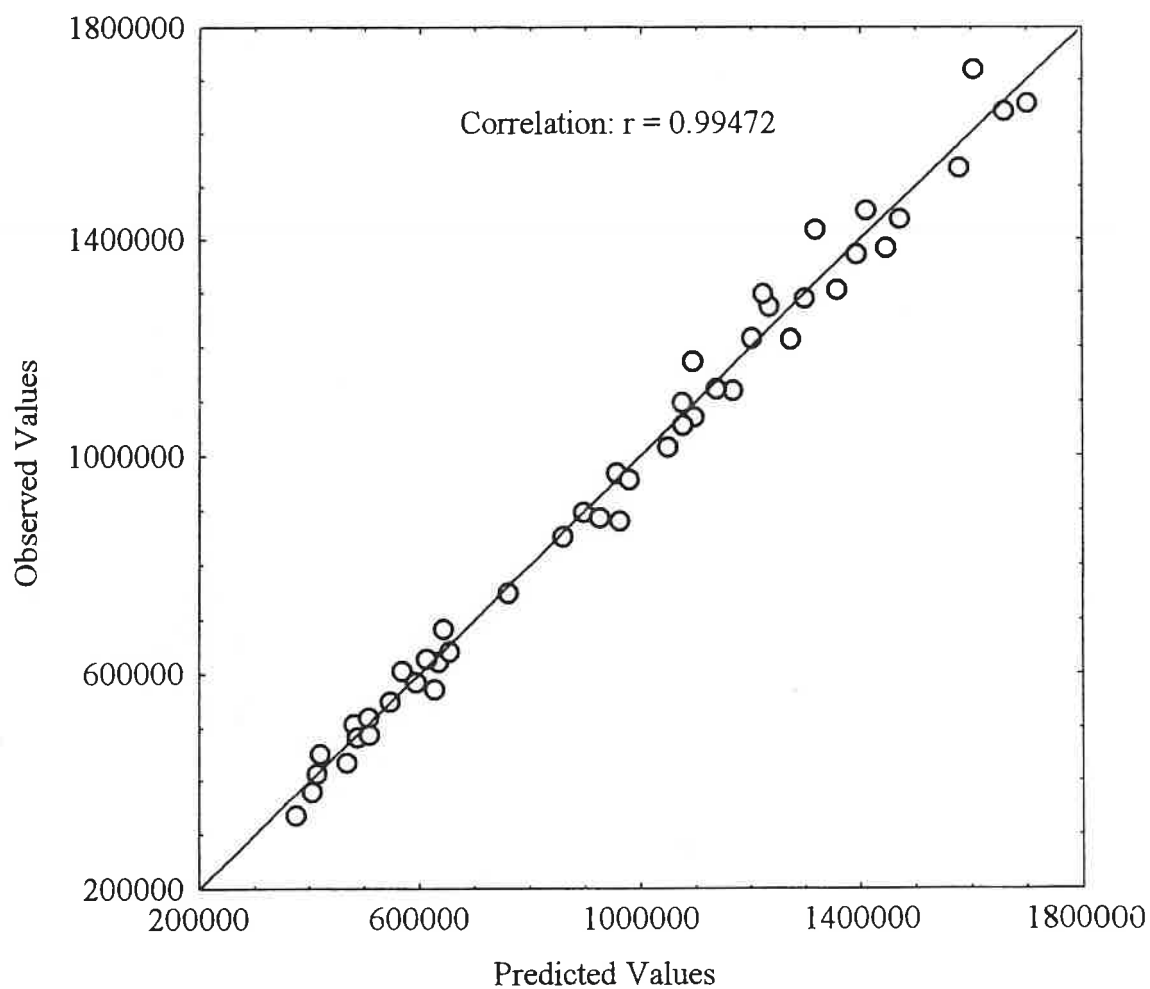
Figure 3 Correlation between predicted and observed values for the relationship shown in *Figure 2*
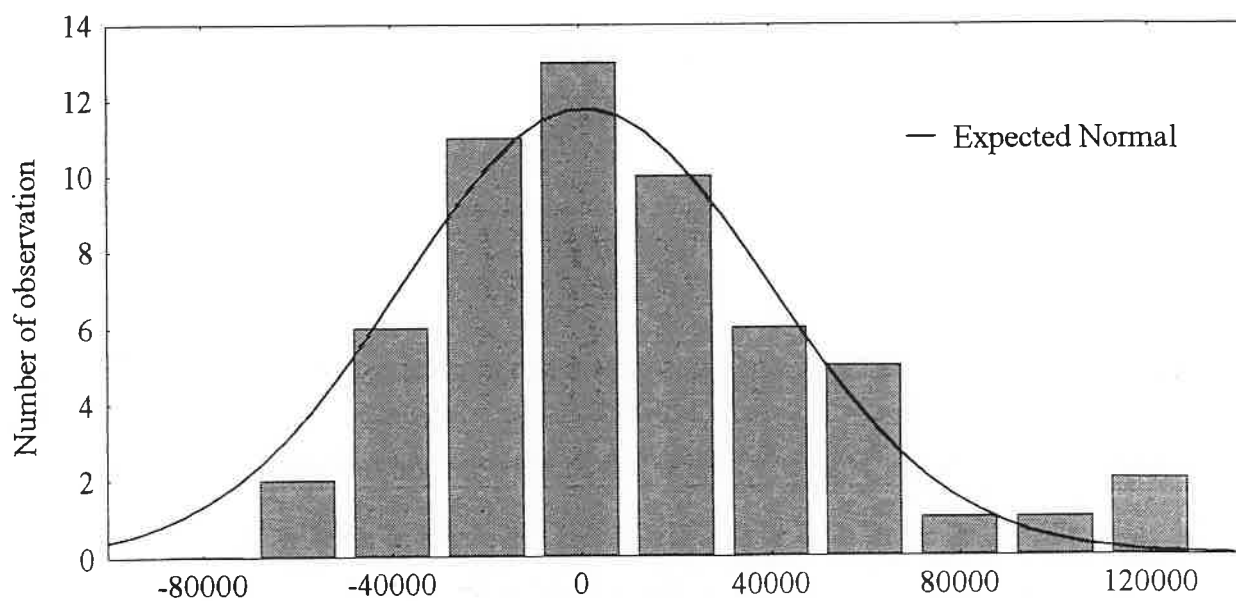
Figure 4 Distribution of remainders of variable average viscometric molecular weight for the relationship from *Figure 2*

Tests have shown that from the raw material used it is possible to produce chitosan of predicted physical and chemical properties which forms the surface on a graph being a visualisation of the formulated mathematical equation. The polymer of a deacetylation degree of over 85% is produced by double deacetylation separated by a washing procedure (*Figure 2*). Time and temperature of deacetylation are evaluated from the relationship linking the described chitosan parameters with the parameters of its production[6].

Correlation between average viscometric molecular weight evaluated from the formulated mathematical equation and the relationship linking average viscometric molecular weight with time and temperature of deacetylation reaction found in previous work[6] are very high - r = 0.99 (*Figure 5*).
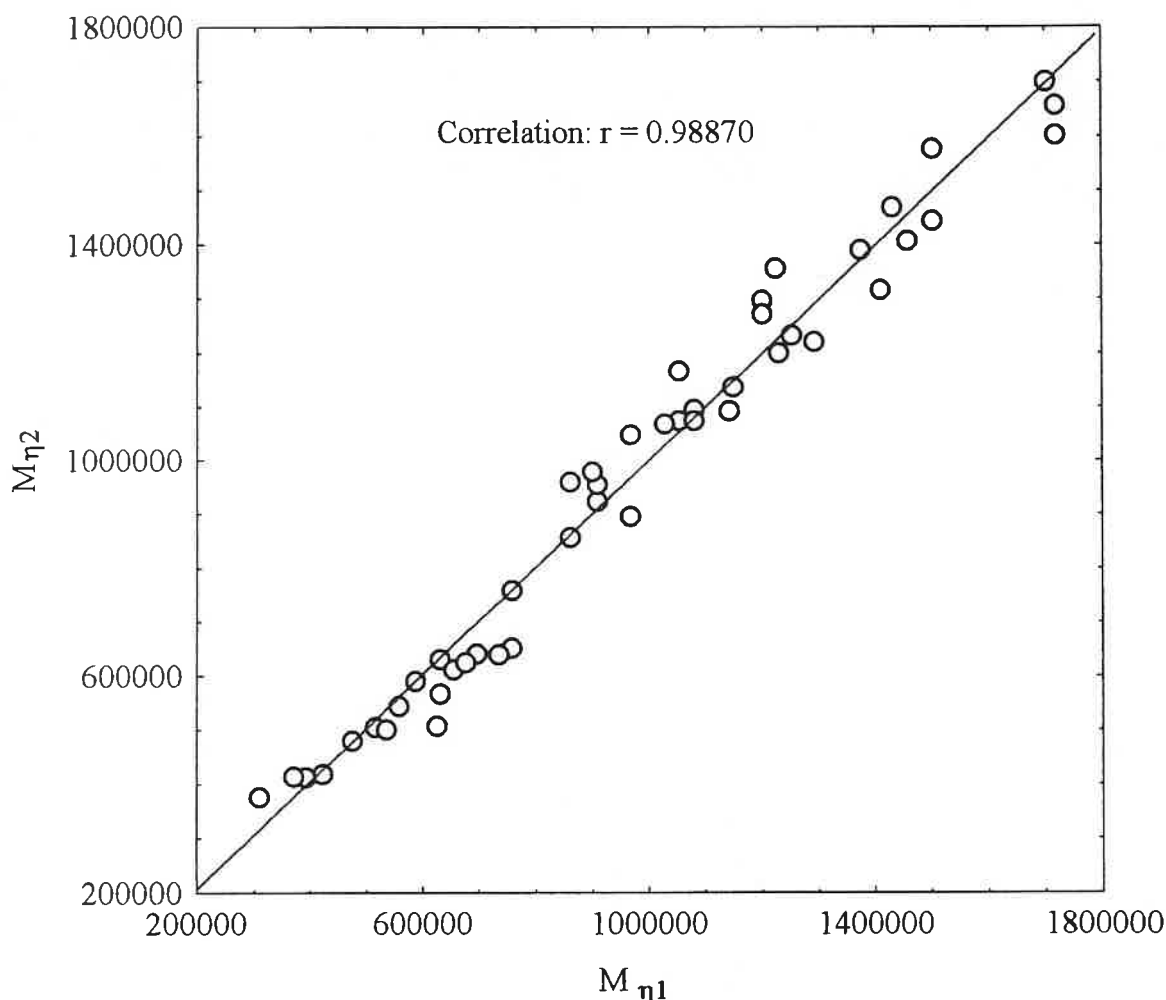
Figure 5 Correlation between average viscometric molecular weight $\overline{M}_{\eta 1}$ and $\overline{M}_{\eta 2}$ where:

$\overline{M}_{\eta 1}$ - average viscometric molecular weight evaluated from the relationship

   linking $\overline{M}_{\eta}$ with time and temperature of deacetylation reaction

$\overline{M}_{\eta 2}$ - average viscometric molecular weight evaluated from the relationship

   linking $\overline{M}_{\eta}$ with deacetylation degree and viscosity of standard chitosan
   solution

Average viscometric molecular weight evaluated from the Mark-Houwink equation for coefficients $K = 1.81 \cdot 10^{-3}$ cm^3/g and $a = 0.93$ increases along with an increase in deacetylation degree, while viscosity of standard solution (1% of chitosan in 0.85% solution of acetic acid) is constant. This is caused mainly by the fact that evaluation of average viscometric molecular weight was made without taking into consideration the influence of the quantity of acetyl groups in polymer chain on value of viscometric coefficients from the Mark-Houwink equation. According to literature data, coefficients K and a are changeable, both or only one of them according to changes in chitosan deacetylation degree[7, 8, 9].

So far there is no standard method of average viscometric molecular weight determination for chitosan changing quality parameters in very wide ranges, e.g. deacetylation step from 60% to 100% and viscosity of standard solution from 10 mPa·s to 3000 mPa·s. The above is confirmed by Roberts and Wang's investigation[9].

Influence of chitosan deacetylation degree on average viscometric molecular weight is larger when viscosity of standard solution is higher (*Figure 2*). Therefore changes of chitosan polymerisation degree as a result of technological process must not be evaluated only by determination of average viscometric molecular weight calculated with the use of Roberts and Domszy coefficients[4]. In the case of double deacetylation, average viscometric molecular weight of the second chitosan produced may be similar to average viscometric molecular weight of first chitosan despite of radical decrease in chitosan standard solution viscosity. For example average viscometric molecular weight of chitosan (1,640,000) determined for polymer of a standard solution viscosity of 1550 mPa·s practically has not changed (1,660,000), while deacetylation degree increased of about 20%. At the same time viscosity of standard solution dropped about three times to the value of 524 mPa·s (point no 1 and 2 *Figure 2*). More information on product properties can be obtained by evaluation of the results of average viscometric molecular weight and viscosity of standard solution determination.

Conclusion

The evaluated mathematical equation is helpful in estimation if there is a chance to produce chitosan of determined chemical and physical properties from raw material available.

Until new Mark-Houwink equation coefficients are found, changes in chitosan polymerisation degree caused by the production process conditions ought to be evaluated based on average viscometric molecular weight and viscosity of chitosan standard solution determination results.

Investigations of the above coefficients ought to be performed for series of chitosans the characteristic properties of which (deacetylation degree, average viscometric molecular weight and viscosity of standard solution) vary in a very wide range.

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Analysis of degree of deacetylation in chitosans from various sources

Marcin H. Struszczyk[a], **Fritz Loth**[b], **Martin G. Peter**[a]

[a]Universität Potsdam, Institut für Organische Chemie und Strukturanalytik, Am Neuen Palais 10, D-14469 Potsdam, e-mail: marcin@iap.fhg.de
[b]Fraunhofer Institut für Angewande Polymerforschung, Kantstraße 55, D-14513 Teltow

Abstract

Chitin from the Northern Atlantic shrimp *Pandalus borealis* and Antarctic krill *Euphausia superba* were deacetylated at various reaction times and temperatures. Average molecular weights were in the range betwen 0.6×10^5 and 2.7×10^5, as found by viscosimetry. Degree of deacetylation (DD) values were determined in a comparative way by application of potentiometric titration, solid state CP/MAS ^{13}C-NMR and IR spectroscopy.

Correlation of the results leads to the conclusion that DD values are determined most reliably by IR and solid state CP/MAS ^{13}C-NMR spectroscopy with computer assisted resolution of the peaks. The spectroscopic methods are especially suitable for analysis of chitosans with DD's < 80%. Potentiometric titration is the method of choice when the DD is higher than 85%.

Keywords: Chitosan, deacetylation degree, calibration coefficient, IR spectroscopy, CP\MAS ^{13}C-NMR, potentiometric titration

Introduction

Chitosan is a cationic polymer which is obtained from chitin [poly-(β-1$\rightarrow$4)-*N*-acetylglucosamine] by deacetylation with highly concentrated sodium hydroxide solution at high temperature. It contanis more than 60% of glucosamine residues. The product of deacetylation ought to be soluble in aqueous solution of mineral and organic acids. Chitosan is also occurs naturally in some fungi. Many methods have been developed for determination of the degree of deactelyation of chitosan. However, there is some uncertainty about the question how to determine DD values most reliably. The aim of this study was to evaluate the IR method by comparison with CP/MAS ^{13}C-NMR spectroscopy and potentiometric titration.

Materials and methods

Chitosan samples were prepared by deacetylation[1] of chitin from shells of Northern Atlantic shrimp, *Pandalus borealis*, and from the armor of Antarctic krill, *Euphausia superba*. The average molecular weight of the chitosan was 0.60 x 10^5 to 2.7 x 10^5, as determined by viscosimetry,[2] and the water retention value (WRV)[3] was in the range between 60 and 170% (Table 1). All samples were dissolved in aqueous acetic acid.

Table 1. Properties of chitosan samples used in the study.

Sample	Ash (wt %)	Moisture (wt %)	M_V (x10^{-5})	WRV (%)
A1	1.2	11.25	2.64	153.9
A2	4.5	11.41	1.87	171.1
A3	1.1	10.41	1.35	99.7
A4	1.1	11.95	1.43	99.8
A5	1.1	11.18	1.11	135.9
A6	1.1	10.73	0.64	88.7
B1	2.6	11.05	2.48	65.2
B2	2.5	9.89	2.27	56.8

A: Chitosan powder from *Euphausia superba*
B: Chitosan flaks from *Pandalus borelis*

Potentiometric titration. The deacetylation degree (DD) of chitosan samples was determined by potentiometric titration in non-aqueous solution (anhydrous acetic acid/1,4-dioxane). The glass indicator and calomel electrode were connected by a salt-bridge with a saturated solution of potassium chloride[4,5].

IR spectroscopy. IR spectra were recorded on a Mattson FTIR spectrometer. Chitosan samples (2 mg) were dried overnight at 60°C under reduced pressure. Then they were mechanically blended with 100 mg of KBr. The thickness of KBr disk was 0.5 mm. It was dried for 24 h at 110°C under reduced pressure before measuring.[6,13] For calculation of DD, the intensity of the amide I band (v = 1650 cm^{-1}) was used as the analytical band, and that of the hydroxyl band (v = 3450 cm^{-1}) as the reference band.[7] The data were evaluated either from the height of peaks or the area under the curve. Background of the spectra was subtracted based on the individual peaks or on the group of peaks, using either the computer software of the instrument or PeakFit for Windows. For calibration, a fully N-acetylated chitin sample was prepared by N-acetylation[8] of a chitin sample (DD 4%, determined by ^{13}C-NMR).

Solid-state ^{13}C - NMR. CP/MAS ^{13}C NMR spectra were recorded with a UNITY 400 (100 MHz) NMR spectrometer at room temperature. Samples of 0.3 cm^3 of powdered chitosan were placed into 5 mm ZrO cups and those sealed with Vespel caps. Spinning rate: 5-6 kHz; repetition time: 3 s; radiofrequency field strength used for dipolar decoupling: 50-70 kHz; linebroadening: 10 kHz; number of transients: 5000; external standard:

Adamantane. DD were calculated from the ratio of the intensities of signals of methyl and anomeric carbons, using computer software obtained with the spectrometer.

Results and discussion

A typical IR spectrum is shown in Fig. 1. Comparison of the spectra of chitosan recorded in KBr and in form of films[9,10] showed that the latter do not yield reproducible data. Therefore, all data given here are based on samples measured in KBr disks. In this study, we have evaluated the IR spectra by three methods as described in the literature. Muzzarelli et al.[7] and Domard[11] used the quotient A_{1650}/A_{3450}, while Sannan et al.[6] recommended A_{1550}/A_{2900}, i.e. the Amide II band as the analytical and the CH band as the reference. Another method has been suggested by Shigemasa et al.[12] who used C-O at 1070 and 1030 cm^{-1} as the reference, and Amide at 1630 and 1660 cm^{-1} as the analytical bands. Furthermore, a calibration coefficient has to be applied, the value which was given by Muzarelli et al.[7] as 0.75, and by Domard[11] as 1.15. In our case, comparison of IR spectra with NMR and potentiometric titration revealed a calibration coefficient of 0.864.

Table 2 shows DD values calculated from the ratio of IR bands A_{1650}/A_{3450} without applying calibration coefficients. It was found that calculations based on the area of the peaks give large variations and thus are not reliable. However, there is a good agreement in the values using peak intensities and backkgroud correction based on individual peaks or groups of peaks.

**Table 2.** DD values obtained for calculation, based on the ratio A_{1650}/A_{3450}.

Sample	Baseline separation based on group of peaks	Baseline separation based on individual peak
A1	51,6	57,8
A2	61,4	66,2
A3	73,3	76,1
A4	94,8	95,7
B2	98,0	99,8

The data shown in Table 2 were evaluated by application of the various calibration coefficients as discussed above, and compared with the DD values obtained by NMR and potentiometric titration, respectively (Table 3). When plotting DD values calculated by different methods against each other in a correlation graph, the line obtained should ideally intersect the origin of the coordinates.

Fig. 3 shows a plot of IR data against those obtained by potentiometric titration. The best correlation was found with a calibration coefficient of 0.864 (individual peak) and 0.751 (group of peaks), provided that DD is in the range between 85 and 100%. This is explained by the increasing solubility of chitosan with increasing DD values.
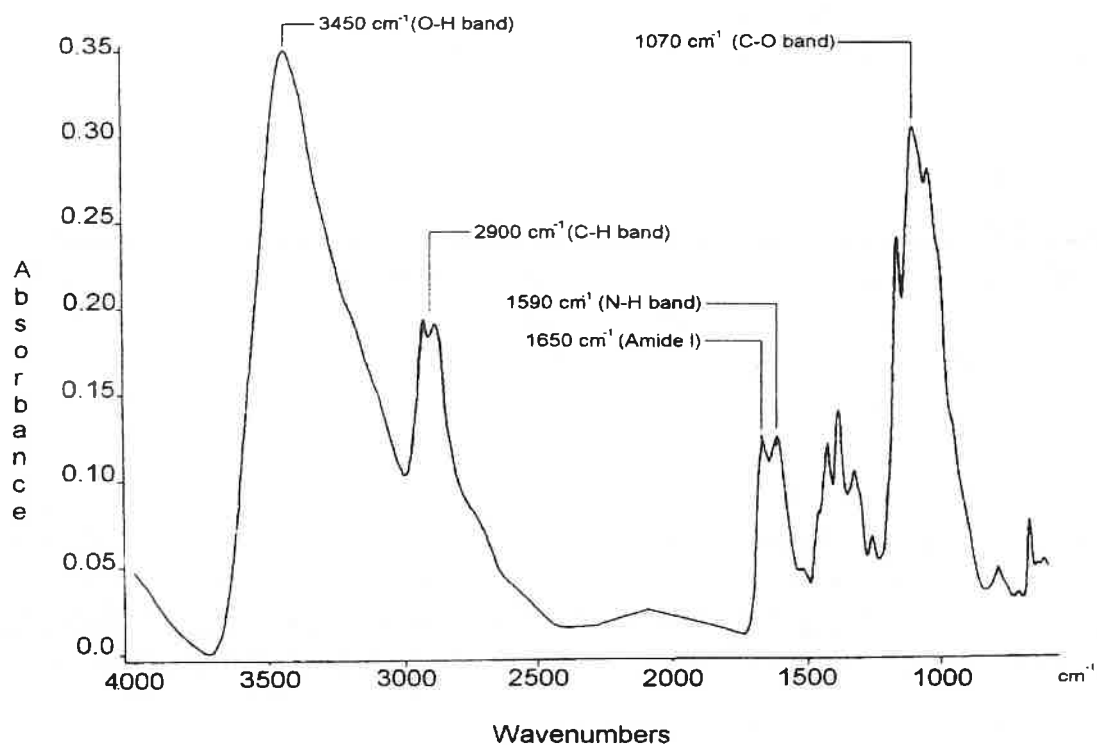
Figure 1. IR spectrum of chitosan
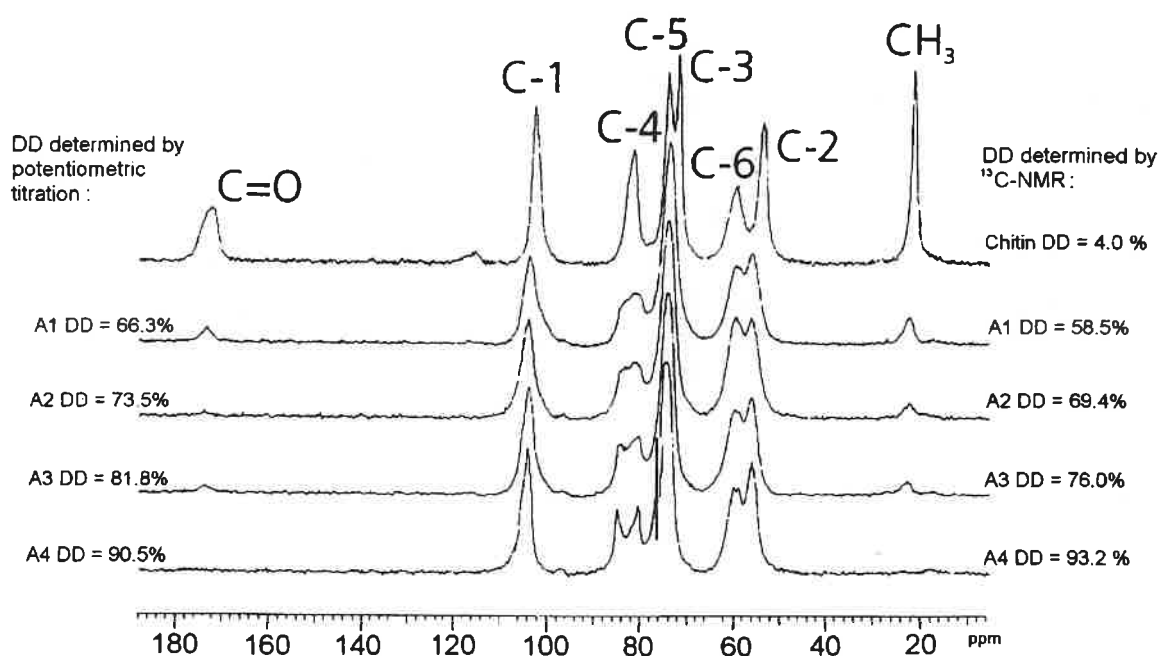
Figure 2. Solid-state ^{13}C NMR spectra of chitin and chitosan

A good correlation of DD values is also found when IR data are compared with NMR data, as shown in Fig. 4. Again, calibration coefficient 0.864 gives the best correlation.

Minke et al.[13] reported that chitin contains two types of amide groups, differing by the mode of hydrogen bonding. One of those results from intermolecular C=O----H-N interactions while the other is due to C-O----H-N intramolecular hydrogen bonds to C(6) hydroxyl groups between neighbouring monosaccharide units. Therefore Shigemasa et al.[12] proposed to use the sum of A_{1650} and A_{1630} cm^{-1} as the analytical, and A_{1070} as the reference bands. However, in our study, this approach gave rather poor correlation in the case of potentiometric titration (data not shown), whereas the correlation with NMR was very good (Fig. 5).

Table 3. DD values (%) of chitosans obtained for ratio A_{1665}/A_{3450} with various calibration coefficients.

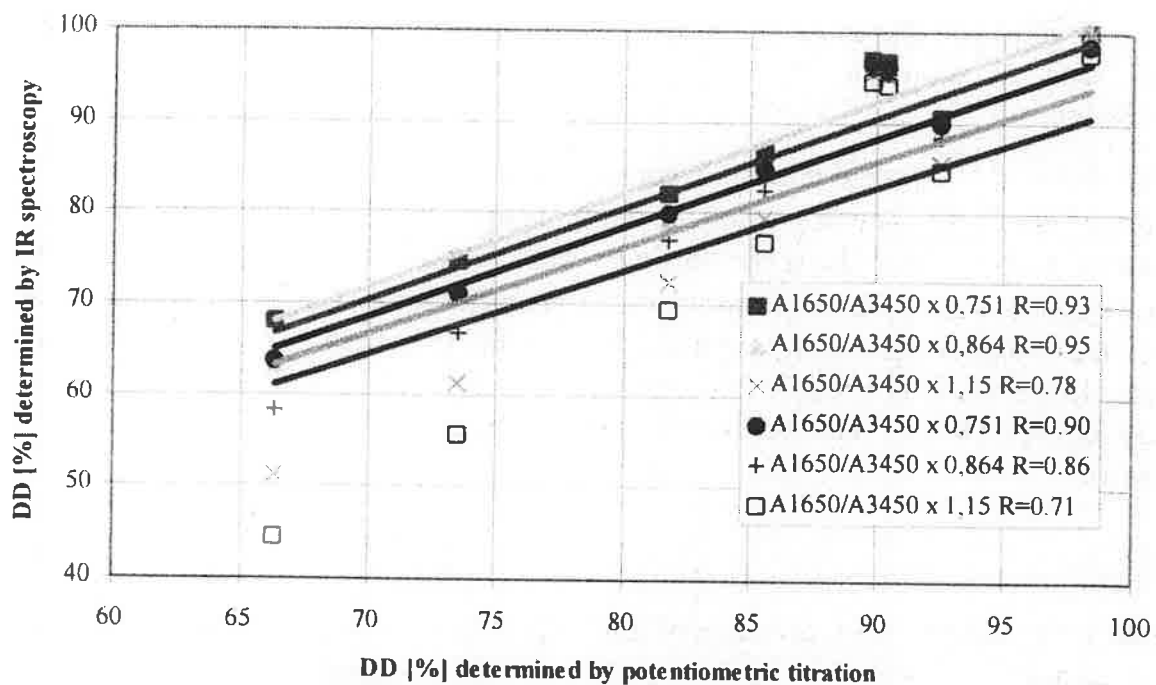| Sample	^{13}C-NMR	potentiometric titration	IR spectroscopy					
			baseline separation based on					
			on individual peak			group of peaks		
Calibration coefficient			0,751 (1)	0,864 (2)	1,15 (3)	0,751 (1)	0,864 (2)	1,15 (3)
A1	58.5	66.3	68.0	63.2	51.1	63.6	58.2	44.3
A2	69.4	73.5	74.6	70.8	61.2	71.0	66.7	55.6
A3	76.0	81.8	82.0	79.3	72.5	79.9	76.9	69.3
A4	93.2	90.5	96.8	96.3	95.1	96.1	95.5	94.0
B1	95.6	89.9	96.9	96.5	95.3	96.4	95.9	94.5
B2	99.2	98.4	99.9	99.8	99.8	98.5	98.3	97.7
A5	79.0	85.6	86.6	84.5	79.5	84.8	82.5	76.8
A6	85.1	92.6	90.6	89.2	85.6	90.0	88.5	84.7

(1) - according to Muzzarelli[7]

(2) - coefficient calculated for the average value of A_{1665}/A_{3450} for fully acetylated chitin
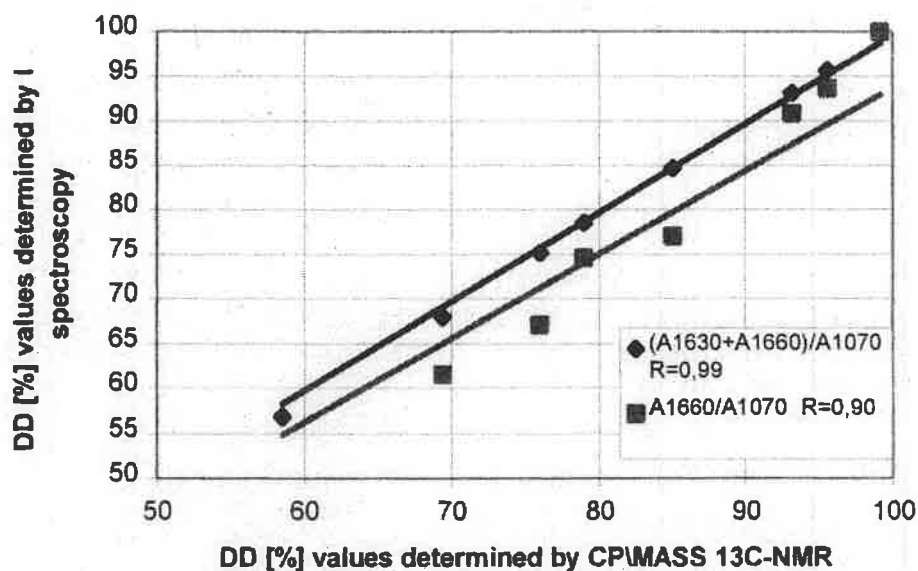
(3) - according to Domard[11]

Conclusions

1. Besides solid-state NMR spectroscopy, best accuracy is obtained using the method of Muzzarelli[7] for calculation of DD values with baseline separation based on group of peaks and chitosan samples prepared as KBr disks.

2. Application of computer software for peak separation is recommended for best results.

3. Because the accuracy of DD depends on various factors, such as the method of baseline separation in evaluation of IR spectra, selection of the reference method (i.e. potentiometric titration or NMR), it is recommended to calibrate the method in each new case.

Figure 3. Plot of DD values determined by potentiometric titration and IR spectroscopy (O, ▲, x : baseline separation based on individual peak; ●, +, ❑ : baseline separation based on group of peaks; **R** : correlation coefficient)
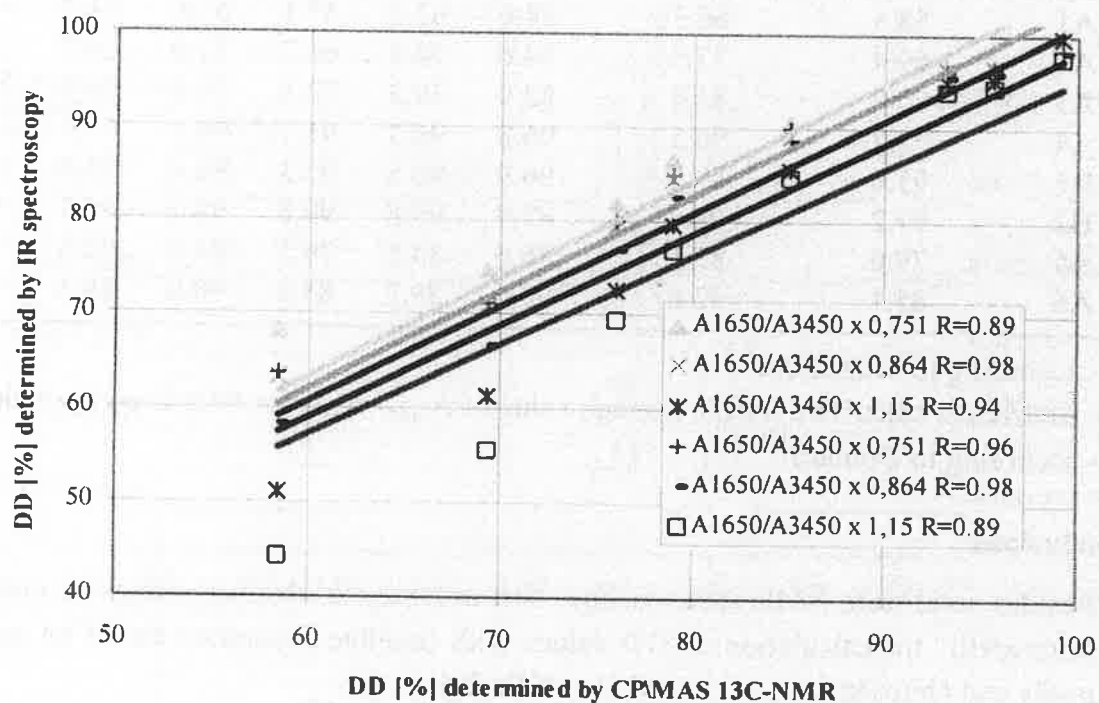
Figure 4. Plot of DD values determined by CP/MAS ^{13}C-NMR and IR spectrosopy (▲, x , ✳ : baseline separation based on individual peak ; +, -, ❑ : baseline separation based on group of peaks ; **R** : correlation coefficient)

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Figure 5. Plot of DD values obtained by NMR and IR spectroscopy according to the method of Shigemasa[12] (peak evaluation by PeakFit 4.0) ; **R** : correlation coefficient

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PREPARATION OF CHITOSAN FROM SQUID (*Loligo spp.*) PEN BY A MICROWAVE-ACCELERATED THERMOCHEMICAL PROCESS

Francisco-Martín GOYCOOLEA, Inocencio HIGUERA-CIAPARA,
Georgina HERNÁNDEZ, Jaime LIZARDI y Karina-Dalila GARCÍA
*Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD), Apartado Postal No. 1735
C.P. 83000 Hermosillo, Sonora (MEXICO), Fax:+52 62 80 00 57
E.Mail: fgoyco@cascabel.ciad.mx*

Abstract

Chemical N-deacetylation of chitin during chitosan preparation from seafood waste, usually requires very drastic processing conditions involving use of concentrated alkali, high temperature and long time treatments. A novel method to prepare chitosan from squid (*Loligo spp.*) pen waste was investigated, aiming to reduce the long processing times typically required to achieve N-deacetylation. Finely ground dry squid pen meal was dispersed in solution of NaOH (30%), subjected to microwave radiation (~2.45 GHz) for 22 min, washed with cold water and dried. The physicochemical characteristics of the chitosan thus obtained, were compared to those of the materials obtained under conventional heterogeneous (NaOH 50%; 90°C; 6 h, bubbling N_2) and under homogeneous (NaOH 11.1% 0°C; 25°C; 77 h) conditions. The microwave treated chitosan was soluble in dilute acetic acid, had a degree of acetylation (DA) of ~12.8 %, as assessed by first derivative UV spectroscopy, lower to that of chitosan obtained under conventional heterogeneous and homogenous conditions, with DA respectively of 16.2 and 36.8%. The microwave accelerated deacetylation reaction brought in a slight reduction in intrinsic viscosity ([η]~695 ml/g), with respect to the conventional products ([η] ~821-1102 ml/g), indicative of chain degradation. X-ray diffraction analysis revealed that the crystalline structure of the microwave-treated squid pen chitosan, was similar to that of the conventionally-produced material and both differed form the typical chitosan L-2 polymorph diffraction pattern, with loss of the (100) equatorial reflection at lattice angle $2\theta \sim 10.6°$, indicative of a more amorphous conformation. Solid-state CP-MAS ^{13}C NMR spectra, confirmed that deacetylation in the microwave-treated sample, proceeded to a greater extent than in the homogeneous conventionally-treated one, and also that similar polymorphic structures were obtained on both materials, since identical chemical shifts and peak features were observed for the different carbon signals.

Keywords: chitosan, *Loligo spp.*, deacetylation, microwave, crystalline, polymorphs, chain-degradation

Materials and Methods

Material. Dried *Loligo spp.* pen waste was obtained from a seafood processing plant in Guaymas Sonora Mexico. It was ground in a Weber hammer mill (mesh 40 ~0.3mm).

Deacetylation processes. Ground loligopen was subjected to chemical N-deacetylation by the following processing conditions: a) Heterogeneous (NaOH 30% 90°C. 6 h. under N_2). reactor IKA-Labortechnike RE-162/P[1]. b) Homogeneous (25°C; 77 h) from previously solubilised alkali chitin (NaOH 11.1% 0°C)[2]. c) Microwave-accelerated reaction. The process is described as follows: dry loligo pen meal was ground on a Weber mill (mesh 40; i.e.~0.3mm), added NaOH 30% in a 500 ml bottle. placed on a microwave-oven digestion system CEM (Mod. MDS- 82D. Matthews, NC, USA) operated at 80% of total maximum power (800 watt), at a frequency of 2.45 GHz for 22 min; cold distilled water (4°C) was added, then it was filtered, thoroughly washed with water and dried at 25°C.

Degree of acetylation (DA). The DA of chitosan samples was determined by first derivative UV[3], using a CARY1 UV-VISIBLE spectrophotometer, with a standard curve of N-acetyl-glucosamine (Sigma Chemicals Inc.).

Intrinsic viscosity [η] and molecular mass (M_v): Relative viscosity (η_{rel} 1.2 - 2.0) of chitosan solutions in AcONa 0.2M/AcOH 0.3M, were measured in an Ubbelohde capillary viscometer immersed in a Koehler water circulator (25°C ± 0.1) (Bohemia NY, USA). Molecular mass was estimated from the relation $[\eta] = 0.069 \times M_v^{0.77}$ [4].

X-Ray diffraction analysis. X-ray powder patterns on chitosans were measured by a Rigaku Geigerflex X-ray diffractometer fitted with a graphite monochromator using $CuK\alpha$ radiation generated at 35kV and 20 mA.

CP-MAS ^{13}C NMR solid-state spectrometry. Single-contact 50.32 MHz ^{13}C CP-MAS (cross-polarisation magic angle spinning) NMR spectra were recorded on a Bruker CXP-200 spectrometer fitted with a Bruker-z32DR-MAS-DB probe. Samples were contained in a ceramic cylindrical rotor and spinned at 4.5 KHz. Contact time for cross polarisation was 2.5 ms and 1400-4000 scans accumulated. Spectra were referenced indirectly to a zero value for tetramethylsilane (TMS).

Results and discussion

Chemical and macromolecular characteristics of the chitosans obtained from the three different processes investigated are compared in Table 1. Use of microwave irradiation in order to accelerate the reaction time needed to achieve deacetylation of squid pen chitin, resulted in time reduction by 16-fold from the conventional high temperature heterogeneous process (6h 90°C), or in nearly 200-fold reduction from the long-time low-temperature homogeneous process (72h 25°C). In preliminary trials. it was found that 22 min and 80% power. were respectively the time and temperature optimum operating conditions to achieve N-deacetylation necessary to render soluble the chitosan. The thus obtained polymer, had a DA~12%, which was considered to be in the terminal region for the completion of the N-deacetylation reaction of squid chitin reaction under heterogeneous conventional conditions (30% NaOH 80°C)[1]. As expected, the homogeneously deacetylated sample had a much higher DA value (DA=36.85%), yet it was soluble in dilute acetic acid. in agreement with previous reports[2]. Despite the short processing time. unavoidably. depolimerization in the microwave-treated sample proceeded to a greater extent than in the conventionally treated samples, as it was noticed from the somewhat lower [η] value of 695ml/g . A possible explanation offered at this stage. is that in the microwave oven the N-deacetylation reaction proceeded in the presence of oxygen, since it was not considered safe to seal the reaction bottle previously purged with N_2. Hence, the microwave treatment might have accelerated as well the depolimerization reaction.

Table 1. Degree of acetylation, intrinsic viscosity and molecular mass of squid pen chitosans obtained under different process conditions.

| Reaction conditions	Degree of acetylation (%) [a]	$[\eta]$ [b] (ml/g)	M_v [c]
NaOH 11.1% 25°C, 72 h	36.85	821	196 400
NaOH 50% 90°C 6 h	16.20	1102	285 200
microwave oven: NaOH 30%, >100°C 22 min	12.08	695	158 000

[a] By first derivative UV [3]

[b] Intrinsic viscosity (25°C ± 0.1, AcONa 0.2 M / AcOH 0.3 M)

[c] Relation $[\eta] = 0.069 \times M_v^{0.77}$ [4]

It was also interesting to notice, that the homogeneous N-deacetylated sample suffered less depolimerization than did the polymer produced in the conventional reactor under heterogeneous conditions (i.e. $[\eta]$ values respectively of 821 and 1102 ml/g). A possible explanation to such discrepancy, was that the homogenous sample was left to react with NaOH 11.1% under quiescent conditions in a sealed bottle purged with N_2, while the heterogeneous one was continuously stirred under a gentle stream of bubbling N_2, thereby being more effective in prevention of chain degradation by β-oxidation. X-ray powder diffraction patterns (Figure 2), revealed that the microwave irradiation process in itself did not have any different or special (i.e. 'non-thermal') effect on the conformation of squid chitosan in the solid state, as both materials shared similar patterns with a single peak at lattice angle $2\theta \sim 19.8°$. Absence of peak at lattice angle $2\theta \sim 10.2°$, could indicate the formation of a different polymorphic structure than the L-2 conformation. Similar x-ray powder diffraction patterns have been observed for chitosans N-deacetylated under homogeneous conditions, but at much higher acetyl contents (55%)[5]. Spectra of ^{13}C CP MAS NMR confirmed that microwave irradiation did not induced the formation of a different polymorphic crystalline structure. Chemical shifts of ^{13}C NMR peaks for the various C signals, matched closely those previously assigned to C-1 (104.7 ppm), C-4 (83.0 ppm), C-5,C-3 (75.0 ppm) and C-6, C-2 (57.8), CH_3 (23.5 ppm) and C=O (175 ppm), for chitosan from various sources[6]. Note that acetyl group carbon signals nearly disappeared in the microwave treated chitosan, while in the homogenous N-deacetylated both peaks are still present. Singlet signals for C1 and C4 peaks observed indicate similar 2-fold helical conformation on the with no obvious special effects associated to microwave irradiation.
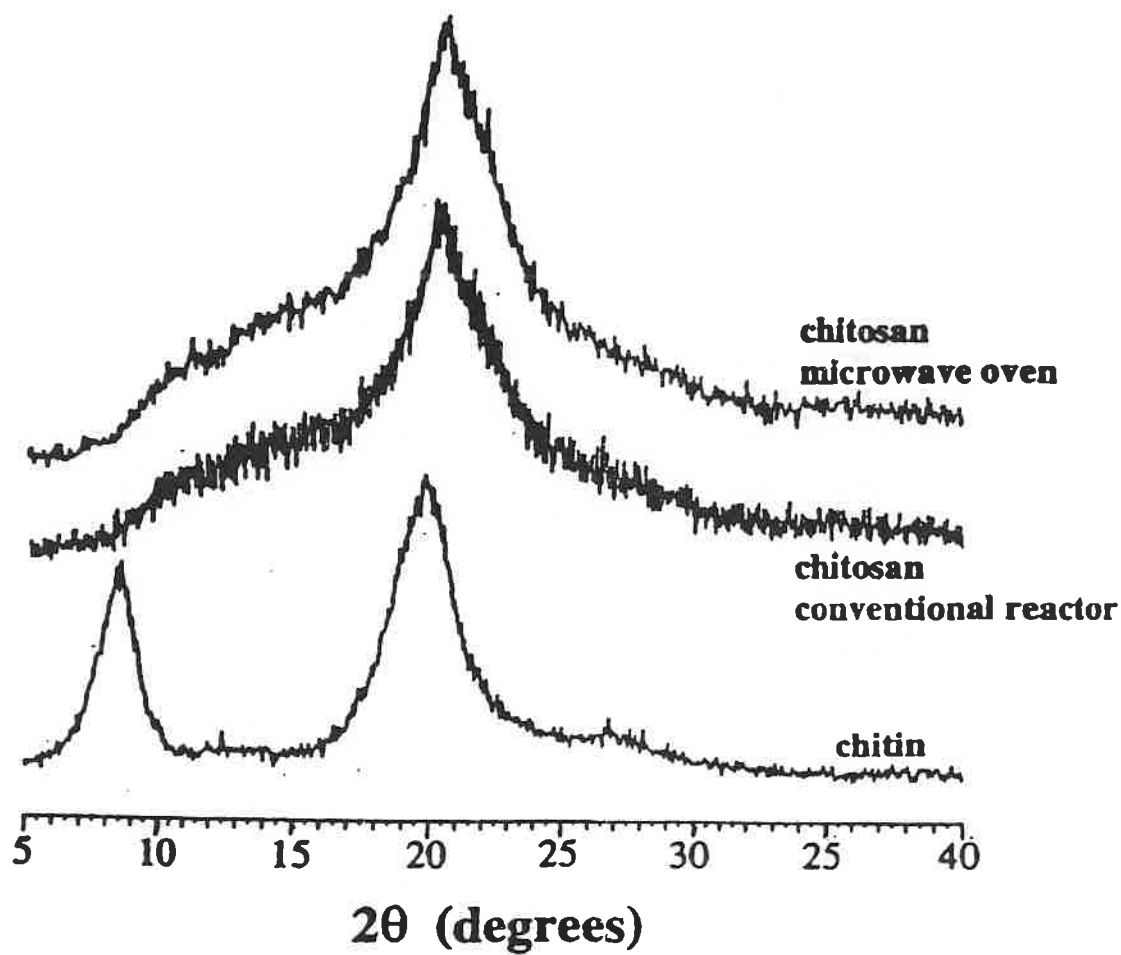
Figure 1. X-Ray diffractograms of squid pen chitin and chitosan powders.
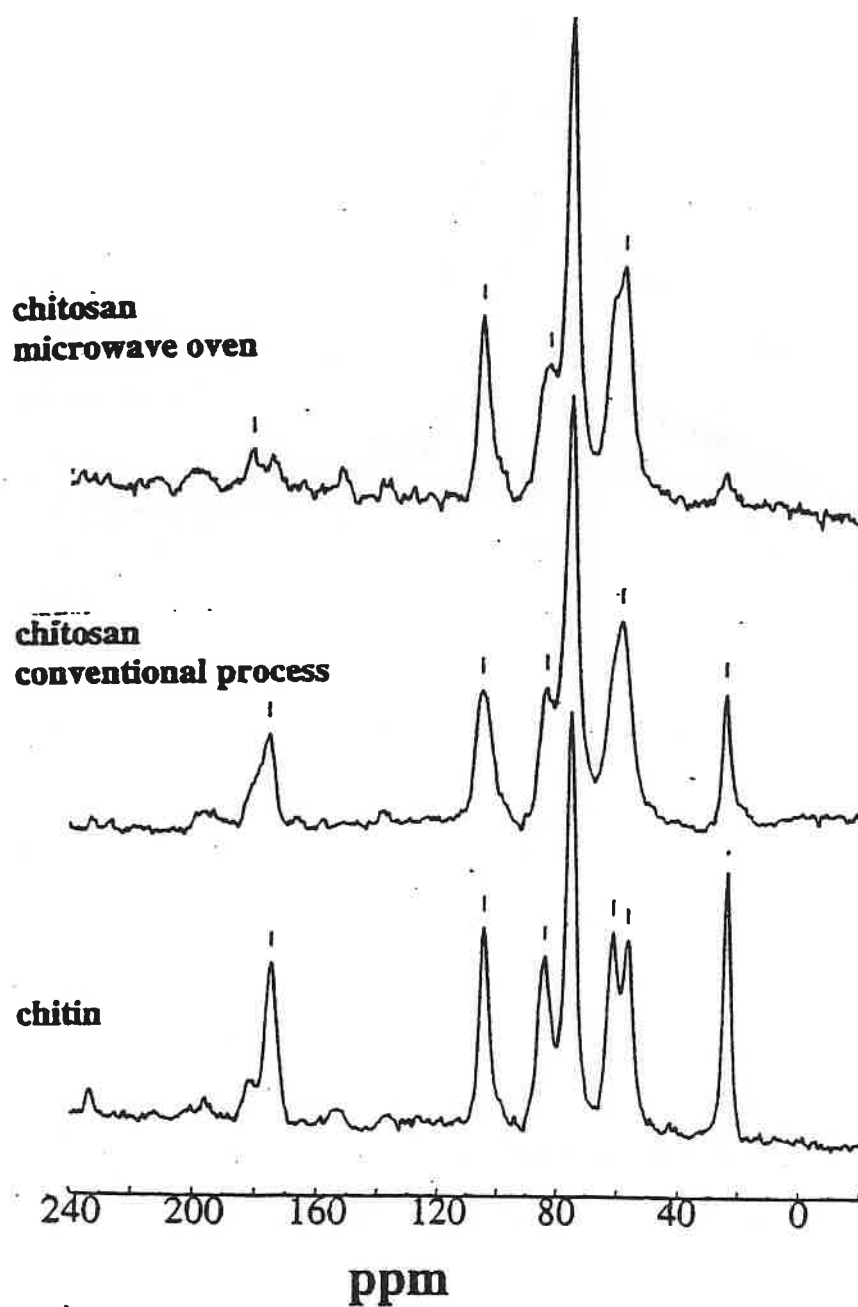
Figure 2. Solid-state CP-MAS ^{13}C NMR spectra (50 Mhz) of squid pen chitiñ and chitosan powders.

Conclusions

Microwave irradiation processing was found a suitable technology for reducing by at least ~16-fold the N-decacetylation reaction time to produce chitosan from squid pen meal. The chitosan polymer thus prepared, had both lower molecular mass and N-acetyl substitution, than the materials obtained by conventional processes, yet they shared similar crystalline and overall structural characteristics. The effect of microwaves on acetyl groups distribution on the polymer chain and other physicochemical properties, need further studying. This process may also prove effective to reduce N-deacetylation time for other chitinous waste sources (e.g. shrimp and crab shell waste).

Acknowledgements

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Analysis of Functions of Chitin prepared from Silkworm *Bombyx mori*

Atsunobu HAGA, Akira SHIRATA
National Institute of Sericultural and Entomological Science,
Ministry of Agriculture, Forestry and Fisheries
1-2 Ohwashi, Tsukuba Ibaraki 305, Japan

Abstract

Insects are the greatest un-utilized resource on the earth. As one of analysis of functions of insect-derived complex molecules such as chitin, antibacterial and antifungal activity of chitin prepared from pupa of silkworm *Bombyx mori* was investigated. Antibacterial assay was carried out using plant pathogenic bacterias (*Agrobacterium tumefaciens, Clavibacter michiganense* pv. *michiganense, Erwinia carotovora* subsp. *carotovora, Pseudomonas cichorii, Xanthomonas campestris* pv. *campestris*) and antifungal assay using plant pathogenic fungi (*Cochliobolus miyabeanus , Fusarium solani f . sp. mori , Roselinia necatrix , Septoria cucurbitacearum , Sclerotinia sclerotiorum*). Sensibility of bacterias and fungi was investigated using fully deacetylated chitosan and water-soluble chitin which were prepared from exuvium of silkworm pupa. Sensibility of bacterias for chitin and chitosan was investigated by measuring of inhibition against for propagation of bacterias, and sensibility of fungi was by measuring of inhibition against for extension of fungi. Consequently fully deacetylated chitosan prepared from silkworm pupa completely inhibited extension of fungus against only mulberry white root rot, *Roselinia necatrix,* for three days of culture. However after 4th day extension of the fungus was recognized because of diffusion. Effect of water-soluble chitin could not be recognaized against the above bacterias and fungi.

Key words : silkworm, *Bombyx mori* , chitin, chitosan, antibacterial activity, antifungal activty, mulberry white roor rot

Introduction

Insects can be regarded as the greatest biological resource that remains unutilized. It is essential to advance the knowledge that has been accumulated from the studies of silkworms on sericulture and silk reeling industry which is an industrial field closely connected to insects. In recent years, it is highly desirable to create new materials by using insects. The development of new technologies for utilization of insect-derived complex molecules such as chitin will play significant role in the creation of new industrial fields in the 21st century. For utilizations of the functions of insect-derived complex molecules such as chitin, antibacterial and antifungal effect of chitin prepared from pupa of silkworm *Bombyx mori* was investigated.

Materials and Methods

Materials : Chitin was utilized that had been prepared from chitin of silkworm pupae separated from thin-shelled silk layers (HAGA, 1996). Chitosan with higher degree of *N*- deacetylation and with lower molecular weight could have stronger antifungal effect

(Uchida, 1988). Therefor the assay of antibacterial and antifungal activity was carried out using full deacetylated chitosan prepared from pupa of silkworm *bombyx mori*, three kinds of chitosan prepared from crab shell chitin that differ in degree of N − deacetylation (DDA of 100 %, 90 %, 87 %) and water−soluble chitin (DDA of 45∼48 % : HAGA, 1996).

Purification of chitin : Chitin was utilized that had been prepared from silkworm pupa by the method of Hackman (Hackman, 1954 ; HAGA, 1996). Demineralization was carried out by treatment with $2N$ HCl for 96 hours by strong stirring in 1000 ml of the solution at 25℃. Deproteinisation by treatment with 500 ml of $1N$ NaOH for 42 hours in the boiled solution and removement of pigments and lipids for 4 hours by extracting with 95% ethanol was carried out (HAGA, 1996).

Deacetylation of chitin : Chitosan was prepared from chitin of silkworm pupae separated from thin−shelled silk layers using heterogeneous reaction by the deacetylation of the chitin. Deacetylation was effected by treating chitin with 50%(w/w) NaOH for 16 hours at 150℃. Purification of the chitosan was effected by dissolving it in 10% acetic acid and reprecipitating the gel with sodium hydroxide. The purification process was repeated until pH of the suspension became neutral. After repeating the purification, the prepicitate was washed with ethanol and dried in vacuum (HAGA, 1996)

Preparation of water-soluble chitin : Chitin was utilized that had been prepared from exuvia of silkworm pupa. Water−soluble chitin was prepared under homogeneous conditions by the method of Sannan *et al.* To 40% NaOH was added the chitin at 25 ℃ in vacuum, the alkali chitin solution with high viscosity was prepared and then left to stand at 25℃ for 71 hours with sttiring (HAGA, 1996).

Determination of degree of deacetylation : FT−IR spectroscopy (JASCO, FT/IR−8900) was used to determine the degree of deacetylation (DDA) of the water−soluble chitin. The DDA was determined from the absorbance of the amide II band and at 1561 cm^{-1} by the use of the 1071 cm^{-1} or 1033 cm^{-1} absorption band as an internal standard (Matsuura *et al.*, 1995).

Assay of antibacteral and antifungal activity : Since water−soluble chitin is soluble in water but full deacetylated chitosan and three kinds of chitosan prepared from crab shell chitin couldn't be dissolved in water, the above chitosans were examined that had been dissolved in aqueous acetic acid. Acetic acid can't inhibit extension of fungi but could do extension of bacterias. As it was found in advance that aqueous acetic acid under 0. 5 % couldn't inhibit extension of bacterias which were utilized in the assay (See Table 1.), the above chitosans were dissolved in 0. 1 % aqueous acetic acid to adjust 100 ppm and 1000 ppm of chitosan solution.

Acitivity of bacterias for chitin and chitosans : Sensibility of bacterias for chitin and chitosan was investigated by measuring of inhibition against for propagation of bacterias.

Acitivity of fungi for chitin and chitosan : Sensibility of fungi was by measuring of inhibition against for extension of fungi.

Results and Discussion

Chitosan prepared from silkworm pupa chitin : The synthesis of chitosan from silk-

worm pupa was carried out using heterogeneous reaction (50%w/w NaOH at 150℃ for 16 hours) by the deacetylation of chitin. The field of the product was 32.7%. In the FT−IR spectrum of the deacetyl−chitin two absorption bands (amid I and amide Ⅱ) have been observed indistinctly. This indicates that the product was fully deacetylated and have extremely high degree of deacetylation. The product, chitosan, have a solubility in 0.1% acetic acids.

Acitivity of bacterias and fungi for chitin and chitosans : Fully deacetylated chitosan prepared from silkworm pupa and three kinds of chitosan with DDA of 100%, 90% and 87% completely inhibited extension of fungus against only mulberry white root rot, *Roselinia necatrix* , for three days of culture. However after 4th day extension of the fungus was recognized because of diffusion (Table 1.). Effect of fully acetylated chitosan and three kinds of chitosan against mulberry white root rot, *Roselinia necatrix* was shown in 1000 ppm solution of chitosan solution but didn't in 100 ppm solution. Effect of water−soluble chitin didn't be shown against the above bacterias and fungi. Fully deacetylated chitosan from silkworm pupa and chitosans with hi100%, 90% and 87%of degree of deacetylation from crab shell have antifungul effect against mulberry white root rot but water−soluble chitin with DDA of 45∼48%didn't. In the fully deacetylated chitosan and water−soluble chitin with DDA of 45∼48% that both were prepared from silkworm pupa, chitosan with higher degree of deacetylation shows more stronger antifungal effect. This indicates that not only in the deacetylated chitin prepared from crab shell and shrimp shell but also in the deacetylated chitin prepared from silkworm pupa, antifungul activity of deacetyulated chitin depends on their degree of deacetylation. In the deacetylated chitosan of silkworm pupa the same antifungal character were shown as crab shell chitosan and shrimp shell chitosan. This is very interest fact in analysis of biomechanizm that chitosans could recognize specific fungus. Since mulberry white root rot is on of serious pathogen in mulberry, technical developments using chitosan could be expected to serve as environmentally protective method to control pathogenic fungus of mulberry.

Table1. Activity of Antibacteria and Antifungi

Bacteria and Fungi		Chitin and Chitosan				
		Silkworm		Crab		
		A	B	C	D	E
Plant pathogenic bacteria						
Agrobacterium tumefaciens	Rose	−	−	−	−	−
Clavibacter michiganense pv. *michiganense*	Tomato	−	−	−	−	−
Erwinia carotovora subsp. carotovora	Chinese cabbage	±	−	±	±	±
Pseudomonas cichorii	Lettuce	±	−	±	±	±
Xanthomonas campestris pv. *campestris*	Rape	−	−	−	−	−
Plant pathogenic fungi						
Cochliobolus miyabeanus	Rice plant	−	−	−	−	−
Fusarium solani f. sp. *mori*	mulberry	−	−	−	−	−
Roselinia necatrix	mulberry	+	−	+	+	+
Septoria cucurbitacearum	Pumpkin	−	−	−	−	−
Sclerotinia sclerotiorum	mulberry	−	−	−	−	−

+ Active   ± Partially active   − Inactive

A : Full Deacetylated Chitosan
B : Water− soluble Chitin
C : Chitosan (Degree of Deacetylation 100%)
D : Chitosan (Degree of Deacetylation 90%)
E : Chitosan (Degree of Deacetylation 87%)

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PROCESSING PARAMETERS IN SCALE-UP OF LACTOBACILLUS FERMENTATION OF SHRIMP BIOWASTE

Mukku Shrinivas RAO and Willem F. STEVENS
Bioprocess Technology Program, Asian Institute of Technology, PO box 4, Klong Luang, Pathumthani 12120, Thailand. Fax: (66-2) 524 6111. E.Mail:mukku@ait.ac.th

Abstract

Lactobacillus fermentation of shrimp biowaste to obtain chitin besides valuable byproducts such as protein, pigments and calcium has emerged to be an attractive alternative to the conventional chemical process. The chemical process involves acid and hot alkali treatments consuming energy and resulting in industrial wastes. The fermentation process using *Lactobacillus plantarum 541* gives hydrolysate which has high protein and pigment content suitable for human consumption. Experiments were conducted to determine the processing parameters which must be considered during scale-up. Parameters that affect the deproteination efficiency and quality of chitin produced include temperature, pH of shrimp and the fermentation mixture at the start and during fermentation, quantity of inocculum, mixing interval or speed, supplementary carbon source, strains being used and duration of fermentation. These parameters must be considered during scale up of fermentation from lab scale to pilot scale processes. Experiments inferred that during lactic acid fermentation pH must be kept below 5.5, intermittent mixing is necessary to release carbondioxide. Supplementing the medium with glucose leads under microoxygenic conditions used to formation of lactic acid, contributing to the desired higher acidity of the medium. Under optimal conditions an efficient solubilization is achieved of protein and pigments with a good flavour, leaving a solid fraction that can be converted into chitin.

Keywords : Chitin, Lactobacillus, Fermentation, Scale-up, Parameters, Shrimp

Introduction

Shrimp is a major sea food in Thailand and due to its high export potential, many companies have involved themselves in shrimp culture and processing producing high value shrimp products. Shrimp's head and skin are not edible and removed during processing, reducing the storage and handling charges. Crude shrimp head and skin material (30-40 % of whole shrimp) has hardly any economical value and is treated as biowaste and sold to feed mills. The local biowaste production in Thailand is estimated at 250,000 T per year. The shrimp biowaste in the tropical region is enriched with 10-20 % calcium, 30-65 % protein and 8-10 % chitin. The chitin, the protein and the pigment in shrimp biowaste have economical and nutritional value but there is a lack of generally available environment friendly technology at the industrial level to produce these components. In addition, there are no standardized quality parameters and specifications for chitin and chitosan, leading to breakage of product-consumer chain. In the conventional process, high amounts of NaOH are used to remove the protein, fat and pigment from the solid chitin containing fraction. Further, HCl treatment is used to remove the calcium carbonate. High amount of these chemicals leads to corrosion of the equipment being used, besides denaturation of other useful by-products. On the other hand fermentation using lactic acid bacteria (Hall and DeSilva, 1994) can recover chitin as well

as other valuable products such as proteins, minerals and pigment. Also less acid is needed in later stage of decalcification. In view of the existing problems in conventional chitin processing, a study has been conducted to determine optimum conditions for fermentative production of chitin, protein, pigment from shrimp biomaterials.

Methods and Materials

The moisture content was measured by drying the shrimp heads in an oven at 105°C for 24 h or until constant weight. For ash content a crucible was weighed and then put in a furnace at 750°C for conditioning. Then 1 g dried waste was put in the crucible and heated for 24 h in the muffle furnace (Sanyo Gallenkamp, UK). Protein content was measured by Biuret protein assay. Standard curve at 570 nm was obtained. Total lipid was extracted from shrimp waste using soxhlet apparatus. The sample before extraction was dried at 100°C for 4 hours, blended into small pieces, put in a thimble with 200 ml of petroleum ether at 65°C for 15 h. After extraction, the solution was concentrated by continuous removal of solvent and finally drying the solvent at 105 °C.

Preparation of inocculum

Lactobacillus plantarum 541 (TISTR collection, Bangkok) was transferred to liquid MRS medium (5.5 % MRS, Difco # 0881-17-5). After 24 h incubation (24 h, 30°C), this culture was used to inoculate shrimp biowaste.

Fermentative bioconversion of shrimp biowaste

The frozen shrimp was obtained from Surapong Sea Foods Co. Ltd, Samutsakorn, Thailand and thawed and chopped before experiment. 10% (v/w) broth was used in all the experiments except stated otherwise.

Deproteination and demineralization

Deproteination after fermentation was carried by subjecting the solid sample to 2% NaOH for 24 h at room temperature. Demineralization was conducted by subjecting the deproteinized sample to 2 % HCl for 24 h.

Effect of temperature, stirring, inocculum size and fermentation time

To determine the effect of temperature on the growth of Lactobacillus plantarum 541, experiments were conducted by inoculating four reactor vessels (Biostat Q , B. Braun) containing 400 ml of MRS broth each. OD values were measured at regular intervals. Stirring effects of culture were studied by subjecting the four vessels at 0, 50, 100 and 150 rpm speeds and observing growth at 30°C. To investigate the effect of inocculum size, 500 g of crushed shrimp biomaterial 1L beakers was mixed with 0, 5(25 ml), 10 (50 ml), and 15 % (75 ml) inocculum. At regular intervals growth and pH were observed. Samples were taken for deproteinization efficiency at 0, 6, 12 and 24 h to see the optimum time.

Effect of acid and glucose on the fermentation

Experiments were conducted with and without acetic acid (HAc) and different amounts of glucose. Three experiments in beakers containing 500 g shrimp biowaste were carried out. The pH measured at regular intervals in all experiments. In the first set of experiments, 1% HAc (v/w) was added to two beakers and the other two were used as control. Then experiments with glucose in form of cassava syrup (70 % glucose) were

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conducted. Two beakers were given 0, 2, 4, 6, 8, 10 or 12% glucose (w/w) syrup respectively. To ensure uniform distribution, the glucose syrup was diluted two times. The volume of the 14 beakers was made equal by adding distilled water. The pH of hydrolysate was measured. The third experiment was conducted with 1% HAc acid and 10% glucose, to confirm the effect of combination treatment. Finally in the fourth experiment, 1 % HAc was added to 12 beakers with 500 g crushed sample and subsequently 0, 2.5, 5 and 10 % glucose syrup, two beakers each.

Fermentation on large scale (Drum Reactor)

Drum reactor (Mukku et al., 1996) was used to conduct large scale tests. The temperature in the reactor was 30°C. To 5 kg crushed biowaste 10 % (v/w) inocculum, 1% acetic acid, 3.5 % cassava glucose syrup were mixed thoroughly but gently. The reactor was revolved at 8 rpm. Experiments were conducted for 20 h with intermittent sampling for pH, protein and bacterial measurement. After fermentation, the hydrolysate was removed and analyzed. The solid residue in the inner drum of the reactor was rinsed with tap water. The solids were dried in an oven at 50 °C and weighed. The protein content and ash content of the residue were obtained. The results are compared with small scale experiments.

Results and Discussion

Effect of temperature

Experiments conducted to study the growth of L. plantarum 541 at 30,35,40 and 45°C revealed that 30°C was the best temperature. The trend is presented in Fig. 1.
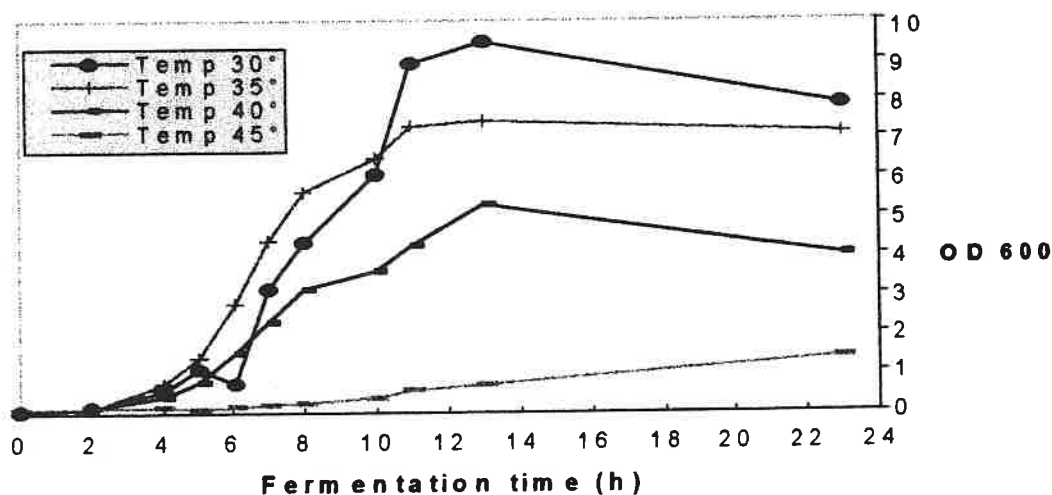

Fig. 1 Effect of Temperature on culture growth of L. Plantarum 541

Shrimp biowaste

The moisture content of the shrimp biowaste was around 75 % w.b. The initial protein content measured by Biuret assay was around 10.1 % w.b. The fat content was 2.3%, initial pH between 7.5 - 8.5.

Standard curve for *Lactobacillus plantarum* 541

A linear relation of Y=1.34 X-0.62 (R Square = 0.99) was obtained where Y is Cell No.(No x 10^9) and X is OD_{600} at 30°C.

pH Changes due to addition of acetic acid

The pH of the initial mixture of shrimp and MRS medium ranges between 7.5-8.5 If the pH is not reduced below 5.5, the biowaste starts to deteriorate producing organic amines and ammonia (Kungsuwan et al. 1996). Besides the Lactic acid bacteria have favorable growth conditions with pH around 5. The lowering of the pH could be achieved by addition of acetic acid. In an experiment, during 20 h fermentation in total 25 ml was added. The pH variations with and without acid addition are presented in Table 1.

Table 1. Variations in pH during fermentation (Temp = 35°C, Shrimp waste = 1 kg)

Duration (h)	Without control (no acid)	With initial control (5ml) Initial = 7.5	With intermittent control with acetic acid Initial pH = 7.97		
			pH	pH after addition	acid (ml)
0	7.95	5.90	7.97	5.30	10
3	7.35	6.45	6.60	5.14	5
6	7.12	6.67	5.82		
9	7.31	6.78	6.59	5.23	5
12	7.31	6.88	6.84		
21	7.31	6.87	6.84		

Fermentation in presence of added glucose

Lactobacillus in microaerophilic environment converts glucose in lactic acid. The lactic acid produced helps in lowering the pH and dissolve the calcium. Alos when glucose is present in sufficient amount lactic acid bacteria will not consume protein. Initially for all samples, the pH followed a similar trend (shown by same line for clarity) but after 8 h, they showed differences. 7.0 % cassava glucose syrup showed excellent results (Fig. 2). This set of experiments inferred that glucose effects in pH reduction usually after 6-9 h.
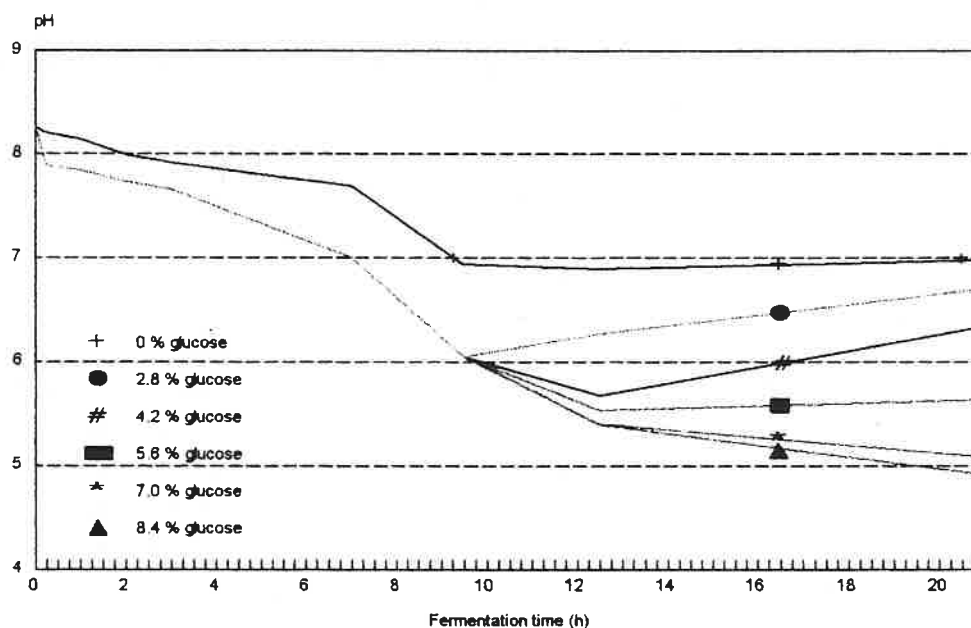
Fig. 2 Effect of different percentages of cassava glucose syrup on pH during fermentation

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Effect of glucose and acetic acid

Subsequently a combination of acetic acid (5 ml i.e. 1 %) and 10 % cassava syrup (70 % conc.) was tried during which the initial pH was brought down to 4.8. The pH remained lower than 6.6 and was after 7 h reduced to 5.5. The hydrolysate had good color and smell. In the combination treatment of different percentages of glucose and acetic acid swelling was observed after 5-6 h, indicating production of CO_2. These experiments indicated that a combination treatment is more effective to reduce the pH. Reduction in the amounts of glucose (5%) resulted in an excellent trend of pH decline as well (Figure 3). These results were not significantly different to that with 10% syrup addition. Though the final pH in glucose experiments without acetic acid addition also reached nearly 5.0, the quality of hydrolysate was better in experiments with acetic acid as the pH during the first few hours also remained lower than 6.5, reducing spoilage.
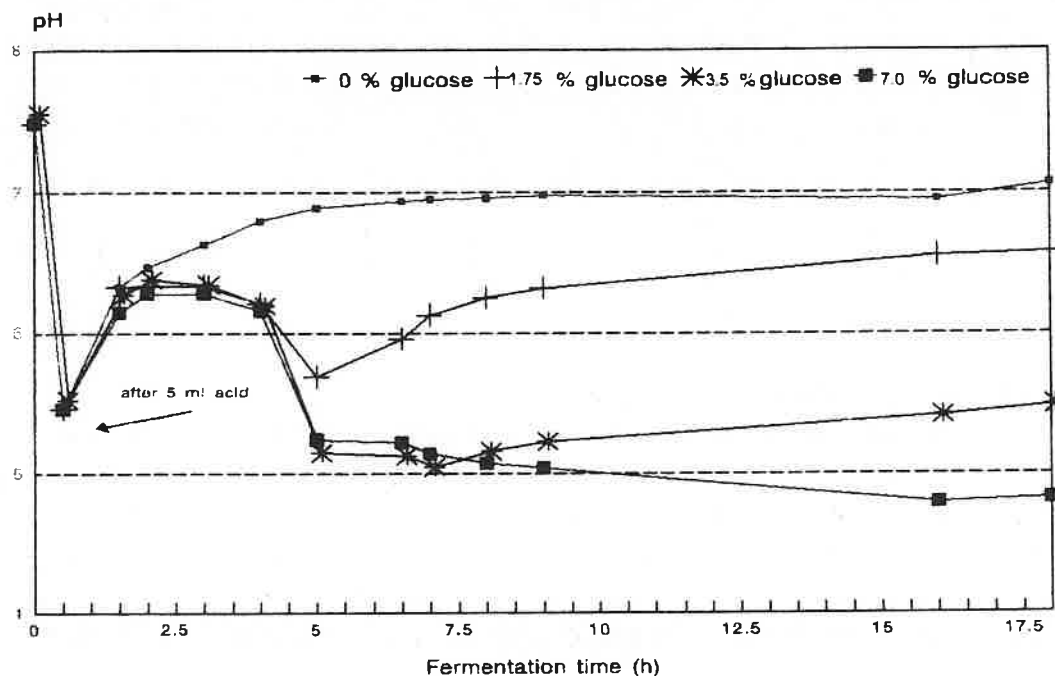

Fig. 3 Effect of combination treatment of HAc (1 %) and cassava glucose syrup on pH

Culture quantity, fermentation time and mixing

In experiments conducted on 1 kg shrimp biowaste with 10 % and 20 % (v/w) inocculum, significant difference was not found on the chitin yield. It was observed that in the residue after fermentation using 20 % inocculum, chitin content was 37.3 % whereas the chitin content with fermentation using 10% inocculum was 35.6 %. These experiments show that after a limit the increase in culture would not give considerable increase in deproteination and therefore chitin extraction. For an industrial scale fermentation, reduction in culture quantity would reduce the overall cost in a long run. Similarly fermentation duration affects the deproteination efficiency.

The acidity produced by lactic acid bacteria reacts with the calcium carbonate present in the shell of the shrimp to produce large quantities of carbondioxide. If proper mixing is not provided, unmixed zones affect the fermentation leading to pockets of high pH material capable of supporting the growth of spoilage organisms (Hall and Reid, 1995). Without mixing, air is trapped in some pockets of the shrimp biowaste accrue. Mixing also helps the bacteria to penetrate into the waste. With acid addition, mixing

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helps the distribution of acid in the waste as the quantity of acid addition is just 1%. Experiment conducted with and without mixing inferred that in the beaker without mixing, the biowaste deteriorated on the top and that swelling was observed due to evolution of carbondioxide. The waste deteriorated when not mixed. Not significant difference was observed on the quantity of protein extracted, but the hydrolysate had an off-odour, indicating spoilage.

Scale up experiments

Experiments on the scaled up experiments on the drum reactor were comparable to small scale. Although the protein extracted was around 3.5 % compared to 8.0 % original, good mixing was observed. The glucose was fixed to 5 % cassava syrup, 1 % acid and 10 % culture, so as to keep the costs low. The time was limited to 20 h. The fermentation was efficient.

Conclusions

Lactobacillus fermentation is a good alternative to extract chitin, protein and minerals from shrimp biowaste, when compared to chemical process even at large scale. The pH should be maintained below 5.5 so that the biowaste does not deteriorate. This could be achieved by adding a combination treatment of 1 % acetic acid and 3.5 % cassava syrup glucose. Temperature should be maintained at 30°C during growth of culture and fermentation with biowaste. Stirring does not significantly help growth of pure culture, but mixing is inevitable with biowaste. Sufficient fermentation is achieved in 20 h and later, the final traces of protein and minerals could be removed by treating with 2 % NaOH and 2 % HCl for 1 h at 80°C.

More work is needed to be done on cheaper growth medium. The time effect must be studied in details, so must be the culture quantity. Especially when the medium is expensive. Cheaper medium for lactobacillus will be tried. The quality of hydrolysate need to be analyzed for further applications and so must be for the crude chitin extracted.

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PARTIAL ACETYLATION OF CHITOSAN AND A CONDITIONING PERIOD ARE ESSENTIAL FOR ELICITATION OF H_2O_2 IN SURFACE-ABRADED TISSUES FROM VARIOUS PLANTS.

Heinrich KAUSS[a], Wolfgang JEBLICK[a], Alain DOMARD[b] and Jürgen SIEGRIST[c]

[a]Universität Kaiserslautern, Fachbereich Biologie, Postfach 3049, D-67653 Kaiserslautern, Germany, Fax: +631-205 2600; E-Mail: kauss@rhrk.uni-kl.de; [b]Laboratoire d'etudes des Matériaux Plastiques et des Biomatériaux (URA CNRS 507), 43 Bd. du 11 Novembre 1918, F-69006, Villeurbanne cedex, France, [c] Universität Hohenheim, Institut für Phytomedizin, D-70593 Stuttgart, Germany

Abstract

Hypocotyls from etiolated seedlings of cucumber were gently abraded at their epidermal surface to render them permeable for water-soluble compounds. Segments were cut and used for elicitation of H_2O_2. Most active were partially acetylated polymeric chitosan and respective chitosan fragments (DP26), produced by depolymerization with nitrite. In contrast, fully deacetylated chitosan and respective fragments as well as defined deacetylated chitosan oligomers exhibited only low activity. With chitin oligomers higher concentrations were required and their activity was not additive to that of partially acetylated chitosan. These results indicate complementary receptors exhibiting high affinity for chitosan and its fragments when some of the glucosamine residues are acetylated. Chitin oligomers are percepted by the same receptors but only with lower affinity. However, freshly abraded segments were not competent for elicitation of H_2O_2 by chitosan but developed this potency when the abraded segments were shaken in buffer overnight ("conditioning"). When salicylic acid, which can induce disease resistance in cucumber hypocotyls, was present during conditioning, competence for H_2O_2 elicitation was further enhanced. The requirement of both partial acetylation of chitosan and a conditioning period was also confirmed for hypocotyls of melon, pumpkin, bean, mungbean and soybean as well as for epicotyls of pea. In contrast, under the conditions used, the enhancement of H_2O_2 elicitation by salicylic acid was detectable only in cucumber, pumpkin and pea but not in the other species.

Keywords: Bean, chitosan acetylation, cucumber, elicitor, H_2O_2, melon, pea, salicylic acid, soybean.

Materials and methods

Growth of etiolated cucumber seedlings was as described[1,2]. The other plants were grown under same conditions. The hypocotyls or epicotyls (pea) were gently abraded at their surface using a slurry (0.5 g/ml) of SiC (800 mesh). Segments (2 cm) were cut and used either immediately, or upon overnight conditioning in buffer in the absence or presence of 100 μM salicylic acid in order to induce competence for H_2O_2 elicitation[1]. H_2O_2 elicitation assays were performed in 3 ml of another buffer (10 mM Bistris/Mes, pH 5.7) with either 10 (cucumber, melon, mungbean), 8 (soybean, pea) or 4 segments (pumpkin, bean). H_2O_2 was determined using the luminol method[1].

Chitosan samples and defined oligomers were prepared as described[3]. Chitosan concentration was standardized as glucosamine content[3]. For depolymerization, the chitosan polymers were solubilized in 6 %, v/v, acetic acid (100 mg/80 ml) and sodium nitrite (17.5 mg/20 ml water) was added dropwise at room temperature within 0.5 h. After a further 0.5 h of stirring, the materials were freeze-dried. The number average DP was determined as described[3]. Chitotetraose was purchased from IsoSep AB, Tulling (Sweden).

Seeds of cucumber (cv Mervita), melon (cv Bastion F_1 Hybride), pumpkin (cv Melonennetz), bean (cv Dufrix), pea (cv Rheinperle) and soybean (cv unknown) were purchased from local stores.

Results and discussion

It was formerly shown for a polymeric elicitor preparation from fungal cell walls[1] and ergosterol[2] that abraded hypocotyls of cucumber exhibit competence for H_2O_2 elicitation only when the segments were incubated after abrasion in buffer overnight. Presence of salicylic acid during this conditioning period greatly enhanced the potency for H_2O_2 elicitation by both elicitors. These features were investigated here with the same plant tissue but using partially acetylated chitosan as an elicitor.

Both polymeric and fragmented partially acetylated chitosan cause a transient H_2O_2 "burst" of similar magnitude (Fig. 1). Thus, binding of the chitosan induces a transient increase in the activity of a plasma membrane located O_2 reducing enzyme complex. The subsequent decrease reflects the rate of steadily operating H_2O_2 degrading enzymes[2].

When a second dose of the same elicitor was applied directly after the burst, we observed only a small second burst and only in case of chitosan fragments (Fig. 1A). In contrast, if the segments were washed after the first burst (Fig. 1B), a second dose of chitosan fragments induced a greater burst. These results indicate that the apparent binding affinity of chitosan to presumed receptors is higher in the case of polymeric chitosan which might result from the fact that in this case one molecule can bind simultaneously to several sites, possibly even cross-linking several receptors. The results in Fig. 1B also include that shortly
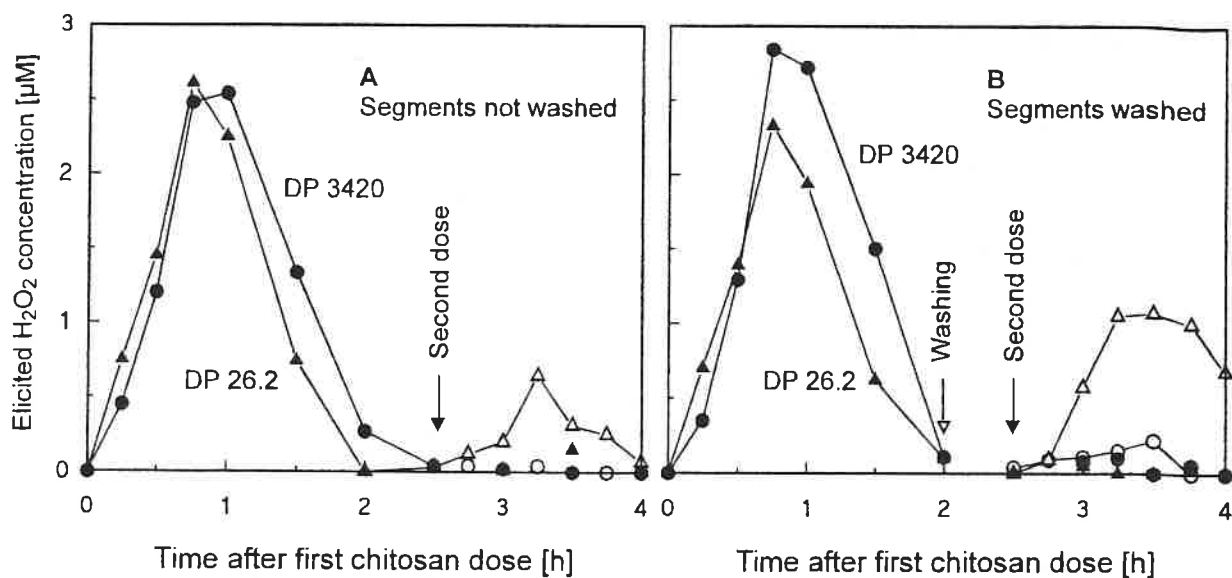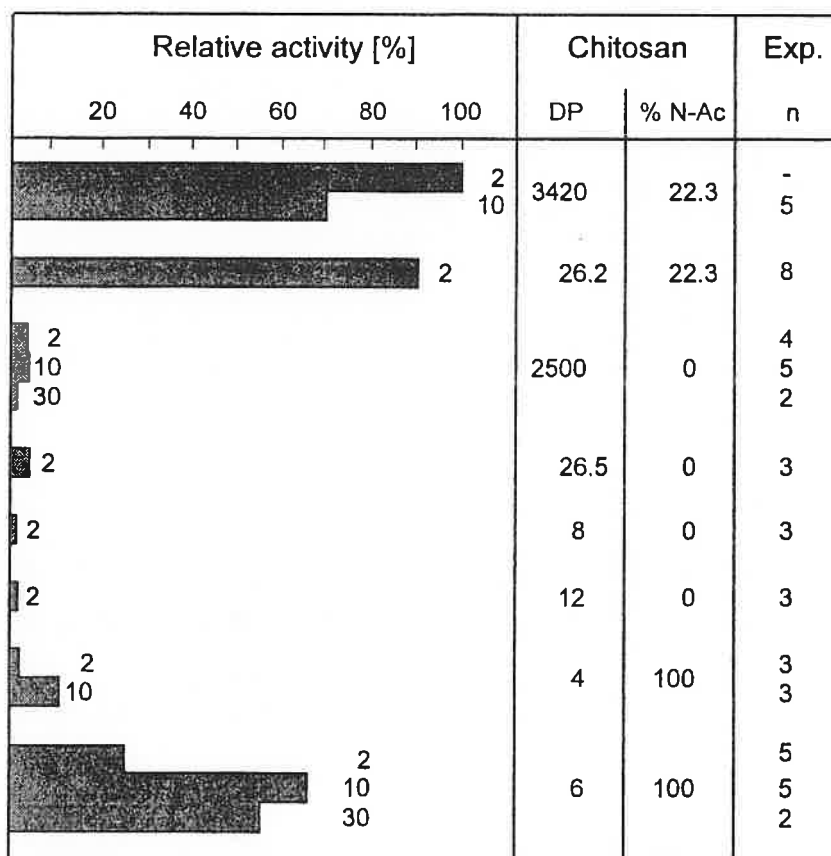
Figure 1. Time-course and reversion of H_2O_2 elicitation induced by partially acetylated chitosan in abraded cucumber hypocotyl segments conditioned in presence of salicylic acid. **A:** The first dose (2 µg/ml) of either 22.3 % N-Ac chitosan (●,DP 3420) or respective fragments (▲, DP 26.2) was added at zero time. A second dose (2 µg/ml, open symbols) of the same materials was added at the arrow directly to the corresponding assays, without washing of the segments. **B:** Same protocol as in A but after the first H_2O_2 burst the segments were washed twice with 3 ml of buffer (open arrow). The second elicitor dose was applied after resuspension (filled arrow). Experiments A and B were performed with the same batch of segments. A representative out of three experiments with similar results is given.

after the first H_2O_2 burst elicited by chitosan fragments, the epidermal cells, at least partially, are still ready for a second stimulation. This observation contrasts elicitation by ergosterol[2] where a classical refractory phase appears to persist for at least 4 h. The difference between polymeric chitosan and its fragments (Fig. 1B) may indicate that the long-term down-regulation of H_2O_2 production in the case of polymeric chitosan is, at least in part, due to a more persistent occupation of the respective receptors rather than to induction of a classical refractory phase. It should also be noted that, in general, the transient nature of elicited H_2O_2 production ("burst") appears to be caused by dephosphorylation of those phosphoproteins which became activated as a consequence of initial elicitor binding (H. Kauss and W. Jeblick, submitted).

H_2O_2 elicitation was high only with partially acetylated chitosan, in case of both high and low DP, whereas fully deacetylated chitosan, respective fragments and defined nonacetylated oligomers exhibited very low activity (Fig. 2).

Relative activity [%]					Chitosan		Exp.
20	40	60	80	100	DP	% N-Ac	n
				2 10	3420	22.3	- 5
				2	26.2	22.3	8
2 10 30					2500	0	4 5 2
2					26.5	0	3
2					8	0	3
2					12	0	3
2 10					4	100	3 3
2 10 30					6	100	5 5 2

Figure 2. Elicitation of H_2O_2 by various chitosan derivatives in abraded cucumber hypocotyls conditioned in presence of salicylic acid. H_2O_2 concentration was determined 45 min after addition of the elicitors, the difference between these values and a control is shown (see Fig. 1). The elicitor concentration used was in any case 2 µg/ml and for some compounds in addition 10 or 30 µg/ml, as indicated by 2, 10 or 30 at the columns. Concentration of chitosans and fragments is given as equivalents of nonacetylated glucosamine whereas the concentration of defined chitosan and chitin oligomers is given on a weight basis. The means from n experiments performed are given relative to the activity of 2 µg/ml partially acetylated chitosan.

With partially acetylated chitosan saturation was reached at about 1.2 µg/ml but routinely 2 µg/ml were used (Fig. 2). When its concentration was raised to 10 µg/ml, the H_2O_2 induction decreased. With chitin oligomers the optimal concentration was above 10 µg/ml (Fig. 2). In one detailed experiment performed, 15 µg/ml chitohexaose were saturating. Interestingly, when the system was saturated with 2 µg/ml of the partially acetylated chitosan and simultaneously chitohexaose was added at 2 or 10 µg/ml, we observed a decrease in H_2O_2 production, indicating that we came higher than the concentration optimum (data not shown). In contrast, the activity of 2 µg/ml chitosan was additive to the activity of 20 µg/ml
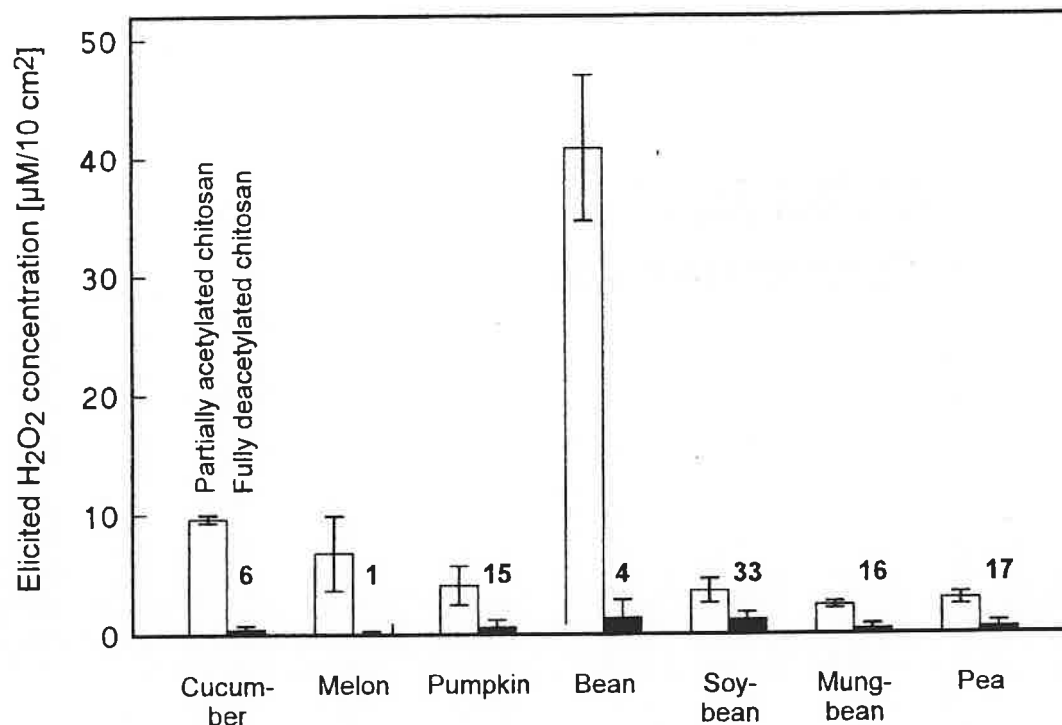
Figure 3. Comparison of H_2O_2 elicitation with partially acetylated chitosan (22.3 % N-Ac, DP 3420) and fully deacetylated chitosan (0 % N-Ac, DP 2500) in various plants. Same experiments as in Fig. 4, conditioning in the presence of salicylic acid. The numbers at the filled column give the percentage of H_2O_2 elicitation by fully deacetylated chitosan: partially acetylated chitosan was set as 100 %.

of a fungal cell wall elicitor of the glucan-type. The latter results prove that the H_2O_2 producing machinery was not rate-liminting. Thus, the presumed receptors specific for partially acetylated chitosan appear to accept also chitin oligomers but with a lower apparent affinity.

Use of chitosan as a common elicitor allowed to compare a variety of plant species in regard to their ability for H_2O_2 elicitation. Similar to cucumber (Fig. 2), all other plants also used in the present report respond with high H_2O_2 production only when partially acetylated chitosan was used (Fig. 3). With fully deacetylated chitosan a low activity was observed in all species. The range was between 1 % (melon) and 33 % (soybean), the means ± SD from 7 species was 13 ± 11 %, compared to partially acetylated chitosan. These results indicate that the receptor requiring acetylation of some of the glucosamine residues within a chitosan chain is wide-spread among plants.

The most striking feature of the cucumber hypocotyl H_2O_2 elicitation system is that freshly abraded tissue is barely competent for H_2O_2 elicitation with a polymeric fungal elicitor[1] and ergosterol[2]. This observation was now confirmed
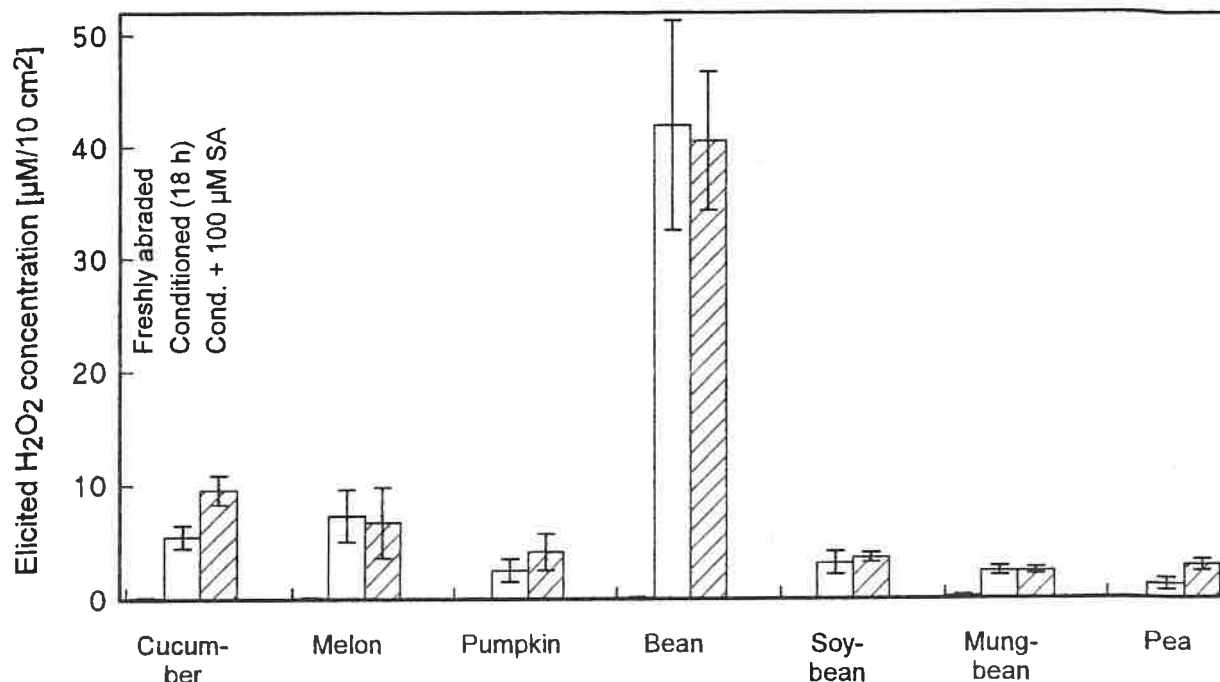
Figure 4. Influence of conditioning in the absence and presence of salicylic acid on elicitation of H_2O_2 by partially acetylated chitosan in abraded hypocotyl segments from cucumber, melon, pumpkin, bean, mungbean and soybean as well as from pea epicotyls. Etiolated seedlings were gently abraded and used shortly after abrasion, or after shaking for 18 h in buffer ("conditioned") without or with salicylic acid (SA, 100 μM). Chitosan of DP 3420 and 22.3 % N-Ac was used at 2 μg/ml as an elicitor. The H_2O_2 concentration was maximal in the case of cucumber at 45 min and with all the other plants at 30 min. The H_2O_2 concentration before elicitation was subtracted. These values were corrected (f = 1.0 - 1.2) for minor differences in the surface area of the various segments used in one assay to allow a better comparison among plants. Means ± SE from 3 independent experiments are given. The comparatively great variability (± SD) is mainly caused by differences between individual experiments in the absolute levels of H_2O_2 production whereas the increase caused by salicylic acid in the case of cucumber, pumpkin and pea was evident in all individual experiments. In the experiments with freshly abraded segments, H_2O_2 production was at the detection limits and with most plants < 1 % of the H_2O_2 elicitation found for segments conditioned with SA. The exception was mungbean (~ 5 %), a result requiring confirmation in future studies.

using partially acetylated chitosan as an elicitor (Fig. 4). Similar to cucumber, an extremely low H_2O_2 elicitation by this chitosan was also evident with freshly abraded hypocotyls of melon, bean and soybean as well as epicotyls of pea (Fig. 4). In all these cases, shaking of the abraded hypocotyl segments in buffer over-night was necessary to result in a pronounced potency to become elicited by partially acetylated chitosan. This holds even true in case of bean hypocotyls which exhibit extremely high rates of H_2O_2 production once conditioned.

In the case of cucumber and fungal elicitor it has been shown that the development of elicitor competence can be inhibited by cycloheximide and puromycin[1], indicating a requirement for protein synthesis. These results and the time-dependence of the effect suggested that H_2O_2 elicitor competence is not constitutive. The results shown in the present report for chitosan as an elicitor as well as for other plant species confirm this notion. This is in contrast to all experience made with suspension cultures which are readily competent for H_2O_2 elicitation (for citations see refs. 1 and 2). It appears that these cultured plant cells are under a constant stress whereas in undisturbed differentiated plant tissue the development of H_2O_2 elicitor competence requires induction by a further stimulus resulting from cuticle abrasion[1,2]. It was speculated that the physiological equivalent of cuticle abrasion might be an unknown event occurring at early stages of the interaction of fungal pathogens with the plant surface. Current research indeed provides first evidence that already the adhesion on the surface of hypocotyls and the subsequent germination of spores from the cucumber pathogen *Colletotrichum lagenarium* are sufficient to provide a stimulus for some induction of H_2O_2 elicitor competence in resistant cucumber hypocotyls (A. Merten and H. Kauss, unpublished results).

Of special interest in regard to plant/pathogen interaction was the observation that in cucumber hypocotyls the development of H_2O_2 elicitor competence can be enhanced by the presence of salicylic acid during the conditioning period. In the case of the fungal elicitor the enhancement was in the mean 4.3-fold[1] and in the case of ergosterol 2.5-fold[2]. Salicylic acid and related chemicals are able to induce disease resistance in the epidermal cells of the cucumber hypocotyls and it appeared likely, therefore, that the enhancement of H_2O_2 elicitation competence represents one of the various mechanisms causing cooperatively the phenomenon of acquired disease resistance. Fig. 4 shows that during conditioning in the presence of salicylic acid also the chitosan-induced H_2O_2 burst is enhanced by 1.7-fold in the case of cucumber. This observation confirms for cucumber the results made with the other two elicitors mentioned above. Similarly, salicylic acid enhanced the H_2O_2 elicitation competence considerably also in pumpkin and pea (Fig. 4). However, a considerable influence of salicylic acid on the induction of H_2O_2 elicitation by chitosan was not found with melon, bean, soybean and mungbean (Fig. 4). Eventhough we have investigated up to now only the end-point of the conditioning process with these plants, these results indicate that the

enhancement effect caused by salicylic acid may not be general among plants. Further experiments have to include time-course studies and also attempts to correlate the elicitor results with infection experiments using the above plants pretreated with inducers of acquired resistance.

Conclusions

The present results indicate the existence of complementary chitosan receptors linked by a signal transduction chain to a H_2O_2 producing enzyme system. These presumed receptors exhibit a preferential binding when some of the glucosamine units of chitosan are acetylated. Such materials occur naturally in fungal cell walls due to the fact that chitosan arises by enzymatic deacetylation of nascent chitin. The requirement of partial acetylation for H_2O_2 elicitation appears to be a feature which is common to several plants and is in agreement with observations on pisatin induction in etiolated epicotyls of pea which exhibits in the low range of the dose-response curves a preference for partially acetylated chitosan oligomers[4]. In contrast, for induction of pisatin in pea pods[5] synthetic non-acetylated chitosan oligomers were considered as potent elicitors although no comparison with partially acetylated samples was made and comparatively high concentrations were required. The latter appears understandable as the intact endocarp surface - as any epidermal layer - likely exhibits a low permeability for water-soluble compounds.

Still another specificity appears to apply for chitosan-induced callose synthesis in suspension-cultured parsley cells. Callose synthesis was induced only by polymeric chitosan and not by fragments, fully deacetylated samples being more potent than partially acetylated ones[3]. In addition, other polycations like poly-*L*-ornithine were also active. These observations suggested that for induction of callose synthesis the polymeric chitosan molecules interact als polycations with general plasma membrane constituents and that in the case of callose induction complementary receptors are not implied.

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