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Immobilization of a protease on modified chitosan beads for the depolymerization of chitosan

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ABSTRACT

Neutral protease was immobilized on chitosan (CS), carboxymethyl chitosan (CMCS), and *N*-succinyl chitosan (NSCS) hydrogel beads. And the biocatalysts obtained were used to prepare low molecular weight chitosan (LMWC) and chitooligomers. Weight-average molecular weight of LMWC produced by neutral protease immobilized on CS, CMCS and NSCS hydrogel beads were 3.4 kDa, 3.2 kDa and 1.9 kDa, respectively. The effects of immobilization support and substrate on enzymatic reaction were analyzed by measuring classical Michaelis–Menten kinetic parameters. The FT-IR, XRD and potentiometric determination results indicated decrease of molecular weight led to transformation of crystal structure, but the degree of *N*-deacetylation and chemical structures of residues were not changed compared to initial chitosan. The degree of polymerization of chitooligomers was mainly from 2 to 7. We observed a strong dependence of the immobilized enzyme properties on the chemical nature of the supports, which leads to different microenvironment of neutral protease and changes the hydrolyzing process.

1. Introduction

Chitosan's biofunctionalities are highly related to its molecular weight and degree of *N*-deacetylation (DD). Fairly recently, evidence is beginning to accumulate that the chitosan with molecular weight ranging 5–30 kDa were shown to possess superior biological activities compared to chitosan. Low molecular weight chitosan (LMWC) of 5–10 kDa were shown to have potential as DNA delivery system (Richardson, Kolbe, & Duncan, 1999). LMWC of 20 kDa were shown to prevent progression of *Diabetes mellitus* and had high affinity for some bacterial lipopolysaccharides than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). Tsai, Wu, and Su (2000) proposed the practical use of LMWC in the milk preservation and oral hygiene. And Tomida et al. (2009) reported that LMWC (<30 kDa) have impressive antioxidant ability to scavenge hydroxyl radicals and to reduce cupric ions than high molecular weight chitosan. Several studies revealed the anti-tumor potential of chitosan was dependent on molecular weight (Harish Prashanth & Tharanathan, 2005).

Generally, low molecular weight chitosan can be obtained by chemical means using HCl, H_2O_2, and $NaNO_2$ that, however, do not lend themselves to easy reaction control and often results in modification of the products. Physical means using sonication and shearing that require special equipment. And by enzymatic means, based either on specific enzymes, such as chitosanases (Hsiao, Lin, Su, & Chiang, 2008) or on unspecific enzymes, including cellulase (Qin et al., 2004), lysozyme (Lin, Lin, & Chen, 2009), lipase (Muzzarelli, Xia, Tomasetti, & Ilari, 1995), amylase (Wu, 2011), papain (Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002; Terbojevich, Cosani, & Muzzarelli, 1996), pepsin (Roncal, Oviedo, Armentia, Fernández, & Villarán, 2007) and pectinase (Shin-ya, Lee, Hinode, & Kajiuchi, 2001).

Among these enzymes, neutral protease, a kind of nonspecific enzyme, had been found to be able to hydrolyze chitosan efficiently and obtain LMWC with different weight-average molecular weight easily by prolonging the duration (Li, Du, & Liang, 2007; Li et al., 2005). But the use of enzymatic means 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 LMWC and COS, which obtained by such enzymatic hydrolysis for biochemical and food area, is limited as a result of an undesirable level of chitosan pyrogenicity caused by the presence of protein of enzyme admixtures (Ilyina, Tikhonov, Albulov, & Varlamov, 2000). The utilization

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of immobilized enzyme offers advantages over free enzyme for the preparation of LMWC and COS free of protein admixtures and is more suitable for biomedical and food applications.

Chitosan and its derivatives are known as ideal support materials for enzyme immobilization (Juang, Wu, & Tseng, 2002; Muzzarelli, 1980) because of their many characteristics like improved mechanical strength, resistance to chemical degradation, avoiding the disturbance of metal ions to enzyme, and antibacterial property and so forth on. And at the same time, using chitosan and its derivatives hydrogel beads as immobilized carrier decreased the contamination of impurity and simplified the purification process (Orrego, Salgado, Valencia, Giraldo, & Cardona, 2010).

It is widely known that immobilization can change, e.g. the accessibility of the enzyme to the substrate or even the affinity of the enzyme towards a specific substrate due to change in the local environmental or altering the mobility of the protein. And immobilization may affect enzyme activity in a way that depends on the enzyme, substrate, products of the reaction, immobilization method and support (Akkava, Sahin, Demirel, & Tümtürk, 2009; Pessela et al., 2007).

The purpose of this paper, therefore, is to study the effect of immobilized support and enzymatic reaction substrate on the catalytic properties and hydrolysis process of the neutral protease immobilized on chitosan (CS), carboxymethyl chitosan (CMCS) and *N*-succinyl chitosan (NSCS) hydrogel beads.

2. Experimental

2.1. Materials

Chitosan was obtained from Aoxing Ocean Biochemical Co. (Zhejiang, China). The chitosan with the DD 75.3% and weight-average molecular weight (M_w) 410 kDa, as initial material from crab shells, was used to prepare the CMCS and NSCS. The chitosan (CS1) with a DD of 91.7% and M_w of 286 kDa, as an initial material from shrimp shells, was used to prepare chitosan with different DD and as the substrate to prepare LMWC. Chitosan with different DD were prepared according to the reference (Chen & Hua, 1996) and CS2 (DD 80.8%) and CS3 (DD 63.2%) were obtained. D-Glucosamine HCl was purchased from Seikagaku Corp. (Japan). All other chemicals were of reagent grade.

The neutral protease, derived from *Bacillus subtilis* As 1.398, was a product of Ningxia XiaSheng Industry Co. (China). The enzyme purification was achieved according to the reference (Su, Wang, Yao, & Yu, 2006) and modified as following: the enzyme solution was supplemented with solid ammonium sulfate to achieve 80% saturation under constant stirring. The solid enzyme was collected by centrifugation and redissolved in water. The solution was then loaded onto the Sephadex G-25 column and deionized water was used as the eluent. The eluate was monitored for protein by measurement of the absorbance at 280 nm. The main active fraction was collected as the purified neutral protease solution, followed by a concentration treatment with PEG-20000. Then the concentrated enzyme solution was lyophilized to get neutral protease powder. Neutral protease purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight of enzyme was calculated using protein markers. The SDS-PAGE (Figure not shown) showed a single band and molecular weight calculated was 33.8 kDa.

2.2. Preparation of CMCS

CS (10 g) suspended in 50% (w/w) NaOH (12 mL) was kept at −20 °C overnight. The frozen alkali CS was unfrozen naturally and then transferred to 2-propanol (120 mL). The mixture was stirred at room temperature completely and $ClCH_2COOH$ (12 g) was added slowly. After the mixture was stirred at room temperature for 2 h, heat was applied to bring the reaction mixture to 60 °C for another 2 h. After the reaction, the mixture was filtered to remove the reaction solvent, and the precipitate was dissolved in deionized water. Next, acetic acid was added to the solution to adjust the pH to 7.0. After it was dialyzed against deionized water for 3 days, the CMCS salt was filtered, and the filtrates were concentrated to about one-twentieth with a rotary evaporator under reduced pressure. The synthesized CMCS was precipitated and washed with ethanol and then collected after it was dried over phosphorus pentoxide *in vacuo*.

2.3. Preparation of NSCS

CS (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 of methanol was added. After the solution was stirred for 1 h, succinic anhydride (30 g) was added to the CS solution. The reaction mixtures became a gel within 30 min. After the solution was stirred for 24 h, the reaction mixture (containing a gel) was filtered to remove methanol and was then dispersed in 400 mL of deionized water. To obtain a sodium salt of the product, and 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 the addition of ethanol. The precipitates were collected after they were over phosphorus pentoxide *in vacuo* to get NSCS.

2.4. Preparation of hydrogel beads

An amount of CS (3 g) was completely dissolved in 100 mL of 1% (v/v) acetic acid. This solution was extruded through a syringe needle into 125 mL of distilled water containing 15 g of NaOH and 25 mL of ethanol under stirring to form spherical gels. The solution was allowed to stand for 3 h; the spherical gels that formed the wet CS beads were removed by filtration and rinsed with distilled water until neutrality was reached. The beads had an average diameter of 2.7 mm.

CMCS and NSCS powder (3 g) were completely dissolved in 100 mL of distilled water. The mixture was extruded drop by drop with a syringe needle into 150 mL of 1% (w/v) $CaCl_2$ solution containing 50 mL of ethanol under stirring to form beads. The beads were allowed to harden in the $CaCl_2$ solution for 3 h. CMCS and NSCS hydrogel beads had average diameters of 1.5 and 2.2 mm, respectively.

2.5. Immobilization of neutral protease

For the activation of the chitosan and its derivatives supports, wet chitosan and its derivatives hydrogel beads (2 g) were treated with 10 mL of GA solution in a shaker for 12 h at room temperature. After they were washed with distilled water until the GA in the washings was not determined at 245 nm, the crosslinked chitosan and its derivatives hydrogel beads were stored at 4 °C until use. The GA-activated chitosan and its derivatives hydrogel beads were then immersed in 10 mL of 0.1 M citrate–phosphate (C–P) buffer solution containing a given amount of neutral protease, and the mixture was gently shaken for 4 h at 25 °C. The supernatant was removed, and the resulting chitosan and its derivatives hydrogel beads were washed with 0.1 M C–P buffer until the protein in the washings was not detected at 280 nm. The immobilized neutral protease were recovered from the solution and then stored at 4 °C. The

optimum immobilization conditions are summarized as following: chitosan and its derivatives hydrogel beads immobilized with 3, 2, and 1% (v/v) GA, enzyme/beads ratio (mg/g) 1.6, 1.2, and 0.6 mg/g at pH of 6.0, 6.0, and 4.0 for CS, CMCS and NSCS hydrogel beads, respectively.

2.6. Preparation of low molecular weight chitosan

Chitosan (10 g) was completely dissolved in 1000 mL 1% (v/v) acetic acid. And then the pH value of chitosan solution was adjusted to 5.4, 5.7, and 5.7 for neutral protease immobilized on CS, CMCS and NSCS with KOH (0.1 M), respectively. After stirred for 1 h, the reactor was kept in a thermostatic shaker and immobilized enzyme was added. The optimum temperature for the hydrolysis reaction for neutral protease immobilized on CS, CMCS and NSCS beads was 55, 55, and 50 °C, respectively. The weight ratio of enzyme protein to substrate was 1:20.

At various intervals, the hydrolysates mixture were taken out and the pH value of the hydrolysates were adjusted to 9 using 10% (w/v) KOH solution. The precipitate was collected by filtration, and washed thoroughly with anhydrous ethanol, then collected after drying over phosphorus pentoxide *in vacuo*. The chitosan hydrolysate prepared from a specified condition is designated as LMWC-time-sup or COS-time-sup, where sup represents the used immobilization support and time represents the hydrolyzing period. For example, an LMWC product produced by hydrolyzing chitosan with immobilized neutral protease on chitosan hydrogel beads for 4 h would be designated as LMWC-4-CS.

2.7. Characterization

The increasing in reducing sugars resulted from a cleavage of glycosidic linkage was monitored by spectrophotometric analysis on the basis of Schales' modified method with D-glucosamine HCl as standard (Imoto & Yagshita, 1971).

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 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. The flow rate was maintained at 1.0 mL/min. The temperature of the column was maintained at 30 °C. The standards used to calibrate the column were TOSOH pullulan (Showa Denko, Tokyo, Japan). All data provided by the GPC system were collected and analyzed using the JiangShen Workstation software package (Dalian, China).

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 5700 spectrophotometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

X-ray diffraction patterns of the degraded chitosan fractions were measured by a Shimadzu Lab XRD-6000 diffractometer and used a CuKα radiation (λ = 1.5404 Å) at 40 kV and 50 mA at 20 °C.

Electrospray ionization-mass spectrometry (ESI-MS) measurement was performed on an Agilent (Santa Clara, USA), model LC/MSD TOF equipped with an ESI source. The hydrolysate of chitosan was infused into the mass spectrometric interface via syringe (Hamilton-Bonaduz, Switzerland, 500 μL) located in a syringe pump (model 11 Plus, Harvard Apparatus, Hugo Sachs Elektronik, Hugstetten, Germany) with a flow rate 5 μL/min. The most important MS parameters were as follows: 300 °C drying gas temperature, 480 L h^{-1} drying gas flow rate, 15 psig nebulizer gas pressure, 4000 V capillary voltage, 60 V skimmer voltage and 215 V fragmentor voltage. The mass-range was set to 300–1300 *m/z*.

2.8. 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 (Tolaimate et al., 2000). The titration was performed with a DELTA-320-S pH meter.

2.9. Estimation of water-solubility

The water-solubility of each LMWC sample was defined as the water-soluble solid content (w/w) in a neutral solution (Lin et al., 2009). Dried LMWC sample of 1 g were accurately weighted and neutralized with 200 mM phosphate buffer at a volume ratio of 1:1. The insoluble solid of the mixture was precipitated by centrifugation at 6000 × *g* for 30 min and washed three times with distilled water to remove solvent residue. Total solids and insoluble solids were dried in an oven at 60 °C until a constant weight was obtained. The water-solubility of LMWC was calculated as:

$$\text{Solubility (\%, w/w)} = \frac{\text{total solid dried weight} - \text{insoluble solid dried weight}}{\text{total solid dried weight}}.$$

The pH-induced cloud points (CP) of LMWC were determined by dissolving LMWC (0.1 g) into 1% (v/v) HAc (100 mL) and adding 0.1 M NaOH until the solution became turbid. The tests were performed at room temperature and the transmittance of the solution at 600 nm as a function of pH value was recorded on a Shimadzu UV-9100 spectrophotometer using a quartz cell with an optical path length of 1 cm (Park, Cho, Chung, Kwon, & Jeong, 2003). Cloud point pH values are defined as the pH when the transmittance was no less than 98%. And pH_{50} is defined as the pH value when the transmittance of the solution at 600 nm reached 50%.

3. Results and discussion

3.1. Reduction in M_w

Fig. 1 shows the GPC curves of LMWC prepared by immobilized neutral protease obtained at various intervals during the batch experiments, respectively. Higher elution volumes correspond to a decrease in the molecular weight of chitosan. As shown in Fig. 1(a) and (b), prolonging the duration increased the extent of degradation. A rapid shift of elution volume to higher ones was seen in the early reaction stage. GPC curves of LMWC prepared by neutral protease immobilized on CS and CMCS hydrogel beads showed similar degradation process and indicated that the enzymatic hydrolysis was an endo-action.

As shown in Fig. 1(c), the GPC curves of all the degraded products prepared by neutral protease immobilized on NSCS hydrogel beads 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 for 4 h, the first peak of GPC curve disappeared and become the only one peak. This is very different from the degradation process as shown in Fig. 1(a) and (b). The bimodal distribution of molecular weight of degraded chitosan indicated a nonrandom degradation

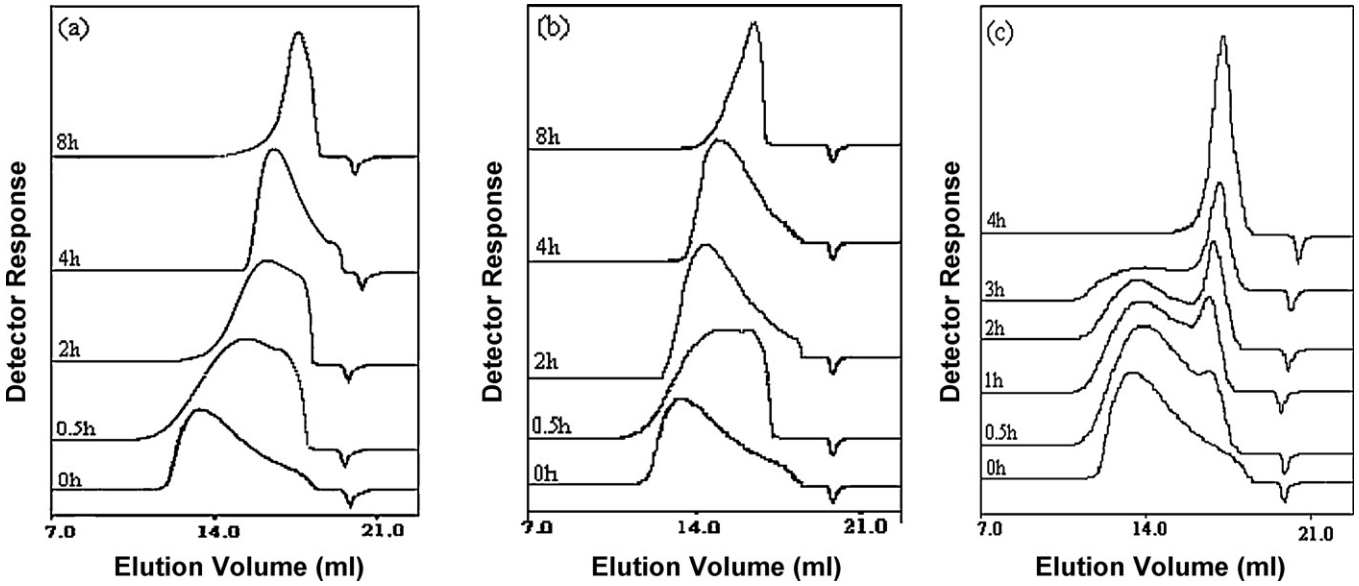

Fig. 1. GPC profiles of enzymatic products of chitosan at different reaction periods. Neutral protease immobilized on (a) chitosan, (b) carboxymethyl chitosan, and (c) *N*-succinyl chitosan hydrogel beads.

of chitosan with immobilized neutral protease on NSCS hydrogel beads and suggested that the chitosan chains contain cleaving points which are more susceptible to neutral protease immobilized on NSCS hydrogel beads than enzyme immobilized on CS and CMCS hydrogel beads.

Fig. 2 indicates the relationship between LMWC's M_w and the hydrolyzing time. Apparently, the M_w of LMWC prepared by three kinds of immobilized neutral protease decreased steeply during the first 2 h. LMWC products obtained by neutral protease immobilized on CS and CMCS hydrogel beads collected at hydrolyzing time of 8 h and LMWC products obtained by enzyme immobilized on NSCS hydrogel beads collected at hydrolyzing time of 4 h, representing the near completion of reaction, produce molecular weight (from largest to smallest) as: LMWC-8-CS (3.4 kDa), LMWC-8-CMCS (3.2 kDa), and LMWC-4-NSCS (1.9 kDa). Neutral protease used in this study was mixtures of endo- and exoenzymes. Fast degradation during the early hydrolysis stage was attributed to endoenzyme activity. Exoenzymes modified the initial LMWC product into smaller fragments by liberating dimmer

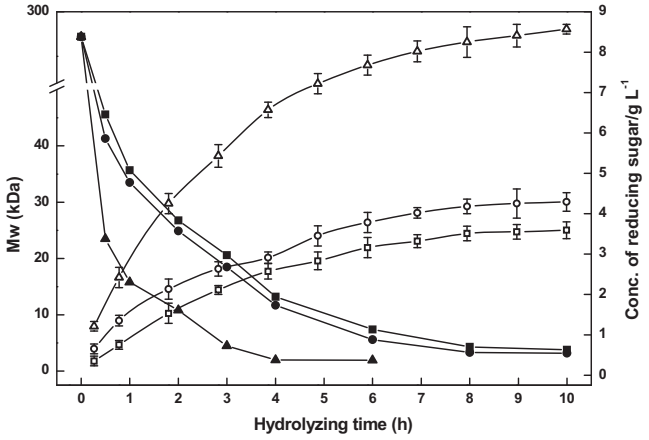

Fig. 2. The weight-average molecular weight (M_w) of chitosan hydrolysates prepared by neutral protease immobilized on (■) chitosan, (●) carboxymethyl chitosan and (▲) *N*-succinyl chitosan hydrogel beads and reducing sugar released in the hydrolysis process by neutral protease immobilized on (□) chitosan, (○) carboxymethyl chitosan and (△) *N*-succinyl chitosan hydrogel beads.

and monomer *N*-acetyl-glucosamine (NAG) or glucosamine flanked on the reducing end of chitosan. Neutral protease immobilized on NSCS hydrogel beads may recognize NAG sites easily, which also helps explain the smaller molecular size of LMWC-NSCS as compared with LMWC-CS and LMWC-CMCS.

In order to further investigate the stability of product molecular size in relation to hydrolyzing time, the amount of reducing sugar was monitored using the Schales' modified method. As shown in Fig. 2, the reducing sugar of LMWC-CS and LMWC-CMCS gradually stopped increasing at 8 h of hydrolyzing time, and LMWC-NSCS kept releasing reducing sugar afterward along the hydrolyzing time and became smaller in molecular size. The extensive degradation of LMWC-NSCS was attributed to the nonspecific digesting behaviour of neutral protease immobilized on NSCS hydrogel beads, which led the digestion of chitosan to be more complete.

In this study, for each of the three kinds of immobilized neutral protease, the same amount of enzyme protein was applied in each hydrolyzing reaction. However, neutral protease immobilized on NSCS hydrogel beads displayed a stronger catalyzing capability than enzyme immobilized on CS and CMCS hydrogel beads, here indicating the chitosan chains contain cleaving points which are more susceptible to neutral protease immobilized on NSCS hydrogel beads. That is to say, the support material leads to different microenvironment of neutral protease and changes the hydrolyzing process.

3.2. Kinetic properties of immobilized neutral protease

In order to clarify the effects and interactions that influence the catalytic reaction of immobilized neutral protease, the kinetic properties of neutral protease immobilized on three kinds of chitosan and its derivatives hydrogel beads using chitosan with different DD as substrates were discussed. And kinetic parameters, K_m and V_{max}, were determined from the Lineweaver–Burk plots (Figure not shown).

3.2.1. Effect of the immobilization support

The microenvironment of immobilized enzyme molecules is a very important factor in enzyme behaviour. K_m and V_{max} values were significantly affected after immobilization onto chitosan and its derivatives hydrogel beads, as shown in Table 1.

Table 1
Kinetic parameters of immobilized neutral protease on chitosaneous hydrogel beads acting on chitosan with different degree of deacetylation.

Kinetic parameters	Substrate	Free enzyme	Immobilized neutral protease on		
			CS	CMCS	NSCS
K_m (mg mL^{-1})	CS1	1.42	1.58	2.43	1.88
	CS2	1.13	1.19	2.15	1.33
	CS3	0.79	0.87	1.87	1.17
V_{max} (mg mL^{-1} h^{-1})	CS1	0.36	0.27	0.72	0.73
	CS2	0.43	0.41	0.85	0.91
	CS3	0.73	0.61	0.94	1.23
Apparent k_{cat}	CS1	0.072	0.054	0.144	0.146
	CS2	0.085	0.082	0.170	0.182
	CS3	0.124	0.122	0.188	0.246
k_{cat}/K_m	CS1	0.051	0.034	0.059	0.078
	CS2	0.075	0.070	0.079	0.137
	CS3	0.157	0.140	0.101	0.210
V_{max}/K_m (h^{-1})	CS1	0.25	0.17	0.30	0.39
	CS2	0.38	0.34	0.40	0.68
	CS3	0.92	0.70	0.50	1.05

Comparison of the K_m value for a given free and immobilized enzyme provides information about interaction between enzyme and its support. As shown in Table 1, the K_m values of three kinds of immobilized neutral protease were higher than that of free enzyme and had the following increasing order: neutral protease immobilized on CS, NSCS and CMCS hydrogel beads, which indicated that the immobilized enzyme had an apparently lower affinity for its substrate than the free enzyme. There are several reasons why a different kinetic behaviour is observed with an enzyme immobilized onto a solid support relative to the free enzyme. Firstly, the immobilization may cause some conformational changes in the enzyme molecules. Secondly, the immobilized enzyme is located in an environment different from that when it is the free solution, and this can have a significant effect on the kinetics. Finally, there is a partitioning of substrate between the solution and support; hence, the substrate concentration in the neighborhood of the enzyme may be significantly different from that in the bulk solution.

The V_{max} values of the free and immobilized neutral protease used for chitosan hydrolysis increased in the following order: neutral protease immobilized on CS hydrogel beads, free enzyme, enzyme immobilized on CMCS and NSCS hydrogel beads. These results might be mainly ascribed to the conformation changes of the enzyme induced by the applied immobilization procedure. Usually, V_{max} decreases with an increase in K_m, but in the case of immobilized neutral protease, there was no obvious decrease in V_{max} though there was a significant increase in K_m.

A better estimation of the catalytic efficiency of an enzyme for a particular substrate is k_{cat}/K_m, in which k_{cat}, the catalytic constant, is defined as $V_{max}/[E]_T$, where $[E]_T$ represents the total molar concentration of the enzyme. In this study, an apparent k_{cat} was obtained using $[E]_T = 5\%$ (w/v) because the enzyme concentration was constant at that level with each hydrolysis sample in the degradation studies (Zhang & Neau, 2002). The k_{cat} and k_{cat}/K_m values are listed in Table 1. A comparison of all the results obtained for the different immobilization supports shows that k_{cat} values followed the order NSCS > CMCS > CS for all the substrates assayed, that is, neutral protease immobilized on NSCS hydrogel beads has highest catalytic activity. The catalytic efficiency values of k_{cat}/K_m followed the same order as the value of k_{cat}, except in the case of the CMCS support with CS3 as substrate, because of the high K_m, the catalytic efficiency was lower.

The term apparent kinetic power (V_{max}/K_m) is defined by the ratio between V_{max} and K_m (Keleti, 1988). This parameter would be the equivalent of catalytic efficiency (k_{cat}/K_m) modified by the quantity of enzyme on each support (immobilization yield) and could be used for choosing the most suitable support (Marín-Zamora, Rojas-Melgarejo, García-Cánovas, & García-Ruiz, 2007). The values of V_{max}/K_m followed (Table 1) the same order as the values of V_{max}, except in the case of the CMCS support with CS3 as substrate, for which a lower kinetic power value was obtained since the K_m values were very high.

3.2.2. Effect of the enzymatic reaction substrate

It has been shown that the stiffness of the chitosan chain is dependent on its chemical composition and these facts also affect the enzymatic hydrolysis. 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.

In order to obtain information about the effect of DD on enzyme–substrate affinity, the K_m values as a function of DD are shown in Table 1. It is clear that chitosan with lower DD showed a lower K_m value indicating their higher affinity towards neutral protease resulting in the maximum depolymerization as evidenced by their V_{max}. At the same time, the V_{max} also increased with decrease in DD.

For all the substrate assayed, the catalytic constant (k_{cat}) values shown by low DD chitosan as substrate were higher than those shown by high DD chitosan as substrate (Table 1). And the results indicate that lower DD chitosan samples experienced a higher catalytic efficiency (k_{cat}/K_m), and would be the sample most susceptible to enzymatic degradation by immobilized neutral protease.

The greater the V_{max}/K_m value, the higher the catalytic efficiency of the enzyme for the substrate. The kinetic power determined suggested that the substrate interaction efficiency of low DD is greater than that of high DD. From this result, it is also suggested that the hydrolysis of partially *N*-acetylation chitosan with the neutral protease immobilized on chitosan and its derivatives hydrogel beads should occur with recognition of GlcNAc units.

As shown in Table 1, the effect of immobilization supports on the kinetic constant of neutral protease depends on the substrate used. Besides being due to substrate–enzyme and substrate–support interactions, the results are coherent with the proposition that variations in kinetic constants are also due to enzyme–support interactions (probably through conformational changes of the enzyme towards more active forms). Therefore, it is possible to regulate the K_m values, which depend on the reaction medium and the substrate's DD, and the apparent catalytic efficiencies (catalytic activity and/or apparent affinity) for a given substrate, by changing the support used for enzyme immobilization.

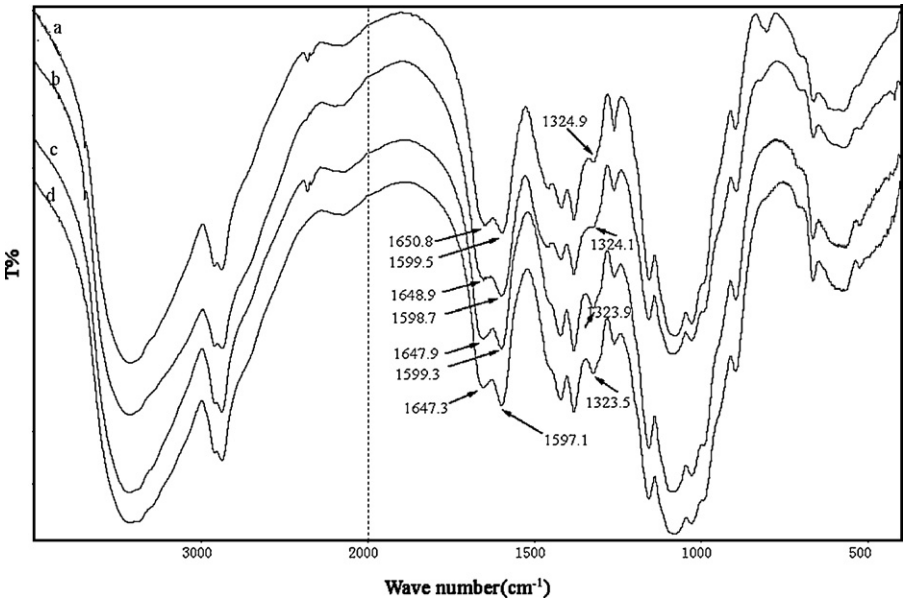

Fig. 3. FT-IR spectra of initial chitosan CS1 (a) and degraded chitosan LMWC-8-CS (b), LMWC-8-CMCS (c), and LMWC-4-NSCS (d). Sample codes correspond to those in Table 2.

3.3. The structure properties of different molecular weight chitosan

3.3.1. FT-IR spectra

Fig. 3 showed the FT-IR spectra of initial chitosan and degraded chitosan LMWC-8-CS, LMWC-8-CMCS and LMWC-4-NSCS. The absorption bands at 1650.8, 1599.5, and 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 (Dong, Xu, & Wang, 2000). The spectrum of LMWC-8-CS, LMWC-8-CMCS and LMWC-4-NSCS is similar to that of initial chitosan, but the amide I band shift 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. And can be seen, there is no significant difference among the amide III of initial chitosan, LMWC-8-CS, LMWC-8-CMCS and LMWC-4-NSCS, which indicated that with the decrease of the molecular weight of chitosan, the DD of hydrolysate did not changed. All these data were consistent well with the data of potentiometric determination of DD (shown in Table 2). The FT-IR spectra suggested that there was no significant difference between the residues of chitosan before and after the hydrolysis by neutral protease immobilized on CS, CMCS and NSCS hydrogel beads.

3.3.2. X-ray diffraction analysis

Fig. 4 showed the X-ray diffraction patterns of chitosan and its 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 (Saito, Tabeta, & Ogawa, 1987). LMWC-4-CS and LMWC-4-CMCS displayed a new characteristic peak at $2\theta = 21.4^\circ$, and the intensity of the characteristic peak at $2\theta = 10.4^\circ$ and 19.8° increased more than that of CS1. Both of the patterns characterized a chitosan polymorph, which is referred to as the 'tendon hydrate polymorph' (Belamie, Domard, & Giraud-Guille, 1997). LMWC-8-CS and LMWC-8-CMCS displayed the other two new characteristic peaks at $2\theta = 15.1^\circ$ and 23.8°, which is referred to as the 'annealed polymorph', just as previously described by Ogawa (Ogawa, 1991), and then was found to be a mixture of the 'tendon hydrate polymorph' and 'annealed polymorph'. Unlike Ogawa, who had to heat the chitosan to 100 °C at least to detect the annealed polymorph, we do not have to reach such a high temperature to observe it. Here, the rise in temperature should not be large enough to allow the occurrence of the annealed polymorph, which is more likely related to the presence of short oligomers. Also, the extent of conversion from the 'tendon chitosan' to the annealed form depends upon the molecular weight of chitosan, so LMWC-8-CS and LMWC-8-CMCS did not show complete conversion to the annealed polymorph because of the lower mobility of its polymer chains. As a consequence, there seems to be an obvious correlation between the depolymerization and the rise in crystallinity due to recrystallization of short chain chitosan.

Compared with initial chitosan, the peak at $2\theta = 10.4^\circ$ of LMWC-2-NSCS disappeared and the intensity of the characteristic peak at $2\theta = 19.8^\circ$ increased. The characteristic peak at small angle of LMWC-2-NSCS disappeared, which corresponded to large spacing, indicated that depolymerization of chitosan lead to decrease of the crystalline perfection. And the peak at $2\theta = 19.8^\circ$ of LMWC-2-NSCS

Table 2
Properties of degraded chitosan by neutral immobilized on chitosan (CS), carboxymethyl chitosan (CMCS), and *N*-succinyl chitosan (NSCS) hydrogel beads.

Support	Sample	Reaction time (h)	M_w ($\times 10^{-3}$)	M_w/M_n	DD (%)
	CS	–	286	6.71	91.7
CS beads	LMWC-4-CS	4	11.2	2.56	92.3
	LMWC-8-CS	8	3.4	1.91	92.3
CMCS Beads	LMWC-4-CMCS	4	9.7	2.36	92.0
	LMWC-8-CMCS	8	3.2	1.98	90.4
NSCS beads	LMWC-2-NSCS	2	10.8	4.18	88.1
	LMWC-4-NSCS	4	1.9	1.28	91.5

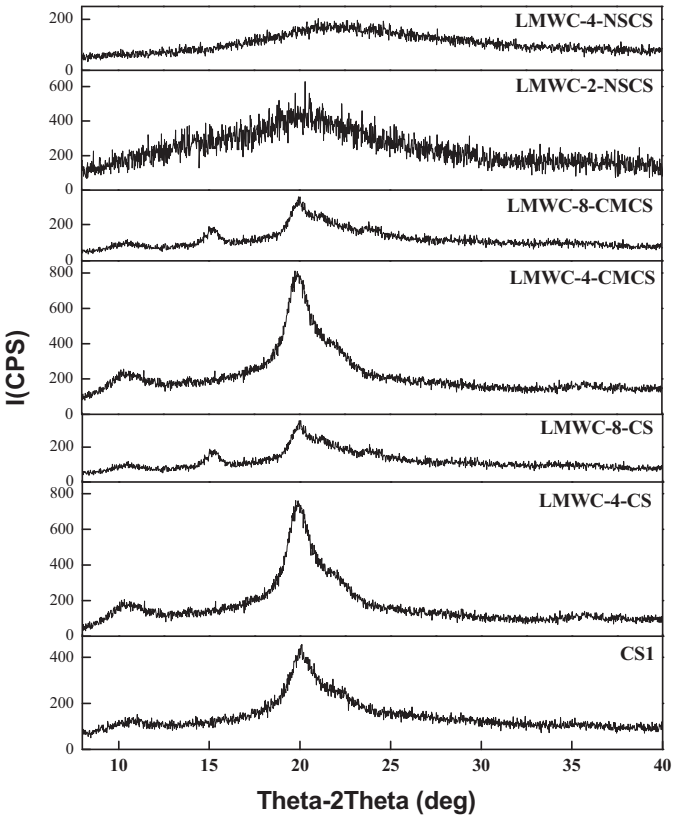

Fig. 4. X-ray diffraction patterns of initial chitosan (CS1) and low molecular weight chitosan prepared by neutral protease immobilized on chitosan, carboxymethyl chitosan and *N*-succinyl chitosan hydrogel beads. Sample codes correspond to those in Table 2.

slightly broadens, which means a decrease in the dimensions of crystalline regions and the number of defects increased with the decrease of molecular weight of chitosan. Because the crystal structure of chitosan depend on the regular distribution of substituting groups along the chain, the transformation of the crystal structure of hydrolysates prepared by neutral protease immobilized on NSCS hydrogel beads indicated that the enzyme selectively cleave the chitosan molecular chain and the distribution of *N*-acetyl groups is not regular. LMWC-4-NSCS had only one major peak and became amorphous. That was to say, the chitosan in amorphous region was first degraded to water-soluble molecules. With deeper degradation, the crystalline structure was destroyed.

The X-ray diffraction patterns of chitosan and its hydrolysates indicated neutral protease immobilized on different supports showed different hydrolysis process, and resulted in chitosan hydrolysate with similar molecular weight shown different crystalline structure.

3.3.3. *ESI-MS analysis of low molecular weight chitosan*

Comparing LMWC molecular size, degree of polymerization (DP) deserves more attention than average molecular weight (Shin-ya et al., 2001), for some prospective functions, such as anti-tumor, are discovered based on DP. DP is the numbers of the residues in polymers, while molecular weight is the sum of mass of all residues in theory. ESI-MS has recently been introduced for analysis of carbohydrate mixtures quickly and sensitively. A great advantage of ESI-MS is the process of soft-ionization causes little or no fragmentation of analytes, allowing the molecular ions of analytes to be identified, even within mixtures, like LMWC. And ESI-MS has been used to determine the type of linkage existing, and to provide sequence and branching data for derivative oligosaccharides.

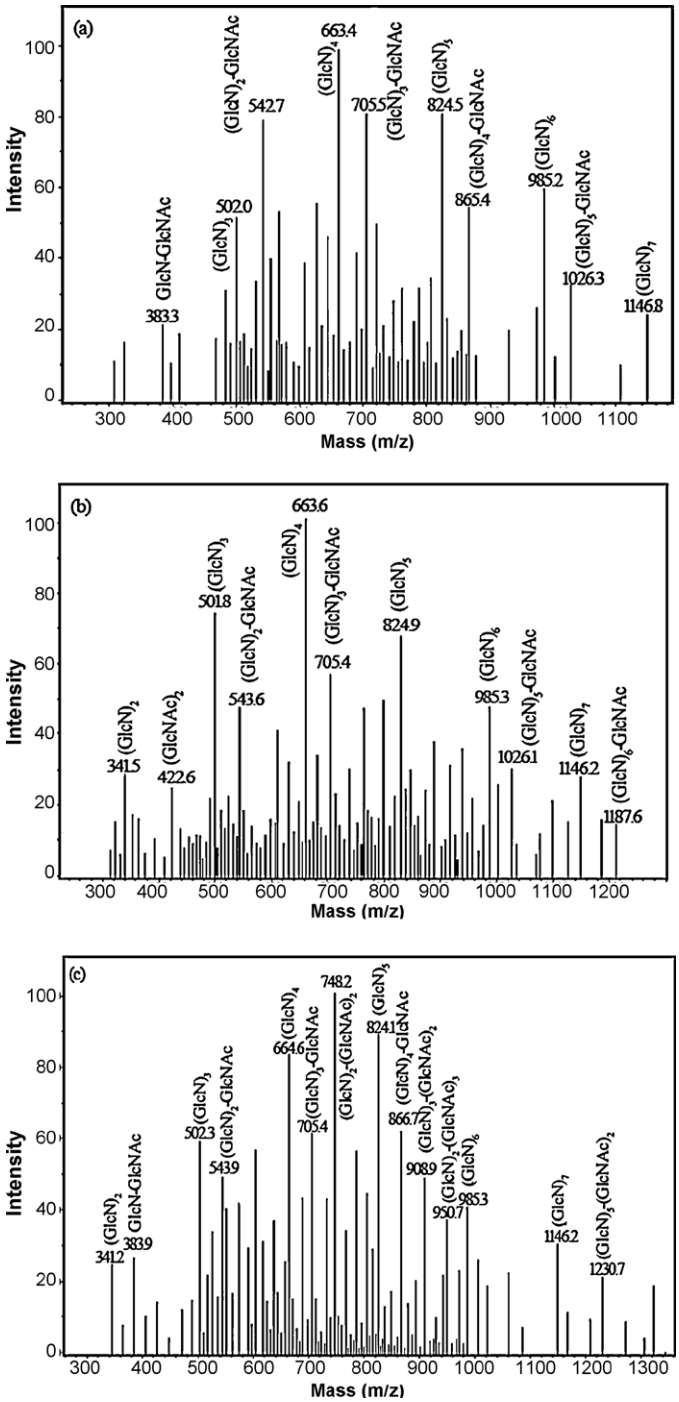

Fig. 5. ESI-MS spectrum of LMWC-8-CS (a), LMWC-8-CMCS (b), and LMWC-4-NSCS (c). Sample codes correspond to those in Table 2.

The ESI-MS was employed for LMWC preparation, and gave some interesting information about the differences on the composition and structure when compared with LMWC prepared by immobilized neutral protease on chitosan and its derivatives hydrogel beads.

The depolymerization of chitosan by immobilized neutral protease was terminated by the addition of 2 M NaOH. The precipitate obtained after centrifugation of the reaction mixture was LMWC and the supernatant contained COS. Fig. 5 shows the ESI-MS spectrum of COS-8-CS, COS-8-CMCS and COS-4-NSCS, respectively. The figures revealed that the product was composed mainly of chitooligomers, especially of DP 2 to 7.

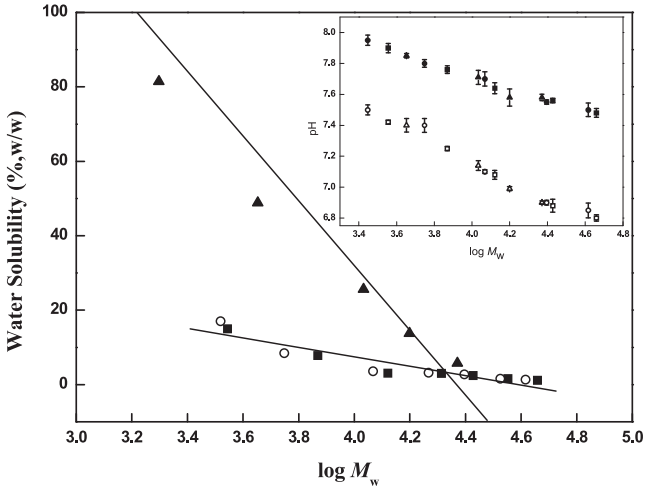

Fig. 6. Relationship between water-solubility and weight-average molecular weight of chitosan. (■) LMWC-CS, (○) LMWC-CMCS, and (▲) LMWC-NSCS. The insert figure: cloud point pH and pH_{50} of different molecular chitosans. Cloud point pH of (□) LMWC-CS; (○) LMWC-CMCS; (△) LMWC-NSCS; pH_{50} of (■) LMWC-CS; (●) LMWC-CMCS; (▲) LMWC-NSCS. The sample concentrations were 0.1% (w/v). The legend is designated as LMWC-support. For example, LMWC prepared by neutral protease immobilized on chitosan hydrogel beads would be designated as LMWC-CS.

As it can be seen, the immobilized neutral protease hydrolyzes chitosan and could produce $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$, $(GlcN)_6$, and $(GlcN)_7$, which indicates that neutral protease immobilized on chitosan and its derivatives hydrogel beads can split the β-1,4-glycosidic linkage 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 neutral protease immobilized on chitosan and its derivatives hydrogel beads can also selectively cleave GlcNAc–GlcN linkage. It is also consistent with the conclusion that the initial chitosan with different DD would affect the enzymatic hydrolysis.

3.4. Water-solubility of chitosan and its degradation

The water-solubility of LMWC prepared by neutral protease immobilized on chitosan and its derivatives hydrogel beads has been determined. Fig. 6 shows the relationship between water-solubility and molecular weight. The water-solubility decreased linearly with logM_w for LMWC. The higher water solubility of chitosan is attributed to the decrease of intermolecular interactions, such as van der Waals forces; the lower the molecular weight, the lower the intermolecular attraction forces. Therefore, the decreasing in water solubility of the chitosan with high molecular weight is probably due to the high molecular weight itself. And, at a similar M_w, LMWC-NSCS showed better solubility as compared with LMWC-CS and LMWC-CMCS. As shown in Figs. 1 and 2, neutral protease immobilized on NSCS hydrogel beads hydrolyzing chitosan showed absolutely different hydrolysis process compared with enzyme immobilized on CS and CMCS. And all the water-solubility data were consistent well with the conclusion of GPC.

The water solubility of chitosan was assayed as a function of pH. A good correlation between pH and transmittance at 600 nm was established for all the chitosans investigated. pH_{50} and the cloud point pH of different molecular weight chitosans are shown in Fig. 6 (insert figure). We observed that cloud point pH and pH_{50} increased with decreasing molecular weight. This was expected because, the presence of rigid crystalline domains, formed by intra- and/or intermolecular hydrogen bonding, was considered to be responsible for the poor solubility of chitosan in high pH solutions. The hydrogen bonding will be disturbed during the depolymerization process, resulting in the improved solubility. It should be noted that these tests were only carried out at one concentration and were simply used to verify that the solubility of chitosan could be improved by decreasing molecular weight.

4. Conclusions

In the study, neutral protease was immobilized on GA-pretreated chitosan and its derivatives hydrogel beads and was used for the preparation of low molecular weight chitosan and chitooligomers. The results reported here demonstrated that different enzyme supports led to different microenvironments of enzyme, and the immobilized enzymes showed different enzyme activity and hydrolysis process. LMWC with similar molecular weight prepared by neutral protease immobilized on different support showed different crystal structure. The degree of polymerization of chitooligomers was mainly from 2 to 7. It seems possible therefore to design selective immobilization supports for a given substrate that maximize the enzyme activity and obtain low molecular weight chitosan and chitooligomers with expected physicochemical properties.

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