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Chitosans depolymerized with the aid of papain and stabilized as glycosylamines

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Abstract

The viscosity of chitosan lactate solutions in the concentration range of interest for practical applications (120–150 g kg^{-1}) was promptly lowered in the presence of papain at pH 3.2 and 25–40 °C. Thermal drying of partially depolymerized chitosan lactate brought about side reactions involving structure alterations, and yielded slightly soluble brilliant brown powders. Full solubility and improved organoleptic properties of the partially depolymerized chitosans were obtained by working up the solutions in such a way as to prevent reactions between the reducing ends and the amino groups, i.e. by incubating the ammonia-precipitated partially depolymerized chitosans at 60 °C under pressure with NH_3 and drying at 40 °C. The pale yellow product (low molecular weight chitosan in glycosylamine form) had long shelf life and was promptly soluble in the presence of glycolic, lactic, ascorbic and hydrochloric acid, less promptly with citric acid. Three commercially available low molecular weight chitosans were also examined for comparison. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Chitosan; Chitooligomers; Papain; Glycosylamine; Chitosylamine

1. Introduction

Partially depolymerized chitosans, with average molecular weight in the order of 10 kDa, seem to have enhanced biochemical significance compared to the chitosans from which they derive, and are more easily handled (Muzzarelli, 2001, 2002). Their superior antibacterial activity has been explained by Liu, Guan, Yang, Li, and De Yao (2001) in terms of inhibition of the transcription from DNA. They were proposed for practical use in milk preservation, cotton textile finishing and oral hygiene (Seong, Kim, & Ko, 1999; Shin, Yoo, & Min, 1999; Tarsi, Muzzarelli, Guzman, & Pruzzo, 1997; Tarsi, Corbin, Pruzzo, & Muzzarelli, 1998; Tsai, Wu, & Su, 2000). Partially depolymerized chitosans modulated plant resistance to diseases (Vasyukova et al., 2001).

When administered to animals, partially depolymerized chitosans stimulate murine peritoneal macrophages, killing of tumour cells (Seo et al., 2000), and antitumour activity (Shon, Ha, Jeong, Kim, & Nam, 2001). In surgery, adhesion prevention was reported by Hellebrekers, Trimbos-Kemper, Trimbos, Emeis, and Kooistra (2000). According to Kondo, Nakatani, Hayashi, and Ito (2000), 20 kDa chitosans prevent progression of *diabetes mellitus* and exhibit higher affinity for lipopolysaccharide than 140 kDa chitosan. Food applications were reviewed by Jeon, Shahidi, and Kim (2000), and drug release by Imai, Shiraishi, and Otagiri (2000).

The depolymerization of chitosan can be done by chemical means (HCl, H_2O_2, HNO_2) that, however, do not lend themselves to easy reaction control, by physical means (sonication, shearing) that require special equipment, and by enzymatic means, based either on specific enzymes, such as chitosanases (Cheng & Li, 2000; Ogawa, Chrispinas, Yoshida, Inoue, & Kariya, 2001; Santos, Guirardello, & Franco, 2001) or on unspecific enzymes (Muzzarelli, 1997; Muzzarelli, Stanic, & Ramos, 1999), including lysozymes, cellulases, lipases, amylases, papain and pectinases (Grigolon, Azevedo, Santos, & Franco, 2001; Muzzarelli, Tomasetti, & Ilari, 1994; Shin-ya, Lee, Hinode, & Kajiuchi, 2001; Terbojevich, Cosani, & Muzzarelli, 1996; Tsai et al., 2000; Zhang, Du, Mitsutomi, & Aiba, 1999).

Among these enzymes, papain, a cysteine protease, is particularly attractive because of its plant origin, wide

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industrial use in meat tenderization, use in medication for wound debridement, and inhibition by human salivary cystatin (Alvarez et al., 2000; Buhling et al., 2000; Lin & Welsh, 1996; Tseng, Tseng, Levine, & Bobek, 2000). Papain, also present in fig latex, is isolated from *Carica papaya* and *C. candamarcensis*, as a crude latex (Caro, Villeneuve, Pina, Reynes, & Graille, 2000; Walraevens, Vandermeer-Piret, Vandermeer, Gourlet, & Robberecht, 1999) that contains some other enzymes, such as lipases (Gandhi & Mukherjee, 2001) and is then purified by crystallization (Monti, Basilio, Trevisan, & Contiero, 2000).

The enzymatically depolymerized chitosans should be prepared and handled with certain precautions. A point of difference from chemically depolymerized chitosans is that those generated by HNO_2 have a 2,5-anhydromannose terminal unit (Casu et al., 1986). An obvious point of difference from plain chitosan is the much higher number of reducing units present in enzymatically depolymerized chitosans. As a consequence, the shorter the chains, the higher the probability that aldehydo groups react with amino groups of partially depolymerized chitosans according to Schiff, Maillard and Amadori reactions that impart undesirable properties to the product, such as brown colour and scarce solubility (Fujimaki, Namiki, & Kato, 1986). In view of this kind of difficulties, certain producers of depolymerized chitosans, currently on the market, associate chitosan with questionable compounds that enhance their solubility, but do not improve their quality (*vide infra*). While these undesirable effects may be minimized by freeze-drying and proper storage conditions in laboratory scale production of oligomers, large-scale production of partially depolymerized chitosans, to be economically viable and technically acceptable, should be based on simple separation processes and thermal drying.

Absence of substantial chemical alteration, good shelf life, prompt dissolution with organic acids, appealing organoleptic properties, efficacy in adsorbing edible oils, colourless solutions, absence of unidentified undeclared compounds, simplicity and sustainability of the production process, and easy formulation with other ingredients, are all industrial requirements for the preparation of depolymerized chitosans.

The present work was undertaken to synthesize glycosylamines from partially depolymerized chitosans and ammonia. Glycosylamines, in which the anomeric hydroxyl group has been replaced by an amino group, are amply present in nature, adenosine being a common example (Binkley, 1988; Pigman, Cleveland, Couch, & Cleveland, 1951). They are synthesized in vitro during the total synthesis of glycoproteins: a substituted chitobiose in the hemiacetal form is converted into a glycosylamine by ammonolysis of the masked aldehyde, and then the glycosylamine is coupled to the peptide in organic media (Cohen-Anisfeld & Lansbury, 1993; Schanzenbach, Ley, Matern, & Peter, 1997; Wang et al., 2001). Glycosylamines are also intermediates in the Diels-Alder and Strecker reactions (Kunz, Hofmeister, & Glaser, 1998; Tietgen, Schultz-Kukula, & Kunz, 2000). A further scope of this work was to evaluate the properties of the chitosans in glycosylamine form, here referred to as chitosylamines, in consideration of the fact that the chemistry mentioned in this paragraph has never been applied to chitosan.

2. Experimental

2.1. Chemicals

High molecular weight crustacean chitosans-20 were supplied by Primex, Drammen, Norway, and by Sirc, Caleppio, Italy; medium molecular weight chitosan-20 (from squid β-chitin) with degree of acetylation 0.20 (by alkalimetry) and p*K* 6.4 was supplied by Daras, Marseille, France. Chitooligomers or partially depolymerized chitosans were Chitooligomer®, RC Bio-chemical, Pusan, Korea; Liposan Ultra®, Vanson, USA; Betasanne®, Sochim International, Milan, Italy. Dietary supplement chitosans were Chitosano-800® and Chitosano-800-F®, Sirc, Caleppio, Italy; Kalo®, Roeder, Turin, Italy. *C. papaya* papain (30,000 USP-U mg^{-1}) was supplied by Calbiochem, Milan, Italy. Other compounds were analytical grade chemicals.

2.2. Instrumental analytical methods

The viscometric measurements were done at the shear rate value of 200 s^{-1} that did not generate excessive mechanical degradation of the polymers. The Haake Rotovisco RV-20 M5 was driven by a computer with Haake software. The thermostatic double-walled NV rotor was housed in an 11 ml cup filled with the solution under study. The Ubbelhode viscometer was also used for some measurements.

Aliquots of a chitosan lactate solution were collected at various periods of time, since the introduction of papain in the gel, immediately frozen and freeze-dried. The reducing capacity of the freeze-dried samples was determined with potassium ferricyanide (0.5 g) in Na_2CO_3 (0.5 M, 1 l) according to the method of Schales (Kurita, Youshino, Nishimura, & Ishii, 1993; Muzzarelli et al., 2001) as follows. The freeze-dried material (25 mg) was dissolved in water (75 ml): a portion (3 ml) was added to the ferricyanide solution (4 ml) and kept at 100 °C for 15 min, cooled at 15 °C for 5 min and read at 420 nm with a Pharmacia colorimeter LKB Novaspec II.

The infrared spectra were recorded with a Bruker IFS 25 spectrometer, or with a Nicolet 20-SX FT-IR spectrometer (DTGS detector) equipped with a Spectra Tech. Multiple Internal Reflectance accessory (DRIFT) for measurements in the solid state.

X-ray diffraction measurements on powder samples were performed with the Bruker AXS General Area Detector Diffraction System (GADDS) equipped with a two-dimensional (2D) gas-filled sealed multiwire

Table 1
Dependence of the velocity constant on the papain concentration for the hydrolysis of chitosan-20 pH 3.2, 25 °C. Reaction time 10 min

Papain concentration (mg/ml)	$(1/[\eta]_t) - (1/[\eta]_0)$ (g/dl)	Velocity constant ($h_{t=0}$) (10^3 min^{-1})
Chitosan 1.85 g l^{-1}		
0.53	0.067	9.0
0.27	0.059	8.0
0.05	0.044	4.4
0.03	0.010	3.4
Chitosan 2.65 g l^{-1}		
0.53	0.080	12.0
0.27	0.073	10.5
0.05	0.057	4.2
0.03	0.050	3.0

detector (scattering-angle resolution of 0.02°). Monochromatized Cu Kα radiation ($\lambda = 0.154$ nm) was used. The powder samples were placed in 0.8 mm diameter Lindemann glass capillaries. The sample–detector distance was 10 cm. The intensity versus scattering-angle spectra were obtained after radial average of the measured 2D isotropic diffraction patterns.

A Philips SEM 505 scanning electron microscope was used: the samples were prepared after cutting them to expose the inner part, fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, dehydrated and submitted to critical point drying and gold coated.

NMR spectra were recorded with a Bruker CXP-300 (75 MHz) spectrometer (polymer concentration 100 mg/0.6 ml D_2O, 25 °C, deuterated acetic acid).

The molecular size determinations were made by gel permeation chromatography with a Beckman Systemgold 116 pump equipped with a Systemgold 406 Analog Interface, a Shodex RI SE-61 detector, a TSK-GEL G-oligo PW column, 7.8 × 300 mm, and a TSK-GEL oligo guard column operated at the flow rate of 0.7 ml min^{-1}. Chitosan samples (10 μl, 5 g l^{-1}) were eluted with a mixture of acetic acid (0.5 M) and sodium acetate (0.2 M) at 25 °C.

Alkalimetry was performed after dissolution of chitosan (free base, 0.5 g) in 0.3 M HCl (20 ml) and titration with the aid of a Radiometer Meterlab pH-meter; the alkalimetric curve was analysed graphically.

Table 2
Kinetic data for the hydrolysis of chitosan-20 by papain at pH 3.2 and 25 °C. $V_i = h[S]$

Chitosan concentration		Velocity constant ($h_{t=0}$) (10^3 min^{-1})	V_i, Initial velocity (10^6 M min^{-1})
g/l	mM		
Papain 0.03 g l^{-1}			
0.56	3.3	2.5	8
1.18	7.0	3.9	27
2.00	11.8	3.4	40
2.65	15.9	3.0	47
3.77	22.2	2.3	52
Papain 0.05 g l^{-1}			
0.57	3.4	3.2	11
1.25	7.4	3.5	26
1.85	10.9	4.4	48
2.65	15.6	4.2	65
3.77	22.2	3.8	84

2.3. Preparative procedures

The depolymerization of chitosan-20 under the action of papain was examined in dilute chitosan solutions (2.65 g l^{-1}) obtained by dissolving chitosan hydrochloride and adjusting the pH value at 3.2 with lactic acid.

The preparation of low molecular weight chitosan lactate was carried out by adding papain (150 mg) to a chitosan lactate salt solution (7.5 g plain chitosan, total gel weight 50 g) and stirring for 90 min. Portions of this solution were either dried at 50 °C, or hydrogenated with $NaBH_4$ and then dried at 50 °C for 48 h at 48% relative humidity. The partially depolymerized chitosan lactate prepared as shown earlier was hydrogenated by slowly adding a 10% $NaBH_4$ solution with the aid of a peristaltic pump up to pH 4.8. After drying at 50 °C for 48 h, the product was found to be promptly soluble, but it turned dark brown in the course of 10 days at 45 °C and 48% relative humidity.

Chitosan was re-acetylated in water–methanol solution with the aid of acetic anhydride as described by Dal Pozzo et al. (2000).

Emulsions were prepared from olive oil with the aid of a Silverson instrument and no emulsifier, and each phase was measured in mm under standard conditions 12 h after preparation.

The Heto Drywinner 6 freeze-drier was used at − 92 °C and 0.1 mPa.

2.4. Preparation of the chitosylamine

Chitosan (7.5 g, Daras 151) suspended in water (43 g) for 2.5 h was reacted with lactic acid (5.3 g) previously dissolved in water (15 g). After dissolution under mechanical stirring (2 h at 25 °C), the solution temperature was raised to 40 °C and pH lowered to 3.2, and papain added (150 mg in 3 g water). The mixture was incubated at 40 °C for 90 min. The partially depolymerized chitosan was precipitated with dilute ammonia (21 ml of 25% ammonia with 130 g water) and the precipitate was washed with one rinse of dilute ammonia. The precipitate was incubated at 60 °C in a sealed container with ammonia solution for 18 h. After decanting, the product was optionally mixed with a sorbitol solution (1.2 g in 3 g water, i.e. 5% w/w) and kept at 40 °C until dry. A blade mill was used to pulverise the pale yellow dry product to the 150–250 μm grain size, fully soluble in 0.2 M HCl within seconds.

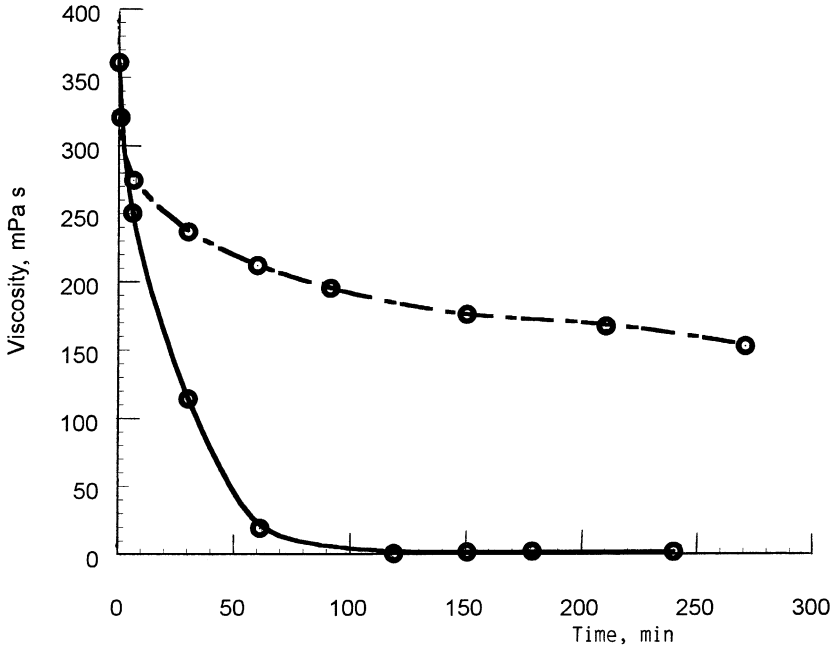

Fig. 1. Viscosity decrease of Primex chitosan-20 (upper curve, 30 g l^{-1}) and re-acetylated Primex chitosan-50 (lower curve, 30 g l^{-1}) due to depolymerization in the presence of papain.

3. Results and discussion

3.1. Depolymerization of chitosan in dilute solutions

For various papain concentrations, the velocity constants at pH 3.2 and 25 °C were obtained from the slopes at $t = 0$, after plotting $(1/[\eta]_t) - (1/[\eta]_0)$ versus time (Table 1). For a 17-fold decrease of the papain concentration, the velocity constant decreased to 1/4 only. In another series of measurements at chitosan concentration 1.85 g l^{-1}, the velocity constant decreased to 1/2.5 only. This relatively modest dependence indicated that the amount of papain could be conveniently kept low.

The kinetic data for the hydrolysis of chitosan-20 by

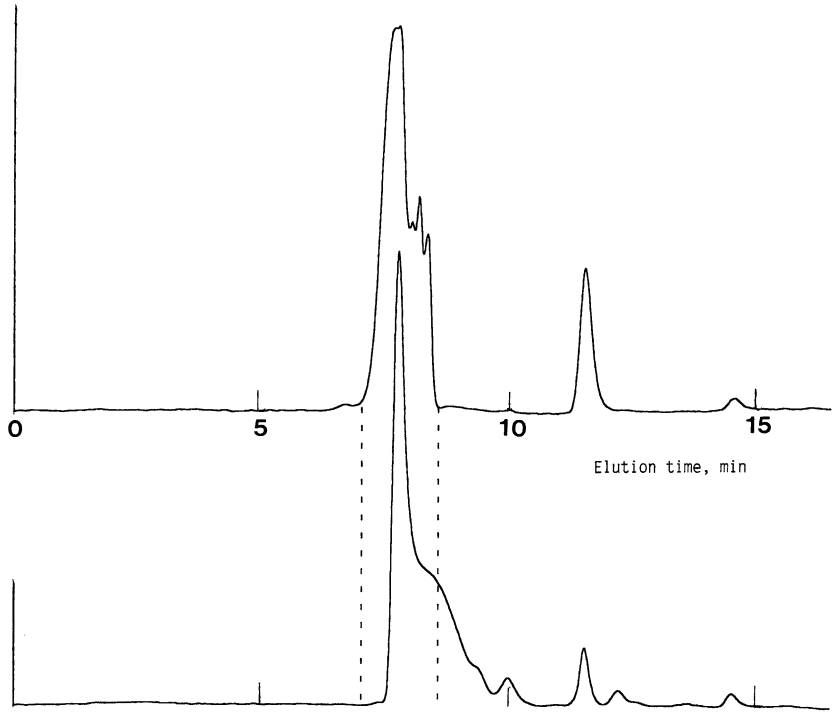

Fig. 2. Chromatographic profiles for chitosan (upper curve) and for partially depolymerized chitosan (lower curve).

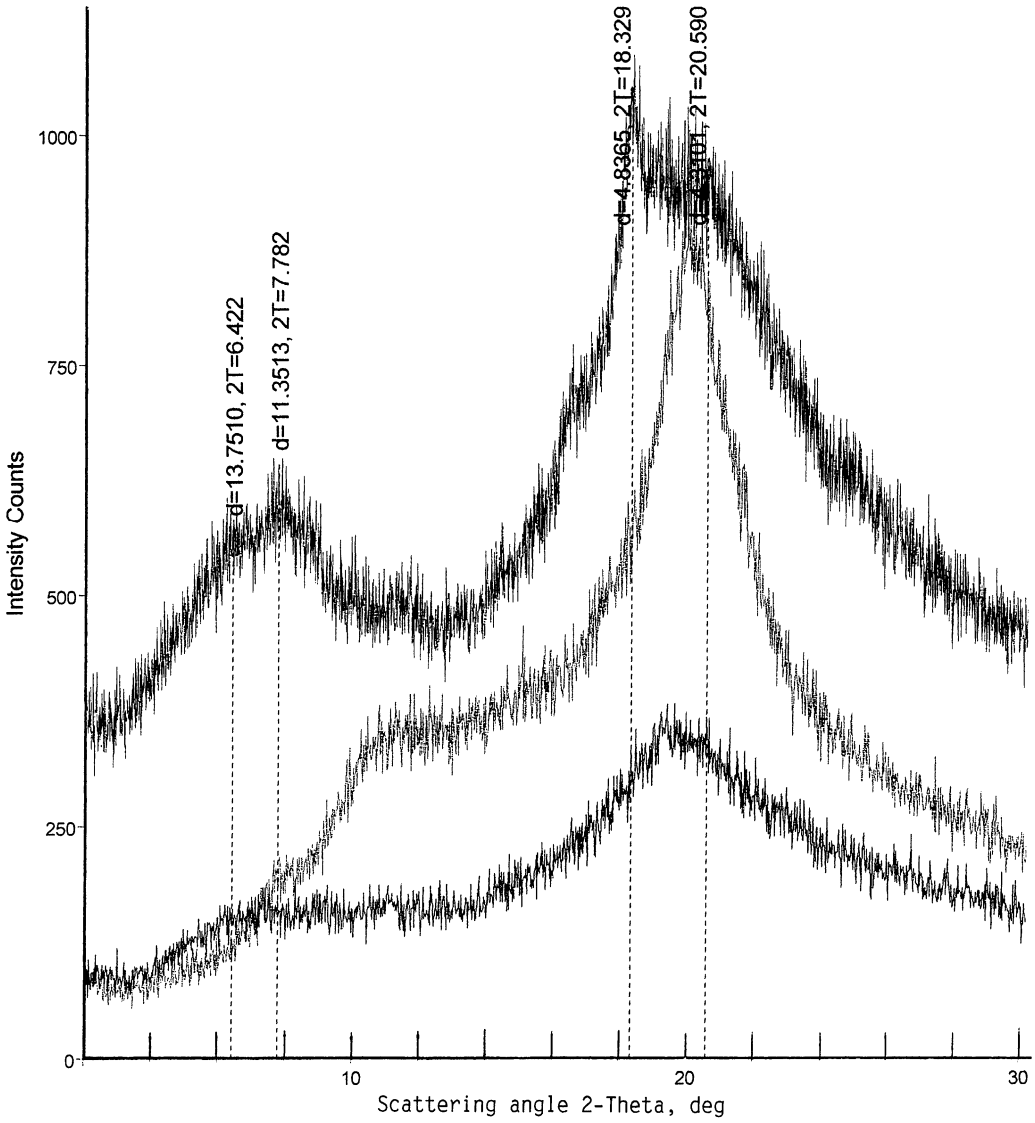

Fig. 3. X-ray diffraction spectra of partially depolymerized Sirc chitosan lactate (upper curve), Sirc chitosan (middle curve), and partially depolymerized Sirc chitosan lactate after hydrogenation (lower curve).

papain at pH 3.2 and 25 °C, collected in Table 2, show that the initial velocity increases with increasing substrate concentration. The calculated K_M was as high as ca. 80–90 mM, indicative of modest substrate to enzyme affinity. Nevertheless, a substantial viscosity drop was observed from 325 to 150 mPa s in 270 min, for Primex chitosan as shown in Fig. 1. Similar results recorded for the Sirc and Daras chitosans, indicated that papain was particularly effective. The samples generated by the 300 min hydrolytic treatment were frozen and then used for chromatographic studies. The papain-treated chitosan gave a chromatographic band showing extended degradation and breakthrough time 7.5 min instead of 7.1 for control (Fig. 2).

Re-acetylated chitosan with degree of acetylation in the range 0.50–0.60, fully soluble over an extended pH range, was even more susceptible to papain than the parent chitosan: in fact the viscosity drop went from 365 to below instrumental detection limit in ca. 100 min (Fig. 1). The corresponding chromatographic elution curve had the breakthrough point at 7.7 min and included more low-molecular weight fractions and oligomers. It was also accompanied by a small band assigned to dimers and monomers at 10.1 min.

3.2. Depolymerization of 15% chitosan gels

While dilute aqueous solutions are convenient for basic investigations, the conditions sought for the large-scale depolymerization of chitosan should involve chitosan-20 concentrations in a much higher range. In a previous work by Muzzarelli et al. (1994), it was shown that the viscosity of chitosan lactate solution (19 g l^{-1}) at 25 °C decreases from 780 to 430 mPa s in 60 min or from 780 to 550 mPa s in 5 min. Interestingly, the plot of the initial

Table 3
Colorimetric data for the reducing capacity of chitooligomers (0.58 mg $\equiv 3.6 \times 10^{-6}$ moles of monomeric unit) towards ferricyanide, and viscosity of the respective 3.3% solutions at 30 °C

Sample	Viscosity (mPa s, 30 °C)	Absorbance (nm)	Consumed ferricyanide (mol)
Control	161	0.909	
25 min	25	0.813	
50 min	20.5	0.781	
75 min	19.5	0.772	
20 h	14.0	0.745	0.35×10^{-6}

velocity versus chitosan lactate concentration was quite steep in the range 1–2% (fig. 2 in Muzzarelli et al., 1994), suggesting potential very fast depolymerization at higher concentrations.

In the present work, we have found in fact that the very viscous 15% gel lost most of its viscosity upon addition of papain and underwent prompt depolymerization. The depolymerized chitosan lactate thus obtained in powder form was soluble in water, but its solubility decreased with protracted curing time up to 8 days. The colour turned caramel and its surface was brilliant, even after milling. The volume taken by this powder was considerably smaller than for the plain parent chitosan with the same granulometry. At the X-ray diffraction spectrometry (Fig. 3), it exhibited an unexpected broad peak at 6.4–7.7 2θ values and absence of peaks in the region 10–11 2θ. The region around 20 2θ was severely perturbed, with new bands peaking at 18.3 and 19.0 2θ, besides a depressed band at ca. 20.6 corresponding to the chitosan band at 20.2 2θ. These spectral alterations indicated structural modifications, presumably due to lactic acid amide formation and cross-linking.

The papain activity is better exerted at 50–55 °C, but the product darkens as soon as it forms because of the Maillard reaction taking place at such a high temperature. Therefore, a reasonable compromise is 40 °C, at which no important darkening takes place. Of course, the action of papain would last longer than 90 min, according to chromatographic evidence obtained over a 12 h period, but after 90 min at 40 °C the depolymerization extent was satisfactory for these purposes.

X-ray diffraction spectrometry indicated that borohydride-treated partially depolymerized chitosan lactate was nearly completely amorphous (Fig. 3, lower curve). The final dry product contained ca. 0.5 g boron kg^{-1}, a concentration relatively high for protracted oral administration.

The reducing capacity data in Table 3, based on the molar ratio chitosan unit/consumed ferricyanide, provided the average degree of polymerization ca. 10, and indicated that 84–96% of the hydrolysis took place in the initial 75 min. Therefore, for practical purposes no more than 60–90 min are necessary to depolymerize chitosan to the said extent.

3.3. Stabilization of the partially depolymerized chitosans in the glycosylamine form

Precipitation and washing with ammonia led *in primis* to the removal of the lactate anion, whose presence would have been detrimental at the end (scarce solubility after 8 days at 40 °C), according to Qu, Wirsen, and Albertsson (1999). Secondly, ammonia prevented the Schiff reaction between terminal aldehydo groups and amino groups of the chitosan thus avoiding darkening and insoluble product formation during thermal drying and subsequent storage. Ammonia formed the glycosylamine of the partially depolymerized chitosans (Fig. 6) under the conditions specified, and the glycosylamine was easily separated from the medium, being a relatively stiff and self-sustaining material (totally different from the NaOH-precipitated chitosan).

One should note that ammonia is an alternative to the chitosan amino groups. The reaction of chitosan aldehydo groups with ammonia yielded a stable glycosylamine type compound. On the other hand, the reaction of chitosan aldehydo groups with the chitosan primary amino groups yields in general an array of side products (Candiano et al., 1988).

The X-ray spectrum of the chitosylamine is similar, but not identical to the chitosan spectrum, a difference being evident in the depression of the 10.26 2θ peak, indicative of some structural alteration.

It is recalled here that the infrared spectrum of a chitosan salt (protonated chitosan) is quite different from the spectrum of the same sample in free amine form obtained by washing with alcoholic NaOH: in the latter two sharply separated bands are observed at 1665 and 1595 cm^{-1}, rather than at 1665 and 1555 cm^{-1} for the protonated form. The infrared spectrum of chitosylamine (Fig. 4) showed alterations compared to the free amine chitosan spectrum, in particular a new band at 1646 was present, together with those at 1664 and 1596 cm^{-1}. After the treatment described, in fact, new amino groups were present at the C1 anomeric carbon, less than one tenth of those at C2.

The chitosylamine capacity for emulsion stabilization was desumed from the data in Table 4. The results for the chitosylamine under study compare favourably with those obtained with dietary chitosans on the market. Remarkably, some of the latter did not dissolve completely, presumably due to the presence of excipients like magnesium stearate in the tablets. Ascorbate was better than chloride for emulsion formation.

3.3.1. Sorbitol as a plasticizer

Sorbitol was studied as a plasticizer for chitosan derived products by Arvanitoyannis, Nakayama, & Aiba (1998). In the present study, sorbitol was found to be suitable as a plasticizer, but glycerol was not. Plasticized chitosans and chitosylamines obtained according to the scheme just described were analysed by infrared spectrometry, X-ray diffraction spectrometry and chromatography. An important finding was that it is difficult to resolve an infrared spectrum

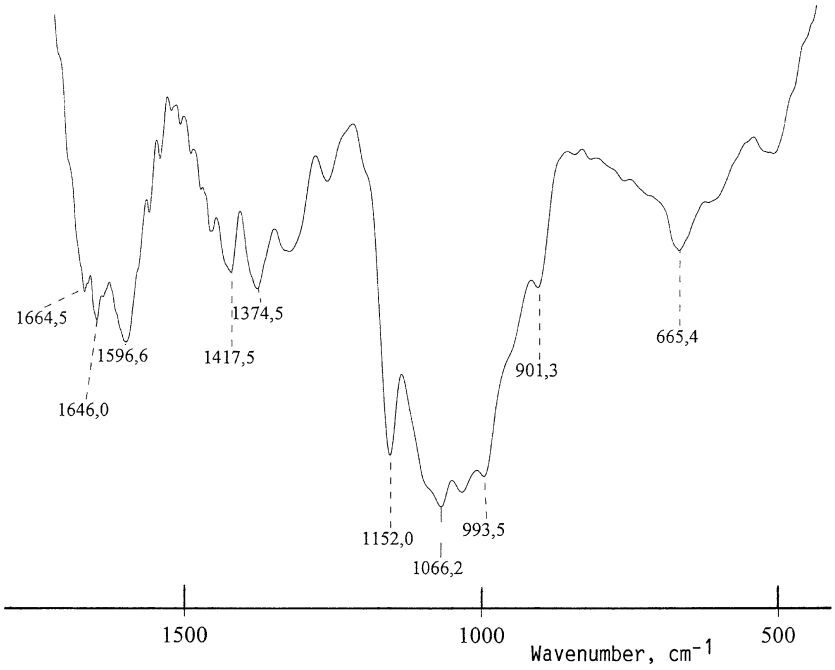

Fig. 4. Infrared spectrum of partially depolymerized Daras 151 squid chitosan in the glycosylamine form.

of the mixture, and that sorbitol in admixture with crustacean or squid chitosans (weight ratios sorbitol to chitosan 1:20 up to 1:3) is not detected by X-ray diffraction spectrometry (Fig. 5). The sorbitol-plasticized chitosylamines (faintly yellow powders), stored at 40 °C and 48% relative humidity for 10 days, were fully soluble in weak organic acid solutions within seconds; the solutions were colourless and odourless.

3.4. Other commercial depolymerized chitosans

3.4.1. Liposan ultra (Vanson)

The alkalimetric data indicated degree of deacetylation 0.78 and p*K* 6.1. The shape of the curve did not provide evidence for any considerable quantity of weak acid accompanying the chitosan.

Table 4
Hight (mm) of the four phases present in a test tube, after stirring for 30 s, as measured after 12 h standing

Mark	Sediment	Water	Emulsion	Oil
Chitosan ascorbate salt				
Sirc chitosan	No	10	24	1
Chitosano-800®	Yes	15	20	0
Chitosano-800-F®	Yes	16	14	6
Kalo®	No	14	20	2
Liposan Ultra®	No	11	24	2
This study, chitosylamine	No	15	20	2
Chitosan chloride salt				
Sirc chitosan	No	45	6	4
Chitosano-800®	Yes	45	4	6
Chitosano-800-F®	Yes	43	7	5
Kalo®	Yes	43	8	4
Liposan Ultra®	No	44	5	6
This study, chitosylamine	No	45	6	3

The infrared spectrum showed typical protonated chitosan bands, though not well defined, at 1640, 1550, 1381, 603 together with bands at 992 and 755 cm^{-1} that were not detected in chitosan spectra, moreover the spectrum was depressed and hardly recognizable, due to the substances that accompanied chitosan.

Electron microscopy showed a prevailing fraction of 100–200 μm particles with smaller particles. Most of the particles showed a smooth surface though onion-skin aspects were frequent. The solution in water was just partial even after a relatively long period of time.

The X-ray diffraction spectrum showed that Liposan Ultra exhibited all peaks typical for chitosan at ca. 10, 20 and 22 2θ values. A pre-clinical study was published by Schiller, Barrager, Schauss, & Nichols (2001).

3.4.2. Chitooligomer 'RC bio-chemical'

The alkalimetric titration of this dark brown product showed the presence of a weak acid, and the curve was difficult to analyse. The p*K* was 6.2–6.4, and the degree of deacetylation >0.90.

The infrared spectrum exhibited a band at 1728 cm^{-1} indicative of the presence of a carboxylic acid. The amide region was poorly defined, containing just one broad band at 1590 cm^{-1}, and in general the spectrum was not so clear as for a regular chitosan salt. ^{13}C-NMR spectrometry showed the presence of all signals typical of chitosan, i.e. CH_3 22.8–22.9, C2 58.5–58.6, C6 62.8, C3 71.1, C5 77.6, C4 79.2, C1 100.3–100.6, CO 184.9 ppm. The latter signal assignable to a carboxyl group was exceptionally high, while the acetyl

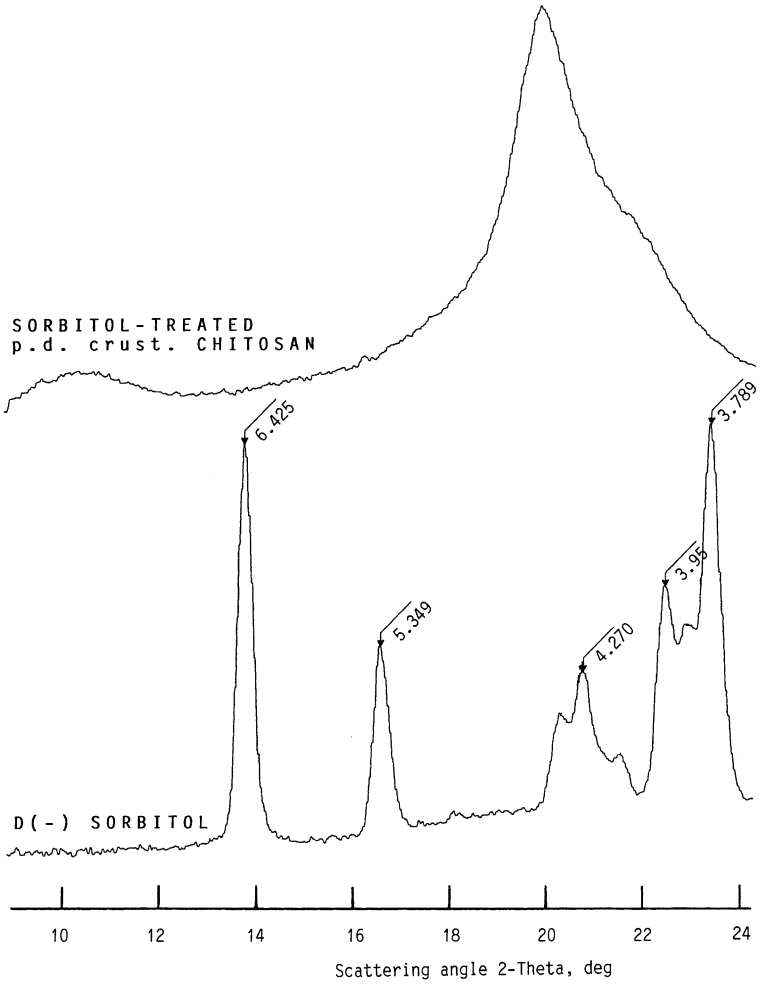

Fig. 5. X-ray diffraction spectra of sorbitol-treated partially depolymerized Sirc crustacean chitosan (upper curve), and D(−) sorbitol (lower curve). Peaks are identified with *d* values.

CO went undetected due to high degree of deacetylation. All other signals were accompanied by spurious signals.

There were intense signals in the interval 1.2–1.5 ppm of the H-NMR spectrum, indicative of the presence of lactate or polylactate, in molar ratio 1:1 with chitosan. Therefore, the product contained 62% chitosan and 37% lactate (w/w). Based on the CH_3 signal, the degree of deacetylation was found to be 0.98, in agreement with alkalimetry.

3.4.3. Betasanne®

Betasanne is a mixture of chitosan with betaine hydrochloride in the ratio 5:4, formulated for patients, who have reduced levels of gastric acid (2 g daily dosage). Betasanne® (2 g) was dissolved in water (100 ml) and filtered on paper to remove insolubles: the solution had pH 1.8, 2.5 mPa s at 25 °C and bad smell. The alkalimetric titration indicated that 0.05 equiv. of HCl were present per gram of Betasanne® powder.

4. Conclusions

With the aid of papain, the partial depolymerization of crustacean and squid chitosans and re-acetylated chitosans is simple, easily controlled and promptly accomplished. Papain (at the chitosan/papain weight ratio 100) is therefore

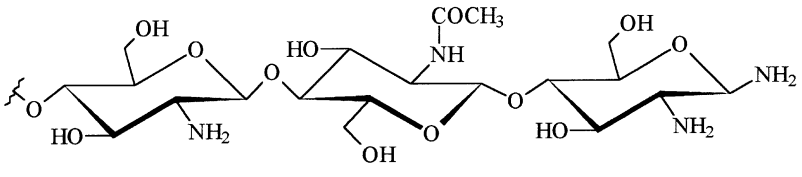

Fig. 6. Chitosan in glycosylamine form (chitosylamine) showing the substitution of –OH with $–NH_2$ at the terminal anomeric C1. Acetylation is random.

suitable for the adjustment of the molecular weight to particular low values, impossible to reach via chemical depolymerization, the latter being hard to stop at the desired extent.

The treatment of chitosan with ammonia removes the lactate anion, whose detrimental presence during drying is well known (Qu et al., 1999), puts the product in an easily handled physical form, and transforms the hemiacetal in glycosylamine. Therefore, the present study offers a straightforward way to the manufacture of high quality preparations from highly concentrated chitosan lactate (150 g chitosan per kg gel) based on the glycosylamine form of the depolymerized chitosan (chitosylamine) that prevents insolubility, dark colouring and short shelf life of the product. In fact, this chemical form of chitosan is not prone to pigment formation during conventional thermal drying, and can be optionally plasticized with sorbitol. The method presented here is simple enough to permit scaling-up with traditional equipment currently used in the food industry. Incidentally, this article appears to be the first report on chitosans in glycosylamine form.

The characteristics of chitosylamines, in plain and plasticized forms, compare most favourably with three commercial 'oligomeric' chitosans and with three chitosan-based dietary supplements.

The re-acetylated chitosans-50 are more susceptible to the enzymatic hydrolysis than plain chitosans-20, and yield of course products with higher degree of acetylation that might be sweeter (Sato, Yoshida, & Otsuki, 2001).

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References

Alvarez, O. M., Fernandez-Obregon, A., Rogers, R. S., Bergamo, L., Masso, J., & Black, M. (2000). Chemical debridement of pressure ulcers: A prospective, randomized, comparative trial of collagenase and papain/urea formulations. *Clinical Research*, *12*, 15–25.

Arvanitoyannis, I. S., Nakayama, A., & Aiba, S. I. (1998). Chitosan and gelatin based edible films: State diagrams, mechanical and permeation properties. *Carbohydrate Polymers*, *37*, 371–382.

Binkley, R. W. (1988). *Modern carbohydrate chemistry*, Basel: Marcel Dekker.

Buhling, F., Fengler, A., Brandt, W., Welte, T., Siegfried, A., & Nagler, D. K. (2000). Novel cysteine proteases of the papain family. *Cellular Pepdidases in Immune Functions and Diseases 2*, *477*, 241–254.

Candiano, G., Ghiggeri, G. M., Gusmano, R., Zetta, L., Benfenati, E., & Icardi, G. (1988). Reaction of 2-amino-2-deoxyglucose and lysine: Isolation and characterization of 2,5-bis(tetrahydroxybutyl)pyrazine. *Carbohydrate Research*, *184*, 67–75.

Caro, Y., Villeneuve, P., Pina, M., Reynes, M., & Graille, J. (2000). Investigation of crude latex from various *Carica papaya* varieties for lipid bioconversions. *Journal of the American Oil Chemistry Society*, *77*, 891–901.

Casu, B., Colombo, M., Compagnoni, T., Naggi, A., Pivari, E., & Torri, G. (1986). Branched chitosans with *N*-acetylglucosamine side chains. In R. A. A. Muzzarelli, C. Jeuniaux & G. W. Gooday, *Chitin in nature and technology*. New York: Plenum Press.

Cheng, C. Y., & Li, Y. K. (2000). An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. *Biotechnology and Applied Biochemistry*, *32*, 197–203.

Cohen-Anisfeld, S. T., & Lansbury, P. T. (1993). A practical convergent method for glycopeptide synthesis. *Journal of the American Chemical Society*, *115*, 10531–10537.

Dal Pozzo, A., Vanini, L., Fagnoni, M., Guerrini, M., DeBenedittis, A., & Muzzarelli, R. A. A. (2000). Preparation and characterization of poly-(ethyleneglycol)-crosslinked reacetylated chitosans. *Carbohydrate Polymers*, *42*, 201–206.

Fujimaki, M., Namiki, M. & Kato, H. (1986). Amino-carbonyl reactions in food and biological systems. Amsterdam: Elsevier.

Gandhi, N. N., & Mukherjee, K. D. (2001). Specificity of papaya lipase in esterification of aliphatic alcohols. A comparison with microbial lipases. *Journal of the American Oil Chemistry Society*, *78*, 161–165.

Grigolon, L. B., Azevedo, A., Santos, R. R., & Franco, T. T. (2001). Enzymatic modification of chitosan by free and immobilized papain. In R. A. A. Muzzarelli, *Chitin enzymology*. Italy: Atec.

Hellebrekers, B. W. J., Trimbos-Kemper, T. C. M., Trimbos, J. B. M. Z., Emeis, J. J., & Kooistra, T. (2000). Use of fibrinolytic agents in the prevention of postoperative adhesion formation. *Fertility and Sterility*, *74*, 203–212.

Imai, T., Shiraishi, S., & Otagiri, M. (2000). A strategy for the immediate release of indomethacin from a sustained-release preparation using a chitosan hydrolysate. *S.T.P. Pharma Sciences*, *10*, 57–62.

Jeon, Y. J., Shahidi, F., & Kim, S. K. (2000). Preparation of chitin and chitosan oligomers and their applications in physiological functional foods. *Food Reviews International*, *16*, 159–176.

Kondo, Y., Nakatani, A., Hayashi, K., & Ito, M. (2000). Low molecular weight chitosan prevents the progression of low dose streptozotocin-induced slowly progressive diabetes mellitus in mice. *Biology and Pharmacological Bulletin*, *23*, 1458–1464.

Kunz, H., Hofmeister, A., & Glaser, B. (1998). Stereoselective syntheses using carbohydrates as carriers of chiral information. In S. Dumitriu, *Polysaccharides* (pp. 539–568). New York: Marcel Dekker.

Kurita, K., Youshino, H., Nishimura, S. I., & Ishii, S. (1993). Preparation and biodegradability of chitin derivatives having mercapto groups. *Carbohydrate Polymers*, *20*, 239–246.

Lin, Y., & Welsh, W. J. (1996). Molecular modelling of substrate-enzyme reactions for the cysteine protease papain. *Journal of Molecular Graphics*, *14*, 62–72.

Liu, X. F., Guan, Y. L., Yang, D. Z., Li, Z., & De Yao, K. (2001). Antibacterial action of chitosan and carboxymethylated chitosan. *Journal of Applied Polymer Science*, *79*, 1324–1335.

Monti, R., Basilio, C. A., Trevisan, H. C., & Contiero, J. (2000). Purification of papain from fresh latex of *Carica papaya*. *Brazilian Archives of Biological Technology*, *43*, 501–507 see also pp. 1458–1464.

Muzzarelli, R. A. A. (1997). Depolymerization of chitins and chitosans with hemicellulase, lysozyme, papain and lipase. In R. A. A. Muzzarelli & M. G. Peter, *Chitin handbook* (pp. 153–163). Italy: Atec.

Muzzarelli, R. A. A. (2001). Chitin enzymology 2001 Italy: Atec.

Muzzarelli, R. A. A. (2002). Chitosan in pharmacy and chemistry Italy: Atec.

Muzzarelli, R. A. A., Tomasetti, M., & Ilari, P. (1994). Depolymerization of chitosans with the aid of papain. *Enzyme and Microbial Technology*, *16*, 110–114.

Muzzarelli, R. A. A., Stanic, V., & Ramos, V. (1999). Enzymatic depolymerization of chitins and chitosans. In C. Bucke, *Methods in biotechnology: Carbohydrate biotechnology protocols.* Totowa: Humana Press.

Muzzarelli, R. A. A., Mattioli-Belmonte, M., Miliani, M., Muzzarelli, C., Gabbanelli, F., & Biagini, G. (2001). In vivo and in vitro biodegradation

of oxychitin–chitosan and oxypullulan–chitosan complexes. *Carbohydrate Polymers*, *48* (1), 15–21.

Ogawa, K., Chrispinas, O. A., Yoshida, N., Inoue, J., & Kariya, K. (2001). Chitosanase, its manufacture, and manufacture of chito-oligosaccharides. Jpn. Kokai Tokkyo Koho, JP 2001 069975.

Pigman, W., Cleveland, E. A., Couch, D. H., & Cleveland, J. H. (1951). Reactions of carbohydrates with nitrogenous substances. I. Mutarotations of some glycosylamines. *Journal of the American Chemical Society*, *73*, 1976–1979.

Qu, X., Wirsen, A., & Albertsson, A. C. (1999). Structural changes and swelling mechanism of pH-sensitive hydrogels based on chitosan and D,L-lactic acid. *Journal of Applied Polymer Science*, *74*, 3186–3202.

Santos, E. S., Guirardello, R., & Franco, T. T. (2001). Purification of chitosanase from *Bacillus cereus*. In R. A. A. Muzzarelli, *Chitin enzymology.* Italy: Atec.

Sato, K., Yoshida, S., & Otsuki, T. (2001). Enzymatic manufacture of *N*-acetylglucosamine as sweetener from chitin or partially deacetylated chitosan. Jpn. Kokai Tokkyo Koho 078795. CA 134: 251308.

Schanzenbach, D., Ley, J. P., Matern, C. M., & Peter, M. G. (1997). Synthesis of glycosylamines and *N*-glycopeptides. In R. A. A. Muzzarelli & M. G. Peter, *Chitin handbook* (pp. 215–220). Italy: Atec.

Schiller, R. N., Barrager, E., Schauss, A. G., & Nichols, E. J. (2001). A randomized, double blind, placebo-controlled study examining the effects of a rapidly soluble chitosan dietary supplement on weight loss and body composition in overweight and mildly obese individuals. *Journal of the American Nutraceutical Association*, *4*, 34–41.

Seo, W. G., Pae, H. O., Kim, N. Y., Oh, G. S., Park, I. S., Kim, Y. H., Kim, Y. M., Lee, Y., Jun, C. D., & Chung, H. T. (2000). Synergistic cooperation between water-soluble chitosan oligomers and interferon-gamma for induction of nitric oxide synthesis and tumoricidal activity in murine peritoneal macrophages. *Cancer Letters*, *159*, 189–195.

Seong, H. -S., Kim, J. -P., & Ko, S. -W. (1999). Preparing chito-oligosaccharides as antimicrobial agents for cotton. *Textile Research Journal*, *69*, 483–488.

Shin, Y., Yoo, D. I., & Min, K. (1999). Antimicrobial finishing of polypropylene nonwoven fabric by treatment with chitosan oligomer. *Journal of Applied Polymer Science*, *74*, 2911–2916.

Shin-ya, Y., Lee, M. Y., Hinode, H., & Kajiuchi, T. (2001). Effects of *N*-acetylation degree on *N*-acetylated chitosan hydrolysis with commercially available and modified pectinases. *Biochemical Engineering*, *7*, 85–88.

Shon, Y. H., Ha, Y. M., Jeong, T. R., Kim, C. H., & Nam, K. S. (2001). Modifying action of chitosan oligosaccharide on 2-amino-3,8-dimethylimidazo [4,5-*f*] quinoxaline (MeIQx)-induced mutagenesis. *Journal of Biochemistry and Molecular Biology*, *34*, 90–94.

Tarsi, R., Muzzarelli, R. A. A., Guzman, A., & Pruzzo, C. (1997). Inhibition of *Streptococcus mutans* adsorption to hydroxyapatite by low molecular weight chitosans. *Journal of Dental Research*, *76*, 665–672.

Tarsi, R., Corbin, B., Pruzzo, C., & Muzzarelli, R. A. A. (1998). Effect of low-molecular-weight chitosan on the adhesive properties of oral streptococci. *Oral Microbiology and Immunology*, *13*, 217–224.

Terbojevich, M., Cosani, A., & Muzzarelli, R. A. A. (1996). Molecular parameters of chitosans depolymerized with the aid of papain. *Carbohydrate Polymers*, *29*, 63–68.

Tietgen, H., Schultz-Kukula, M., & Kunz, H. (2000). Glycosylamines as auxiliaries in stereoselective syntheses of chiral amino compounds. In A. Ricci, *Modern amination methods* (pp. 103–128). Weinheim: Wiley-VCH.

Tsai, G. J., Wu, Z. Y., & Su, W. H. (2000). Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. *Journal of Food Protection*, *63*, 747–752.

Tseng, C. C., Tseng, C. P., Levine, M. J., & Bobek, L. A. (2000). Differential effect toward inhibition of papain and cathepsin C by recombinant human salivary cystatin SN and its variants produced by a baculovirus system. *Archives of Biochemistry and Biophysics*, *380*, 133–140.

Vasyukova, N. I., Zinov'eva, S. V., Il'inskaya, L. I., Perekhod, E. A., Chalenko, G. I., Gerasimova, N. G., Il'ina, A. V., Varlamov, V. P., & Ozeretskovskaya, O. L. (2001). Modulation of plant resistance to diseases by water-soluble chitosan. *Applied Biochemistry and Microbiology*, *37*, 103–109.

Walraevens, V., Vandermeer-Piret, M. -C., Vandermeer, A., Gourlet, P., & Robberecht, P. (1999). Isolation and primary structure of the CCI papain-like cysteine proteinases from the latex of *Carica candamarcensis* hook. *Biological Chemistry*, *380*, 485–488.

Wang, Z. G., Zhang, W., Visser, M., Live, D., Zatorski, A., Iserloh, U., Lloyd, K. O., & Danishefsky, S. J. (2001). Toward fully synthetic homogeneous glycoproteins. *Angewandte Chemie International Edition*, *40*, 1728–1732.

Zhang, H., Du, Y., Mitsutomi, M., & Aiba, S. -I. (1999). Preparation of chitooligosaccarides from chitosan complex enzyme. *Carbohydrate Research*, *320*, 257–260.