Effect of Immobilized Neutral Protease on the Preparation and Physicochemical Properties of Low Molecular Weight Chitosan and Chito-oligomers

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ABSTRACT: Neutral protease was immobilized on glutaraldehyde-pretreated *N*-succinyl chitosan hydrogel beads and the biocatalyst obtained was used for the preparation of low molecular weight chitosan and chito-oligomers with molecular weight of 1.9–23.5 kDa from commercial chitosan. Factors affecting the chitinolytic hydrolysis were described. The degradation was monitored by gel permeation chromatography. The structure of degraded chitosan was characterized by Fourier transform infrared, X-ray diffraction and liquid chromatography-mass spectrometry. Immobilized neutral protease showed optimal depolymerization at pH 5.7 and 50°C. The degree of deacetylation of the hydrolysates did not change compared to that of the initial chitosan. The decrease of molecular weight led to transformation of crystal structure but the chemical structures of residues were not modified. The degree of polymerization of chito-oligomers was mainly from 3 to 8. The method allows cyclic procedures of immobilized enzyme and *N*-succinyl chitosan support utilization, and is suitable for a large-scale production of the low molecular weight chitosan and chito-oligomers free of protein admixtures. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 4185–4193, 2006

Key words: enzyme; low molecular weight chitosan; degradation; characterization

INTRODUCTION

The depolymerization products of chitosan, low molecular weight chitosan and chito-oligomers, have received increased attention for their interesting properties, including their inhibitory effects on the growth of bacteria and fungi,[1,2] their ability to induce disease resistance-response genes in higher plants, and as an elicitior of defense mechanisms in plant.[3] Low-molecular weight chitosan with an average molecular weight more than 5 kDa prevented the rise of serum cholesterol of rats fed cholesterol-enriched diets for 14 days.[4] And chito-oligomers are now proven to be potent angioinhibitory and antitumor[5,6] agents, as shown by inhibition of angiogenesis and inducing apoptosis as a function of DNA fragmentation.[7] Chitotriose exhibits inhibitory effect toward angiotensin converting enzyme (ACE) and hence could be used as an antihypertensive agent.[8]

Because of their potential biological activities, many methods, such as chemical and enzymatic method, have been developed to prepare chitosan degradation products. Enzymatic process seem to be generally preferable to chemical reactions, since the hydrolysis course and product distribution are subject to more facile control,[9] in spite of the faster rate of chemical reaction.[10] However, use of chitosanase, the specific enzyme, is too expensive to be commercialized for the production of low molecular weight chitosan and chito-oligomers.[11]

In our previous work, neutral protease, a kind of nonspecific hydrolytic enzyme, had been found to be able to hydrolyze chitosan efficiently and obtain low molecular weight chitosan with different weight-average molecular weight and chito-oligomers easily by prolonging the duration.[12]

But the use of enzymatic hydrolysis has been limited due to their unstable nature and the resulting requirement of stringent conditions, such as a particular pH and temperature. And in hydrolysis reactions, purified enzymes can be rather costly and to discard them after each use is not economical. The application of low molecular weight chitosan and chito-oligomers obtained by such enzymatical hydrolysis in biochemical and food area is limited. Because of the presence of protein of the enzyme admixtures will result in an undesirable level of chitosan pyrogenicity.[13] Preparation of low molecular weight chitosan and chito-oligomers

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using immobilized enzyme can overcome all these disadvantages. In our previous work, the immobilization of neutral protease on *N*-succinyl chitosan hydrogel beads resulted in a high recovery of enzymatic activity and promoted a high stabilization.[14]

In this study, we investigated the hydrolytic properties of immobilized neutral protease on *N*-succinyl chitosan hydrogel beads. Factors affecting enzymatic hydrolysis of chitosan were studied. The products with different molecular weights were characterized by gel permeation chromatography (GPC), Fourier transform infrared (FTIR), X-ray diffraction (XRD), and liquid chromatography-mass spectrometry (LC-MS). The modes of action of immobilized enzyme and kinetic study on the enzyme activity have been discussed, and the relation between the molecular weight and the physicochemical properties of degraded products is investigated in detail. An understanding of physicochemical properties is essential for better application and the utilization of immobilized neutral protease offers advantage over free enzymes for the preparation of low molecular weight chitosan and chito-oligomers free of protein admixtures and more suitable for biomedical and food applications.

EXPERIMENTAL

Materials

Chitosan was obtained from Yuhuan Ocean Biochemical Co. (Zhejiang, China). The chitosan with the degree of the *N*-deacetylation (DD) 75.3% and weight-average molecular weight (M_w) 410 kDa, as initial material from crab shells, was used to prepare the *N*-succinyl chitosan. Chitosan (CS5) with DD 91.7% and $M_w = 286$ kDa, as initial material from shrimp shells, was used to prepare chitosan with different degree of deacetylation and as the substrate to prepare low molecular weight chitosan and chito-oligomers. Chitosans with different DD were prepared according to the reference[15] and the molecular parameters were listed in Table I. All other chemicals were of reagent grade.

The neutral protease, derived from *Bacillus subtilis* As1.398, was a product of Ningxia XiaSheng Industry Co. (China).

TABLE I
The Molecular Parameter of the Investigated Chitosan and Kinetic Parameters of Each Kind of Chitosan as Substrate

Initial chitosan	DD (%)	M_w ($\times 10^{-4}$)	K_m (mg/mL)	V_{max} (mg/mL h)
CS 1	63.2	29.4	0.88	1.23
CS 2	75.3	28.9	1.09	1.05
CS 3	80.8	28.6	1.33	0.91
CS 4	85.6	28.7	1.56	0.83
CS 5	91.7	28.6	1.88	0.73

Synthesis of *N*-succinyl chitosan

Chitosan (10 g) was treated with 200 mL of 5% (v/v) acetic acid and stirred at room temperature. To the viscous solution formed, 800 mL methanol was added. After stirring for 1 h, succinic anhydrides (30 g) was added to the chitosan solution. The reaction mixtures became a gel within 30 min. After stirring for 24 h, the reaction mixture was filtered to remove methanol, followed by dispersing in 400 mL of deionized water. To obtain a sodium salt of product, an adequate amount of NaOH was added to the reaction mixture to give a clear solution of pH 8–10. The solution was dialyzed against deionized water for 3 days and then filtered. The filtrates were concentrated to about one-twentieth with a rotary evaporator under reduced pressure and precipitated by adding anhydrous ethanol. The precipitates were collected after drying over phosphorus pentoxide in vacuum to get *N*-succinyl chitosan powder.

Preparation of *N*-succinyl chitosan hydrogel beads

N-succinyl chitosan powder (3 g) was completely dissolved in 100 mL distilled water. The mixture was extruded drop by drop using a syringe needle into 150 mL 1% (w/v) $CaCl_2$ solution containing 50 mL ethanol under stirring to form beads. The beads were allowed to harden in the $CaCl_2$ solution for 3 h. *N*-succinyl chitosan hydrogel beads have average diameter of 2.2 mm.

Immobilization of neutral protease to *N*-succinyl chitosan hydrogel beads

For the activation of the enzyme immobilization supports, 2 g of wet *N*-succinyl chitosan hydrogel beads were treated with 10 mL 1% (v/v) glutaraldehyde citrate-phosphate (C-P) buffer (0.1*M*, pH 4.0) solution with constant shaking for 12 h at room temperature, followed by washing with distilled water. After washing with distilled water until the glutaraldehyde in the washings was not determined at 245 nm, the crosslinked *N*-succinyl chitosan hydrogel beads were stored at 4°C until used. The glutaraldehyde-activated beads were immersed in 10 mL C-P buffer (0.1*M*, pH 4.0) containing 12 mg of neutral protease, and the mixture was gently mixed with a shaker at 25°C for 4 h. The supernatant was removed, and the beads were washed with the same buffer until the protein in the washings was not detected at 280 nm. The immobilized enzyme was recovered from the solution and then stored at 4°C. Under these optimum conditions, the immobilized neutral protease yielded a protein loading of 122 μg/g-wet *N*-succinyl chitosan hydrogel beads and a specific activity of 32.5 μmol/min mg protein.

Batch hydrolysis of chitosan by immobilized neutral protease

Unless otherwise stated, the degradation was carried out as follows: the chitosan powder (0.1 g) was introduced in a reactor containing liquor adjusted to the desired pH with acetic acid. Chitosan to liquor ratio 1 : 100 was used. After the mixture was stirred for 1 h, the reactor was kept in a thermostatic shaker and immobilized enzyme was added. The weight ratio of enzyme protein to substrate was 1 : 20. Details of the reaction condition are given in the text. At various intervals, 0.5 mL of the reaction mixture was taken out and filtered through 0.45 μm Millipore filters. The filtrate was analyzed by gel-permeation chromatography.

Preparation of low molecular weight chitosan and chito-oligomers

Chitosan (10 g) was completely dissolved in 1000 mL 1% (v/v) acetic acid. After dissolved completely, the pH value of the chitosan solution was adjusted to pH 5.7 using 10% (w/v) KOH solution. The solution in the reaction vessel was placed in a thermostatic shaker at 50°C. Immobilized enzyme (equals to 0.5 g free neutral protease) was added to initiate reaction.

After 30 min, 1 h, 2 h, 3 h, 200 mL of the mixture were taken out and the pH value of the hydrolysate was adjusted to 9 using 10% (w/v) KOH solution. The hydrolysate was then subjected to vacuum filtration. The precipitate was collected and washed thoroughly with deionized water and anhydrous ethanol, and was finally collected by drying over phosphorus pentoxide in vacuum to get samples LC1, LC2, LC3, and LC4. The filtrate was concentrated to about one-twentieth with a rotary evaporator under reduced pressure at 40°C, and then precipitate was obtained by adding anhydrous ethanol to the mixture. The precipitate was washed thoroughly with anhydrous ethanol. The water-soluble portion of hydrolysates was collected after drying over phosphorus pentoxide in vacuum to get samples LC-W1, LC-W2, LC-W3, and LC-W4.

After 4 h, the immobilized enzyme was taken out by filtration and the pH value of the hydrolysate was adjusted to 9 with 10% (w/v) KOH solution. After the removal of the precipitate by filtrating, the supernatant were concentrated to about one-twentieth with a rotary evaporator under reduced pressure at 40°C and precipitated by adding anhydrous ethanol. The precipitate was washed thoroughly with anhydrous ethanol and collected after drying over phosphorus pentoxide in vacuum to get samples LC-W5.

Characterization

To determine the optimal pH and temperature of chitosan enzymatic hydrolysis by free and immobilized neutral protease, the enzymatic hydrolysis reaction were carried out over the pH range 4.2–6.3 and temperature range 25–80°C. The increasing in reducing sugars resulted from a cleavage of glycosidic linkage was monitored by spectrophotometric analysis on the basis of Schales' modified method[16] with D-glucosamine HCl as standard.

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) of sample were measured by a gel-permeation chromatography (GPC). The GPC equipment consisted of two TSK Gel connected columns (TSK G5000-PW and TSK G3000-PW (Tokyo, Japan)), TSP P100 pump (Thermoquest, San Jose, CA) and RI 150 refractive index detector (Thermoquest, San Jose, CA). The eluent was 0.2*M* CH_3COOH/0.1*M* CH_3COONa. Eluent and chitosan sample solutions were filtered through 0.45 μm Millipore filters. The flow rate was maintained at 1.0 mL/min. The temperature of the column was maintained at 30°C. The sample concentration was 0.4 mg/mL. The standards used to calibrate the column were TOSOH pullulan (Showa Denko, Tokyo, Japan) of defined molecular weight ranging from 5.9 to 40.4 kDa. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package (Dalian, China). And the weight-average molecular weight was calculated by the following equation:

$$10\mathrm{g}(M_w) = -0.4315V_e + 10.5308$$

FTIR spectra were recorded with KBr pellets on a Nicolet FT-IR 5700 spectrophotometer. Chitosan and its degraded products were mixed with potassium bromide (1 : 200) and compressed into pellets. The resolution of the infrared spectra was 4 cm^{-1}, and for each spectrum there were 16 scans, which were recorded from 400 to 4000 cm^{-1} in transmittance mode.

X-ray diffraction patterns of the degraded chitosan fractions were measured by a Shimadzu Lab XRD-6000 diffractometer and used a Cu$K\alpha$ radiation ($\lambda = 1.5404$ Å) at 40 kV and 50 mA at 20°C. The relative intensity was recorded in the scattering range (2θ) of 8–40°.

The degree of polymerization (DP) of chito-oligomers was determined by liquid chromatography-electrospray ionization mass spectrometry. Chromatography for separation of chito-oligomers was carried out by applying the sample to a 4 μm (250 mm × 4.6 mm, i.d.) High-performance Carbohydrate Column (P/N: WAT 044355, Waters, Milford, MA, USA), using a 2690 Alliance high-performance liquid chromatograph (Waters, Milford, MA, USA). The combination of the mobile phase, prepared by mixing acetonitrile: water in the ratio of 60 : 40 (v/v), and a flow rate of 0.5 mL/min was found to be adequate for the sample analysis. Separation was performed at 40°C. Electrospray ionization mass spectrometry (ESI-MS) measurement was performed on a Waters ZMD mass spectrometer equipped

with a gas nebulizer probe. The capillary was typically held at 2.80 kV and the source temperature was maintained at 105°C. Nitrogen was used as the desolvating gas at flow rates of 300 L/h and the desolvation temperature was 180°C. Spectra were obtained in negative mode in the mass range 350–1800 Da.

Potentiometric determination of the degree of deacetylation (DD)

The chitosan (0.1 g) was dissolved in a known excess of 0.1*M* HCl acid (10 mL). From the titration of this solution with a 0.1*M* NaOH solution, a curve with two inflection points was obtained. The first and second inflection points are the equivalence points of the titration of excessive hydrochloric acid and the titration of protonated chitosan, respectively. The difference of the volumes of these two points was corresponding to the acid consumed for the salification of amine groups and allows the determination of the degree of deacetylation of the chitosan.[17] The titration was performed with a DELTA-320-S pH meter. Under continuous stirring, the standard NaOH solution was added stepwise and the volume of added NaOH and pH values of solution were recorded. The titration was terminated when the pH value of the solution reached a value of 13.0. Three replicates were performed for each sample. The degree of deacetylation determined by potentiometric titration was calculated using the following equations[18]:

$$\mathrm{DD} = \frac{(V_2 - V_1) \times C \times 16 \times 10^{-3}}{m \times 0.0994} \times 100\%$$

where V_2 is the second inflection point along the abscissa when the pH value of chitosan solution is plotted against V of titrant solution (mL), V_1 is the first inflection point along the abscissa (mL), C is the concentration of titrant solution (mol/L), m is the dry weight of chitosan and degraded chitosan powder (g), 16 is the molar mass of $—NH_2$ of chitosan, 0.0994 is the theoretic content of $—NH_2$ in chitosan.

RESULTS AND DISCUSSION

Reaction condition of immobilized enzymatic hydrolysis

Free and immobilized neutral protease reacted with chitosan in different pH solution for 1 h at 50°C. The optimum pH was determined and showed in Figure 1. The free neutral protease showed the optimum pH at 5.4. The pH optimum of immobilized neutral protease on *N*-succinyl chitosan hydrogel beads shifted in the alkaline region relative to the pH of free enzyme and showed the optimum pH at 5.7. Chitosan is a polycationic substance, but when it is changed into *N*-succinyl chitosan by introducing $—CO(CH_2)_2COONa$ groups onto $—NH_2$ along chitosan molecular chain, amphoteric polyelectrolyte containing both cationic and anionic fixed charges were prepared. Therefore, in studies of the immobilized enzyme, the optimum pH shifts toward higher one can be accounted for solely by electrostatic potential of the support.[19] The immobilized neutral proteases were less sensitive to pH changes than free enzyme.

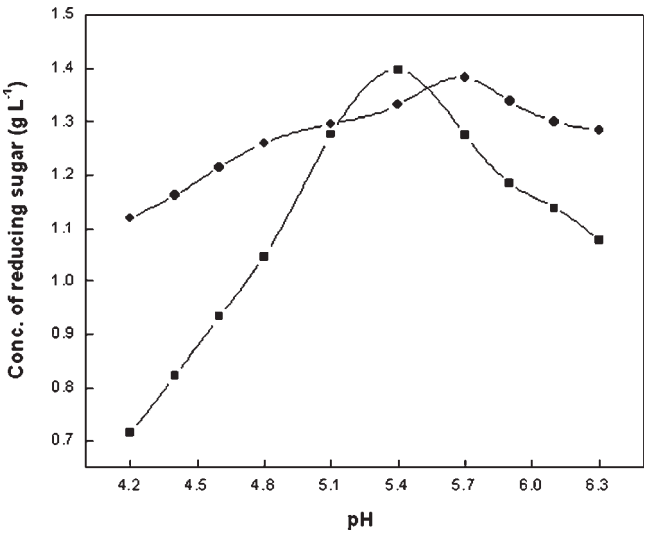

Figure 1 Effect of reaction pH on the relative activity of free (■) and immobilized neutral protease on *N*-succinyl chitosan hydrogel beads (●). Reactions were performed at 50°C for free and immobilized enzyme at various pH values for 1 h.

Free and immobilized neutral protease reacted with chitosan solution (pH 5.4 for free neutral protease and pH 5.7 for immobilized enzyme) at different temperatures for 4 h. The results are shown in Figure 2. It was observed that the optimum temperature for hydrolysis reaction for the free as well as the immobilized neutral protease remained the same at 50°C. And the loss of the activity of immobilized enzyme is lower than that of free enzyme at the investigated temperature range. The enzyme support generally has a protecting effect at high temperatures at which enzyme deactivation occurs. The conformational flexibility of the enzyme is affected by immobilization. The immobilization of enzyme causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation by raising the temperature.[20]

GPC measurements showed the information on degradation process. Figure 3 illustrates GPC curves of chitosan and its degraded products obtained at various intervals during the batch experiments. Obviously prolonging the duration increased the extent of degradation.[21] The GPC curves of all the degraded products appeared a new peak at higher elution volume. And with the deeper of depolymerization, the area of the peak corresponding to the high molecular weight chitosan decreased while that corresponding to the low molecular weight chitosan increased. After hydrolysis

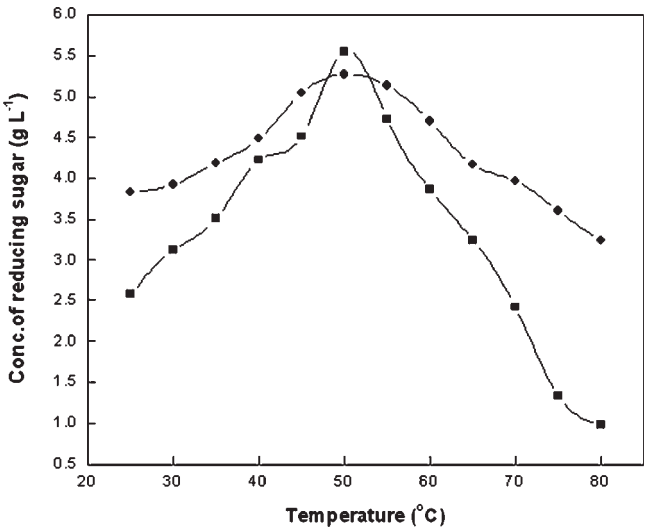

Figure 2 Effect of reaction temperature on the relative activity of free (■) and immobilized neutral protease on *N*-succinyl chitosan hydrogel beads (●). Reactions were performed in chitosan (CS5) solution pH 5.4 for free neutral protease and pH 5.7 for immobilized enzyme on *N*-succinyl chitosan hydrogel beads at various temperature values for 4 h.

for 4 h, the first peak of GPC curve disappeared and become the only one peak. This is very different from the degradation process of chitosan by free neutral protease, during which all the GPC curves appeared only one peak and with the deeper of depolymerization of chitosan the peak of the elution curve only shifted toward higher elution volumes.[12] The bimodal distribution of molecular weight of degraded chitosan indicated a nonrandom degradation of chitosan with immobilized neutral protease and suggested that the chitosan chains contain cleaving points which are more susceptible to neutral protease immobilized on *N*-succinyl chitosan hydrogel beads than free enzyme.

The influences of DD on chitosan enzymatic susceptibility

It has been shown that the stiffness of the chitosan chain is dependent on its chemical composition[22] and these facts also affect the enzymatic hydrolysis.[23] DD plays an important role in the action of enzyme on the chitosan polymer and the DD of substrate would cause a change in the affinity between enzyme and substrate. The effect of DD of substrate on the affinity between enzyme and substrate was tested by using increasing concentrations of chitosan substrate from 0.5 to 4% in pH 5.7 HAc–NaAc buffer and incubating at 50°C for 2 h. And chitosans with different DD but similar weight-average molecular weight were used as substrate and the relationship between the substrate concentration and rate of reaction can be described by the known Michaelis-Menten equation.

$$\upsilon = \frac{\upsilon_{max}\, S}{K_m + S}$$

where υ_{max} is the maximum rate of reaction and K_m is the Michaelis constant.

The Michaelis constant K_m and the maximum rate of reaction υ_{max} values of immobilized neutral protease to chitosan substrate with different degree of deacetylation are determined from the Lineweaver-Burk plot:

$$\frac{1}{\upsilon} = \frac{1}{\upsilon_{max}} + \frac{K_m}{\upsilon_{max}}\frac{1}{S}$$

From the slope and intercept of the plot of $1/\upsilon$ versus $1/S$ (Fig. 4), the K_m and the maximum rate of reaction υ_{max} values were obtained and listed in Table I. The plots indicated that the enzymatic hydrolysis of chitosan with neutral protease immobilized *N*-succinyl chitosan hydrogel beads obeys well with the Michaelis-Menten kinetics. K_m is the measure of the strength of ES complex; a higher K_m indicates weak binding, whereas the lower value, strong binding. The K_m value is considered to be a measure of an overall affinity between enzyme and chitosan, it is clear that lower DD of chitosan resulted in a dramatically decrease in K_m, which suggested that the hydrolysis of partially *N*-acetylation chitosan with the neutral protease immobilized on *N*-succinyl chitosan hydrogel beads should occur with recognition of GlcNAc units. And the bimodal molecular weight distribution of degradation

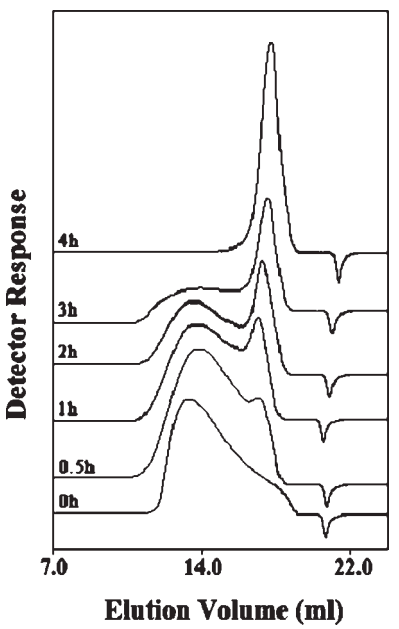

Figure 3 GPC profiles of enzymatic products of chitosan at different reaction periods. The concentration of chitosan (CS5) was 1% (w/v), the weight ratio of immobilized neutral protease to substrate was 1 : 20, reaction condition was 50°C, and pH 5.7.

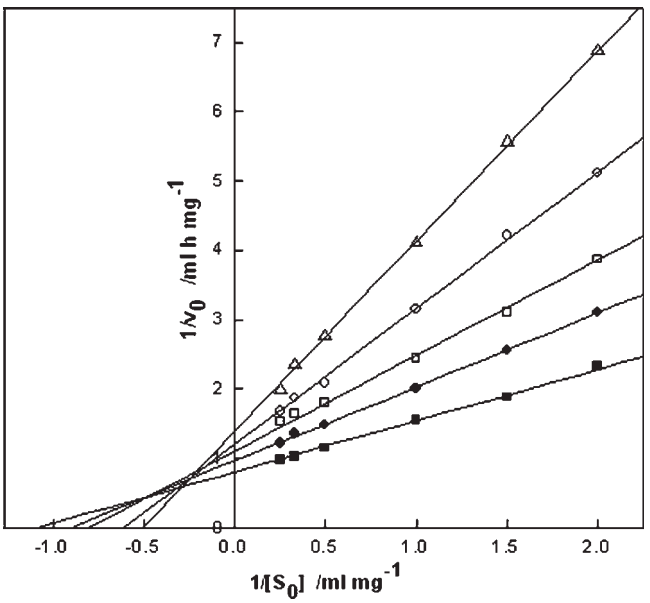

Figure 4 Lineweaver-Burk plot for immobilized neutral protease using chitosan with different degrees of deacetylation (DD) as the substrate. The parameters of investigated chitosan were listed in Table I, degradation condition was 50°C, pH 5.7, 2 h. (Δ) CS1, (○) CS2, (□) CS3, (●) CS4, (■) CS5.

samples in Figure 3 can also due to the influence of *N*-acetyl groups. At the same time, the υ_{max} values also increased with decrease in DD.

The structural properties of different molecular weight chitosan

The DD of degraded chitosan are listed in Table II. The DD of the water-insoluble portion (LC1 ~ LC4) of hydrolysate decreased while that of the water-soluble portion (LC-W1 ~ LC-W4) increased after degradation. And the DD of LC-W5 did not change compared with the initial chitosan. It also suggested that the enzyme might selectively cleavage some glycosidic bonds. The distribution of the acetyl groups in initial chitosan is not even in that the commercially available chitosan, which is generally produced by heterogeneous *N*-deacetylation of chitin. Therefore, neutral protease immobilized on *N*-succinyl chitosan hydrogel beads hydrolyzes chitosan at its susceptible cleaving points and resulted change the DD of different portion of hydrolysates.

Figure 5 shows the IR spectra of initial chitosan and degraded chitosan LC-W5. The absorption bands at 1650.8, 1599.5, 1324.9 cm^{-1} in initial chitosan are attributed to the amide **I**, N—H bending mode of $—NH_2$ and amide **III** band, respectively.[24] The spectrum of LC-W5 is similar to that of initial chitosan, but the amide **I** band shifts to low wave number, this suggested that carbonyl groups had more opportunity to form stronger hydrogen bonds in that the scission of polymer chains led to the increasing mobility of molecule. As can be seen, there is no significant difference between the amide **III** of initial chitosan and LC-W5, which indicated that with the decrease of the molecular weight of chitosan, the degree of deacetylation of hydrolysate did not changed. The IR spectra suggested that there was no significant difference between the residues of chitosan before and after the hydrolysis by neutral protease immobilized on *N*-succinyl chitosan hydrogel beads. And the DD of LC-W5 did not change. All these data were consistent well with the data of potentiometric determination of DD.

Figure 6 shows the X-ray diffraction patterns of chitosan and its water-insoluble portion of hydrolysates. The wide-angle X-ray diffraction (WAXD) pattern of initial chitosan shows its characteristic peaks at $2\theta = 10.4^\circ$ and 19.8°, which coincided with the pattern of the ''L-2 polymorph'' of chitosan reported previously.[25] Compared with initial chitosan, the peak at $2\theta = 10.4^\circ$ of LC1 and LC3 disappeared and the intensity of the characteristic peak at $2\theta = 19.8^\circ$ increased. The characteristic peak at small angle of LC1 and LC3 disappeared, which corresponded to large spacing, indicated that depolymerization of chitosan lead to decrease of the crystalline perfection. To compare the

TABLE II
Properties of Degraded Chitosan Obtained by Immobilized Neutral Protease on *N*-Succinyl Chitosan Hydrogel Beads

Sample	M_w ($\times 10^{-3}$)	M_w/M_n	DD (%)	Yield (%)	Protein content in hydrolysates (%)
CS	286	6.71	91.7	–	–
LC1	23.5	5.31	90.8	90.3	–
LC-W1	2.2	1.32	94.5	1.1	–
LC2	15.8	4.77	89.9	81.1	–
LC-W2	2.1	1.30	93.3	6.8	–
LC3	10.8	4.18	88.1	66.2	–
LC-W3	2.1	1.30	92.7	15.1	–
LC4	4.5	3.32	86.9	44.3	0.001
LC-W4	1.9	1.30	92.7	28.1	0.001
LC-W5	1.9	1.28	91.5	65.4	0.002

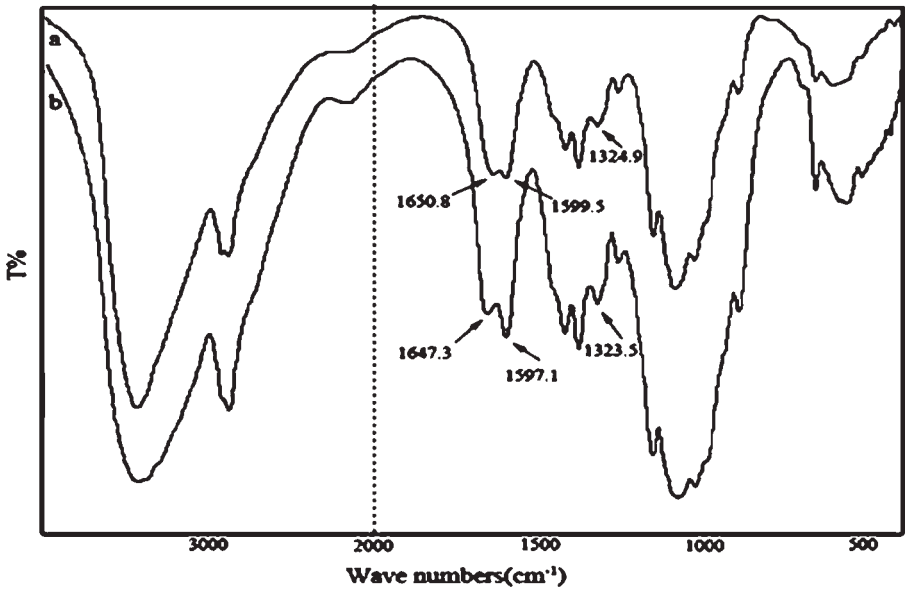

Figure 5 FTIR spectra of chitosan (a) and degraded chitosan LC-W5 (b). LC-W5 was prepared in such condition: pH 5.7, 50°C, the weight ratio of immobilized neutral protease to substrate was 1 : 20, the concentration of chitosan (CS5) solution was 1% (w/v), immobilized neutral protease and CS5 solution reacted for 4 h.

X-ray diffraction patterns of LC3 with LC1, the peak at $2\theta = 19.8^\circ$ of LC3 weakens and slightly broadens, which means a decrease in the dimensions of crystalline regions in LC3 and the number of defects increased with the decrease of molecular weight of chitosan.[26] Because the crystal structure of chitosan depend on the regular distribution of substituting groups along the chain,[27] the transformation of the crystal structure of hydrolysates prepared by neutral protease immobilized on *N*-succinyl chitosan hydrogel beads indicated that the enzyme selectively cleave the chitosan molecular chain and the distribution of *N*-acetyl groups is not regular. LC-W5 had only one major peak and became amorphous.[28] That was to say, the chitosan in amorphous region was first degraded to water-soluble molecules. With deeper degradation, the crystalline structure was destroyed.

Liquid chromatography coupled with mass spectrometry (LC-MS) with electrospray ionization (ESI) mass spectrometer has been used for structure

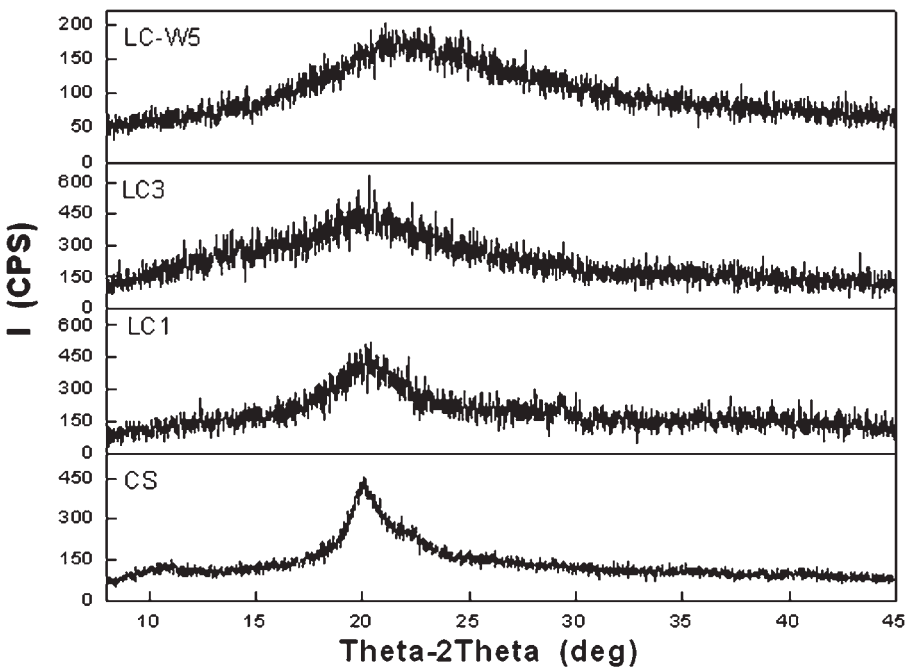

Figure 6 X-ray diffraction patterns of initial chitosan and degraded chitosans. Sample codes correspond to those in Table II.

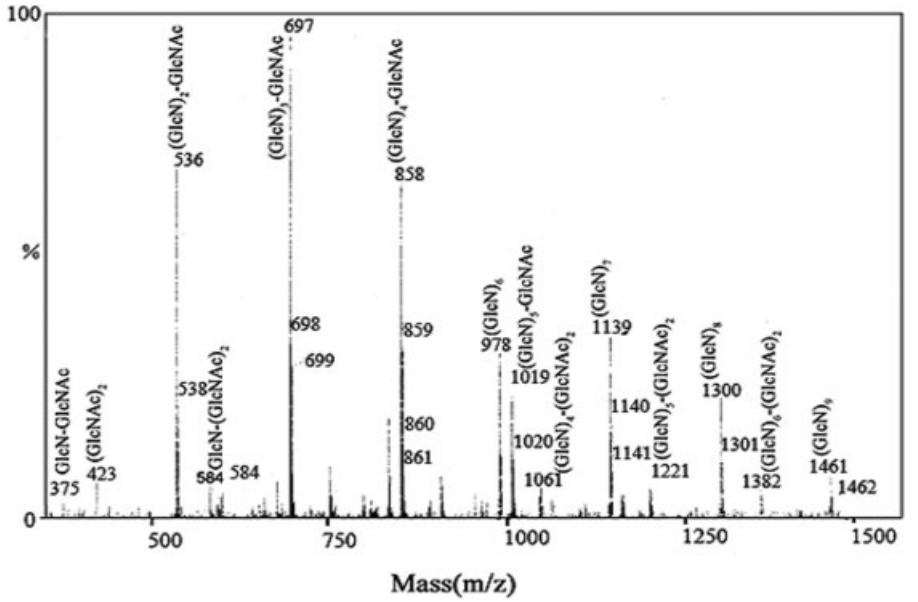

Figure 7 Liquid chromatography-electrospray ionization mass spectrum of LC-W5. LC-W5 was prepared in the same condition as shown in Figure 5.

characterization of saccharide[29] and the LC-MS analysis can provide the information of molecular weight for the structural elucidation.[30] Although the relative amount of each oligomer product could not be determined from relative intensity in the LC-MS spectrum since a linear correlation between the relative ion-intensity and the quantification of the product has not yet been established, it has been accepted that the relative ion-intensity can reflect the quantification of the products.[31] Figure 7 shows the LC-MS spectrum of LC-W5 which revealed that the product was composed mainly of chito-oligomers, especially of degree of polymerization (DP) 3 to 8. In the LC-MS spectrum, chito-oligomers contained deprotonated molecules $(M\text{-}nH)^-$ ion because the electrospray ionization mass spectrometry experiment used negative ion mode. As it can be seen, the immobilized neutral protease hydrolyzes chitosan and could produce $(GlcN)_6$, $(GlcN)_7$, $(GlcN)_8$, and $(GlcN)_9$, which indicates that the immobilized enzyme can split the β-1,4-glycosidic linkages of GlcN–GlcN. And the immobilized enzyme also produced mixture of hetero-oligomers, each of which carries one or two GlcNAc residue. These results suggested that the immobilized neutral protease can also selectively cleave GlcNAc–GlcN linkage.[32] It is also consistent with the conclusion that the initial chitosan with different DD would affect the enzymatic hydrolysis.

Protein content in degraded chitosan

Regulatory requirements concerning the use of low molecular weight chitosan and chito-oligomers in humans will be far more demanding.[2,33] It has been reported that the purity of low molecular weight chitosan and chito-oligomers influences its toxicological profile.[34] And only an ultrapure grade of degraded chitosans was safe to be applied in various biological and physiological systems.[35] The utilization of low molecular weight chitosan and chito-oligomers prepared by free enzyme for biomedical and food purpose was limited as the result of an undesirable level of chitosan pyrogenicity caused by the presence of about 0.1% (w/w) of the proteins of the enzyme complex.[13] The protein content in hydrolysates prepared by immobilized neutral protease on *N*-succinyl chitosan hydrogel beads were listed in Table II. The results showed that the low molecular weight chitosan and chito-oligomers samples obtained had very low content of protein admixtures (<0.002%). This mean the utilization of immobilized enzyme offers advantage over free enzymes for the preparation of low molecular weight chitosan and chito-oligomers free of protein admixtures and more suitable for biomedical and food applications.

CONCLUSIONS

Neutral protease can be of great value for preparing low molecular weight chitosan and chito-oligomers. In this study, neutral protease was immobilized on glutaraldehyde-pretreated *N*-succinyl chitosan hydrogel beads. Our experiments have shown that the immobilized neutral protease on *N*-succinyl chitosan beads lead to different microenvironment of enzyme and is more susceptible to GlcNAc-unit in chitosan chain than free enzyme. The optimal hydrolysis temperature and pH was 50°C and 5.7, respectively. The reduction

in molecular weight of the resulting chitosan led to the transformation of the crystal structure and no change of the total degree of deacetylation. IR spectra confirmed that the chemical structures of residues were not modified. The degree of polymerization of chito-oligomers was mainly from 3 to 8. And the utilization of immobilized enzyme offers advantages over free enzyme for the preparation of low molecular weight chitosan and chito-oligomers free of protein admixtures and more suitable for biomedical and food applications.

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