Chitosaneous Hydrogel Beads for Immobilizing Neutral Protease for Application in the Preparation of Low Molecular Weight Chitosan and Chito-Oligomers

Jin Li,[1,2] Yumin Du,[2] Liping Sun,[2] Hongbo Liang,[2] Tao Feng,[2] Yuan'an Wei,[3] Pinjia Yao[3]

[1]*College of Environmental Science and Engineering, Ocean University of China, Qingdao 266003, People's Republic of China*
[2]*Department of Environmental Science, Wuhan University, Wuhan 430079, People's Republic of China*
[3]*College of Life Sciences and Technology, Guangxi University, Nanning 530005, People's Republic of China*

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ABSTRACT: Enzyme hydrolysis with immobilized neutral protease was carried out to produce low molecular weight chitosan (LMWC) and chito-oligomers. Neutral protease was immobilized on (CS), carboxymethyl chitosan (CMCS), and *N*-succinyl chitosan (NSCS) hydrogel beads. The properties of free and immobilized neutral proteases on chitosaneous hydrogel beads were investigated and compared. Immobilization enhanced enzyme stability against changes in pH and temperature. When the three different enzyme supports were compared, the neutral protease immobilized on CS hydrogel beads had the highest thermal stability and storage stability, and the enzyme immobilized on NSCS hydrogel beads had the highest activity compared to those immobilized on the other supports, despite its lower protein loading. Immobilized neutral protease on all the three supports had a higher K_m (Michaelis-Menten constant) than free enzyme. The V_{max} (maximum reaction velocity) value of neutral protease immobilized on CS hydrogel beads was lower than the free enzyme, whereas the V_{max} values of enzyme immobilized on CMCS and NSCS hydrogel beads were higher than that of the free enzyme. Immobilized neutral protease on CS, CMCS, and NSCS hydrogel beads retained 70.4, 78.2, and 82.5% of its initial activity after 10 batch hydrolytic cycles. The activation energy decreased for the immobilization of neutral protease on chitosaneous hydrogel beads. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 3743–3750, 2006

Key words: bioengineering; degradation; enzymes; hydrogels

INTRODUCTION

Low molecular weight chitosan (LMWC) and chito-oligomers have distinct biological and physiological activities.[1–5] They are widely used in functional foods, medicine, and cosmetics, and so on. LMWC and chito-oligomers can be obtained by either chemical hydrolysis or enzymatic hydrolysis.[6] The use of chemical hydrolysis leads to either complete depolymerization or chemical modifications of glucose ring and others,[7] whereas the specific hydrolysis enzyme, chitosanase, is unavailable in bulk quantities for commercially viable applications.[8] In our previous work, neutral protease was found to be able to hydrolyze chitosan (CS) efficiently, and we were able to obtain LMWC with different weight-average molecular weights (M_w's) and chito-oligomers easily by prolonging the duration.[9]

Unfortunately, the use of enzymatic hydrolysis has been limited due to the unstable nature of the enzymes and the resulting requirement of stringent conditions, such as a particular pH and temperature. In hydrolysis reactions, purified enzymes can be rather costly, and to discard them after each use is not economical. Immobilized enzymes typically exhibit greater stability over a broader range of pH and temperature values.[10] In addition, the immobilization of enzymes contributes to the development of continuous processes and is adaptable to a variety of configurations and specific processes carried out in reactors.

Moreover, the utilization of LMWC and chito-oligomers, which are produced by an enzyme method for biomedical and food purposes, is limited as a result of an undesirable level of CS pyrogenicity caused by the presence of about 0.1% (w/w) of the proteins of the enzyme complex.[11] The utilization of immobilized enzyme offers advantages over free enzyme for the preparation of LMWC and chito-oligomers free of protein admixtures and is more suitable for biomedical and food applications.

Correspondence to: Y. Du (duyumin@whu.edu.cn)
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CS and its derivatives are known as ideal support materials for enzyme immobilization because of their many characteristics, such as nontoxicity, hydrophilicity, biocompatibility, biodegradability, and antibacterial properties.[12] Moreover, they possess hydroxyl and amino groups, which link with enzymes easily[13] and can be crosslinked frequently with glutaraldehyde (GA) to make enzyme supports that have greater mechanical strength and are more applicable for use in biochemical engineering. The properties of immobilized enzymes are governed by the properties of both the enzyme and support material. The interaction between the two lends an immobilized enzyme specific physicochemical and kinetic properties that may be decisive for its practical application, and thus, a support judiciously chosen can significantly enhance the operational performance of the immobilized system.[14]

In this study, we immobilized neutral protease onto crosslinked chitosaneous hydrogel beads and demonstrated the hydrolytic properties for the production of LMWC and chito-oligomers. A comparative study of the immobilized enzyme activity on different enzyme supports was done. The three immobilized supports used in this study to form hydrogel beads were CS, carboxymethyl chitosan (CMCS), and *N*-succinyl chitosan (NSCS). The properties of the immobilized neutral protease, kinetic parameters, pH, and the thermal stability, storage stability, and operational stability of the immobilized neutral protease were also investigated.

EXPERIMENTAL

Materials

CS was obtained from Yuhuan Ocean Biochemical Co. (Zhejiang, China). CS with a degree of *N*-deacetylation (DD) of 75.3% and a M_w of 410 kDa, as an initial material from crab shells, was used to prepare the CMCS and NSCS. CS with a DD of 91.7% and a M_w of 286 kDa, as an initial material from shrimp shells, was used as the substrate for estimating the enzyme activity. M_w was measured by gel permeation chromatography; DD was determined by the potentiometric titration method. Bovine serum albumin and Coomassie brilliant Blue G250 were purchased from Sigma Chemical Co. (St. Louis, MO). D-glucosamine HCl were purchased from Seikagaku Corp. (Tokyo, Japan). All other chemicals were reagent grade.

The neutral protease, derived from *Bacillus subtilis* A.S.1.398, was a product of Ningxia XiaSheng Industry Co., Ltd. (Ningxia, China).

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*.

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, 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 the addition of ethanol. The precipitates were collected after they were over phosphorus pentoxide *in vacuo* to get NSCS.

Fourier transform infrared (FTIR) spectra were obtained with a Nicolet FTIR 360 spectrophotometer (Thermo Electron Scientific Instruments Corp. USA). The samples were prepared by the mixture of the compounds with KBr (ca. 0.5% w/w) and the pressing of the diluted mixture to form a clear semitransparent pellet. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

Preparation of chitosaneous 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.

Immobilization of neutral protease to chitosaneous beads

For the activation of the chitosaneous supports, wet chitosaneous hydrogel beads (2 g) were treated with 10 mL of GA solution in a shaker for 12 h at room temperature. After they were with distilled water until the GA in the washings was not determined at 245 nm, the crosslinked chitosaneous hydrogel beads were stored at 4°C until use. The GA-activated chitosaneous 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 chitosaneous 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 enzymes were recovered from the solution and then stored at 4°C.

To optimize preparation of neutral protease-containing chitosaneous hydrogel beads, the effects of the following factors during the immobilization step were studied: pH of coupling buffer solution (2–8), GA concentration (0.5–5% v/v), enzyme/hydrogel bead (E/B) weight ratio (0.2–2 w/w), and immobilization time (2–8 h).

Measurement of neutral protease activity

The activity of free and immobilized neutral proteases were determined by spectrophotometric analysis on the basis of Schales' modified method[15] with D-glucosamine HCl as a standard.

Free neutral protease (5 mg) or immobilized enzyme (equal to 5 mg of enzyme protein) was added to 10 mL of a 1% CS solution prepared by 0.2*M* acetic acid-acetic sodium (HAc-NaAc) buffer and incubated in a water bath for 1 h. The specific activity (μmol/min mg of protein) was equivalent to the micromoles of reducing sugar released per minute per milligram of protein at optimum conditions.

Protein assay

We estimated the amount of immobilized enzyme protein by subtracting the amount of protein determined in supernatant after immobilization from the amount of protein used for immobilization. The protein content in the solutions was determined by the method of Bradford,[16] with bovine serum albumin as a standard.

Properties of the free and immobilized neutral protease

The activity assays were carried out over a pH range of 4.2–6.3 and a temperature range of 25–80°C to determine the optimum pH and temperature of free and immobilized enzyme. The activity of pH profiles was determined at various pH values in 0.2*M* HAc-NaAc buffer at 50°C for free and immobilized neutral proteases on NSCS hydrogel beads and at 55°C for immobilized neutral protease on CS and CMCS hydrogel beads. Activity of temperature profiles was determined at the indicated temperature in 0.2*M* HAc-NaAc buffer at pH 5.4 for the free and immobilized neutral proteases on CS beads and at pH 5.7 for immobilized neutral protease on CMCS and NSCS hydrogel beads. The results of pH and temperature of the medium are presented in a normalized form, with the highest value of each set assigned the value of 100% activity.

The effect of substrate concentration on the activity was tested with increasing concentrations of CS substrate from 0.5 to 4% in the corresponding activity HAc-NaAc buffer pH values and with incubation at the corresponding activity temperature. The K_m and V_{max} values of the free and immobilized neutral proteases were determined with the Lineweaver–Burk plot method.

The pH stability of the free and immobilized neutral proteases was ascertained by the measurement of the residual activity of enzyme exposed to various pH's (1.0–13.0) at 25°C for 2 h. The buffers used were 0.1*M* KCl–HCl (pH = 1.0), 0.1*M* C–P (pH = 2.0–8.0), 0.1*M* glycin–NaOH (pH = 9.0–10.0), 0.1*M* Na_2HPO_4–NaOH (pH = 11.0), and 0.1*M* KCl–NaOH (pH = 12.0–13.0). The thermal stability of free and immobilized neutral proteases was determined by the measurement of the residual activity of enzyme exposed to various temperatures (20–70°C) in 0.1*M* C–P buffer (pH = 6.0) for 2 h. Activity of the samples was performed at optimum conditions.

The activity of the free and immobilized neutral proteases after storage in 0.1*M* C–P buffer (pH = 6.0) at 5 and 25°C was measured in a batch operation mode at optimum conditions.

The retention of the immobilized enzyme activity was tested as described in the activity assays of neutral protease. After each reaction run, the enzyme–chitosaneous beads were removed and washed with 0.1*M* C–P buffer (pH = 6.0) to remove any residual substrate on the chitosaneous hydrogel beads. The beads were then reintroduced into fresh reaction medium, and enzyme activities were detected at optimum conditions.

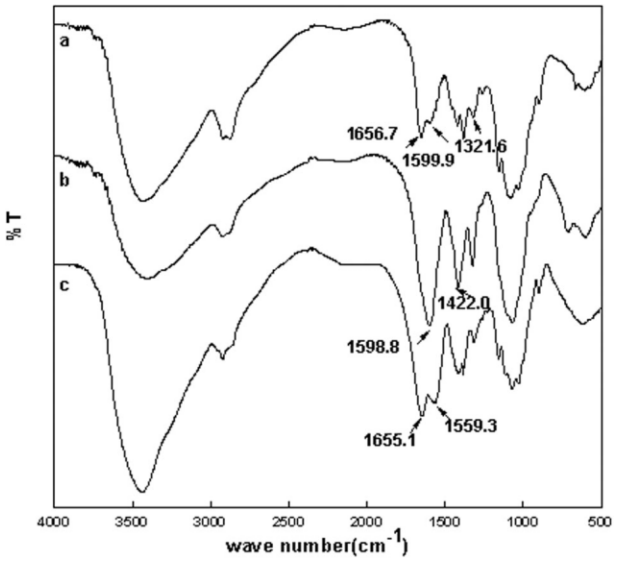

Figure 1 FTIR of (a) CS, (b) CMCS, and (c) NSCS. % T refers to Transmittance (%).

RESULTS AND DISCUSSION

IR spectra of immobilized supports

The IR spectra of the three chitosaneous immobilized supports, CS, CMCS, and NSCS, are shown in Figure 1. The characteristic peak of the amino groups was at 1599.9 cm^{-1}, and amide I and amide III bands at 1656.7 and 1321.6 cm^{-1} appeared in CS.[17] In the spectrum of CMCS in Na salt, two strong peaks at 1598.8 and 1422.0 cm^{-1} were observed due to the asymmetrical and symmetrical stretching of —COO^- groups, respectively[18]. The secondary amide vibration band at 1500–1540 cm^{-1} did not appear in the spectrum of CMCS, which was attributed to the modification of —NH_2.[19] All these indicated that the carboxymethyl groups were on the —OH position. Through comparison of the IR spectra, it was clear that the CS modified with succinic anhydride presented additional medium intensity bands, one at 1655.1 cm^{-1} and the other at 1559.3 cm^{-1}, which were the characteristic bands derived from carbonyl groups, which were formed during the opening of the anhydride as it reacted with the amino group of CS.[20]

Enzyme immobilization

To optimize the conditions of neutral protease immobilization on chitosaneous hydrogel beads, the effect of the pH of the coupling buffer solution (2–8), GA concentration (0.5–5% v/v), *E/B* ratio (0.2–2 w/w), and immobilization time (2–8 h) on protein loading and specific enzyme activity were investigated.

GA is a bifunctional reactive agent mainly capable of reacting with the surface amine groups of the enzyme and chitosaneous supports through the formation of Schiff bases and Michael adducts.[21] The method of immobilizing neutral protease on chitosaneous hydrogel beads with GA involves two reactions. The first reaction introduces aldehyde group onto chitosaneous hydrogel beads, which reacts with the amino group of enzymes. Because the reaction between CS and GA was pH-dependent, an aqueous solution of GA was used during the activation step. The effect of the pH of the coupling solution between activated CS and the enzyme was studied. The optimum pH of the immobilization of neutral protease on chitosaneous hydrogel beads is shown in Table I. The activity of the immobilized enzyme on CS, CMCS, and NSCS hydrogel beads was assumed to be maximum at pHs of 6.0, 6.0, and 4.0, respectively. If the pH was too low, most of the amine groups were positively charged, and the positively charged amine had a very low reactivity with aldehyde to give a low immobilization yield. The activity of the immobilized enzyme was also reduced by a decrease in the activity of neutral protease above the optimum immobilization pH. At the optimal immobilization pH, the intramolecular and intermolecular interactions of the immobilized enzyme molecules were at their height, and the enzyme was also in its optimum globular conformation and protected itself from unfolding phenomena.[22] Such phenomena can also be explained by the fact that the possible polymerization of GA is pH-dependent.[23]

As shown in Table I, the activity of the immobilized enzyme were assumed to be maximum when the concentration of GA was 3, 2, and 1% (v/v) for immobilization on CS, CMCS, and NSCS hydrogel beads, respectively. At low concentrations of GA, it is probable that the aldehyde groups present on the support crosslinked the amine groups of the CS, and the few reversible bonds (Schiff bases) involving the enzyme molecules were not sufficient to prevent their leakage. When the concentration of GA was increased, the increase in the aldehyde groups reached a point of saturation that allowed complete adsorption of the equilibrated enzyme. The activity was reduced by po-

TABLE I
Optimum Conditions for Neutral Protease on CS and CS Derivative Beads

Support	GA (%)	Immobilization pH	Immobilization Time (h)	*E/B* ratio (mg/g)	Protein loaded (μg/g of support)	Specific activity (μmol min^{-1} mg^{-1} of protein)
CS	3	6.0	4.0	1.6	687	6.8
CMCS	2	6.0	4.0	1.2	321	16.4
NSCS	1	4.0	4.0	0.6	122	32.5

lymerization between the enzyme and GA in the solution beyond that concentration, probably due to the toxicity of this reagent toward neutral protease. Complete washing of the activated chitosaneous hydrogel beads to free it of residual GA before contact with the enzyme solution was deemed essential for the achievement of a high activity yield.

The effects of E/B ratio on immobilization were investigated, and the optimum E/B ratios were shown in Table I for a constant bead weight of 2 g (wet weight). When the E/B ratio was above the optimum value for immobilized neutral protease, a reduction in the relative activity was observed, and the results may have been due to enzyme–enzyme interactions and steric hindrance. When E/B ratios were below the optimum values, the available aldehyde groups of the beads were not saturated with enzyme molecules.

Last, the effect of immobilization time on enzyme activity was also studied. For all three kind of enzyme supports, the optimal contact time was 4 h. At longer contact times, the enzyme was deactivated, which may have been due to conformational and surface modifications induced by GA, whereas at shorter contact times, there was a decrease in residual activity that may have been due to the partial release of the enzyme into solution.

The optimum immobilization conditions are summarized in Table I. As shown in Table I, the highest protein loading and enzyme activity occurred in chitosaneous hydrogel beads immobilized with 3, 2, and 1% GA and immobilization times of 4 h at pH's of 6.0, 6.0, and 4.0 for CS, CMCS and NSCS hydrogel beads, respectively. When the three kind of enzyme supports were compared, the immobilized neutral protease on CS beads had the highest protein loaded but the lowest specific enzyme activity, whereas the immobilized protease on NSCS beads had the lowest protein loaded and showed the highest specific enzyme activity.

Characterization of free and immobilized neutral protease

Immobilization is often accompanied by changes in enzymic activity, optimum pH and temperature, affinity to the substrate, and stability. The extent of these changes depends on the enzyme, carrier support, and immobilization conditions.

The activities of free and immobilized neutral proteases were assayed at various pH values. The optimum pH was determined and is shown in Figure 2. The free and immobilized neutral proteases on CS hydrogel beads showed the optimum pH at 5.4. The pH optimums of immobilized neutral protease on CMCS and NSCS hydrogel beads shifted in the alkaline region relative to the pH of free and immobilized enzyme on CS hydrogel beads and showed an optimum pH at 5.7. CS is a polycationic substance, but when it is changed into CMCS and NSCS by the introduction of —CH_2COONa or —$CO(CH_2)_2COONa$ groups onto —OH or —NH_2 along CS molecular chain, respectively, amphoteric polyelectrolyte containing both cationic and anionic fixed charges were prepared. So, in studies of the neutral protease immobilized on CS and its derivations, the optimum pH retained the same value or shifted toward a higher one that could have been due solely to electrostatic potential of the support.[24] The immobilized neutral proteases were less sensitive to pH changes than free enzyme.

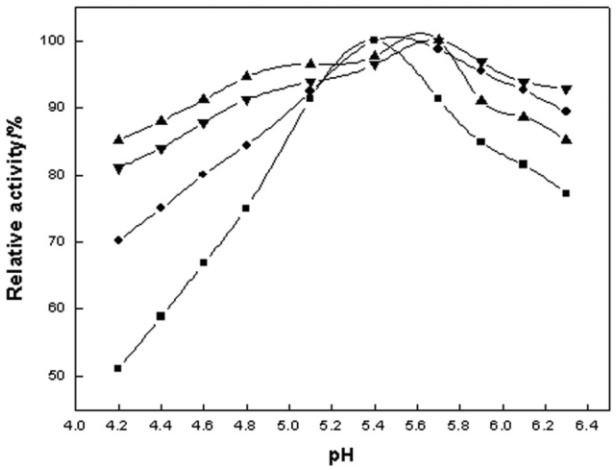

Figure 2 Effect of reaction pH on the relatively activity of (■) free and immobilized neutral proteases on (●) CS, (▲) CMCS, and (▼) NSCS. Reactions were performed at 50°C for free and immobilized neutral proteases on NSCS beads and at 55°C for immobilized neutral protease on CS and CMCS in HAc-NaAc buffer (0.2*M*) of various pH values.

The effects of reaction temperature on the activity of free and immobilized neutral proteases were investigated and are shown in Figure 3. The optimum temperature for the hydrolysis reaction for the free and immobilized neutral proteases on NSCS hydrogel beads remained the same at 50°C. The immobilized neutral protease on CS and CMCS beads showed the optimum hydrolysis temperature at 55°C. The loss of the activity of immobilized enzyme was lower than that of free enzyme in the investigated temperature range. The immobilization of enzyme caused an increase in enzyme rigidity, which is commonly reflected by an increase in stability toward denaturation with increasing temperature.[25] The high activity of immobilized enzymes at low temperatures was probably a result of the favored adsorption of enzymes on the chitosaneous supports.[26]

The kinetics of the hydrolytic activity of free and immobilized neutral proteases were investigated with various initial concentrations (0.5–4%) of CS as the substrate. The Lineweaver–Burk plot of the immobi-

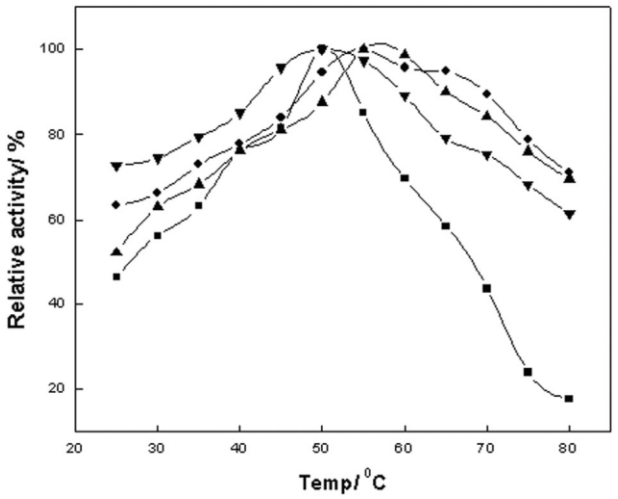

Figure 3 Effect of reaction temperature on the relatively activity of (■) free and immobilized neutral proteases on (●) CS, (▲) CMCS, and (▼) NSCS. Reactions were performed in HAc-NaAc buffer (0.2*M*) at pH 5.4 for free and immobilized neutral proteases on CS beads and at pH 5.7 for immobilized neutral protease on CMCS and NSCS beads at various temperatures.

lized neutral protease on CS, CMCS, and NSCS hydrogel beads are shown in Figure 4. The plots indicated that the enzymatic hydrolysis of CS with immobilized neutral protease obeyed Michaelis–Menten kinetics well. K_m and V_{max} values were significantly affected after immobilization onto CS, CMCS, and NSCS hydrogel beads, as shown in Table II. The change in the affinity of the enzyme to its substrate was probably caused by structural changes in the enzyme introduced by the immobilization procedure or by lower accessibility of the substrate to the active site of the immobilized enzyme. Comparison of the K_m for a given enzyme in both the free and immobilized states provided information about the interaction between the enzyme and its support. Neutral protease was immobilized on a variety of CS derivatives, which provided different chemical microenvironments for the immobilized neutral protease. The CS was modified to produce carboxymethyl and succinyl derivatives, which thus changed the close association of the enzyme with the carbohydrate-based polymer. The chain linkage may have had some effect on the enzyme flexibility, and the composition of the chain may have altered the hydrophilicity of the immediate enzyme environment. As shown in Table II, the K_m values of various immobilized enzyme were bigger than that of free enzyme and had the following increasing order: free enzyme, immobilized enzyme on CS beads, immobilized on NSCS beads, and CMCS hydrogel beads, which indicated that the immobilized enzyme had an apparently lower affinity for its substrate than the free enzyme. This may have been caused by the steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding, or diffusional resistance to solute transport near the particles of the support. The V_{max} values of the four kinds of enzyme used for CS hydrolysis increased in the following order: neutral protease immobilized on CS beads and free enzyme immobilized on CMCS and NSCS hydrogel beads. When we compared the kinetics of the neutral protease immobilized on CMCS and NSCS hydrogel beads, we observed that the two had nearly identical V_{max} values but different K_m values.

Further, the activation energies (E_a's) for free and immobilized neutral proteases were also evaluated and are shown in Table II. The E_a's for free and immobilized neutral proteases on CS, CMCS, and NSCS hydrogel beads were 17.30, 4.80, 3.10, and 1.85 kcal/mol, respectively. E_a's of the immobilized neutral protease on the chitosaneous supports were lower than that of the free enzyme, which meant that the immobilized enzymes possessed higher enzymatic activity and were more temperature-insensitive. The observed decrease in E_a confirmed that there was mass-transfer control for the immobilized enzyme rather than kinetic control.[27] Also, this would tend to indicate that there was a conformational change not only in the immobilized neutral protease but also in the substrate when the enzyme was combined with the substrate. At the same time, a part of binding energy decreased the bond energy of the substrate and formed an electron tensile force, which resulted in a decrease in the E_a, and the enzyme-catalyzed reactions became more easy.[28]

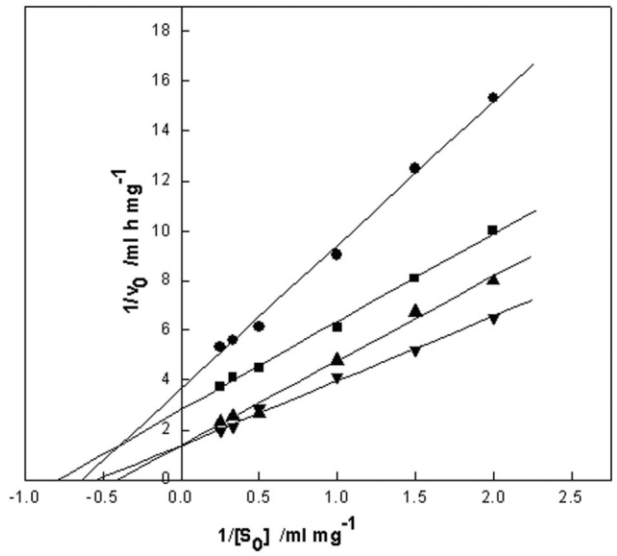

Figure 4 Lineweaver–Burk plots for (■) free and immobilized neutral proteases on (●) CS, (▲) CMCS, and (▼) NSCS hydrogel beads. Reactions were performed at their optimum reaction conditions. [V_0] refers to initial hydrolysis rate; [S_0] refers to substrate concentration at reaction time 0.

TABLE II
Enzymatic Properties of Free and Immobilized Neutral Proteases

Property	Free enzyme	Immobilized neutral protease on CS	CMCS	NSCS
Optimum pH	5.4	5.4	5.7	5.7
Optimum temperature (°C)	50	55	55	50
K_m (mg/mL)	1.27	1.58	2.43	1.87
V_{max} (mg $mL^{-1} \cdot h^{-1}$)	0.36	0.27	0.72	0.73
E_a (kcal/mol)	17.30	4.80	3.10	1.85

We compared the pH stabilities of both the free and immobilized neutral proteases by immersing them in buffers with a pH range of 1.0–13.0 for 2 h at 25°C and then determining the activity at their optimum conditions. The results are indicated in Figure 5. The free enzyme remained stable in the pH range 5.0–6.0, whereas the immobilized neutral protease on CS, CMCS, and NSCS hydrogel beads were stable in pH ranges from 5.0 to 9.0, 4.0 to 7.0, and 4.0 to 7.0, respectively. These results show that the pH stability of the immobilized neutral protease was greater than free enzyme.

We investigated the thermal stability by incubating the free and immobilized neutral proteases at temperatures ranging from 20 to 70°C for 2 h and then determining the activity at their optimum reaction conditions. The results are shown in Figure 6. The residual activity of the immobilized neutral proteases and the free enzyme dropped to 90% at the temperatures mentioned, 40 and 30°C, respectively. At 70°C, immobilized neutral protease on CS, CMCS, and NSCS hydrogel beads retained activities of about 81.1, 75.8, and 72.5%, respectively; whereas the activity retained by the free enzyme was only 13.5%. These results showed that thermal stability of immobilized neutral protease was greater than the free enzyme, which may have been due to the reduction of conformational flexibility in immobilized enzyme.[29] Immobilized enzyme on chitosaneous supports was supposed to preserve the tertiary structure of the enzyme, and it protected the enzyme from conformational changes caused by effects of the environment.

Free and immobilized neutral proteases were stored in a 0.1*M* C–P buffer (pH = 6.0) at 5 and 25°C, and activity measurements were carried out for a period of 30 days. Figure 7 shows the results of storage stability experiments of free and immobilized neutral proteases. The results indicated that the enzyme stored at 5°C was more stable than those stored at 25°C. At 5°C, the free enzyme lost about 50% of its activity within 12 days, whereas the immobilized neutral protease on CS, CMCS, and NSCS hydrogel beads protected about 89.6, 85.3, and 81.7%, respectively, of their activities within 30 days. At 25°C, the free enzyme lost about 50% of its activity within 5 days, whereas the immobilized neutral protease on CS, CMCS, and NSCS hydrogel beads protected about 82.6, 79.3, and 76.8%, respectively, of their activities within 30 days.

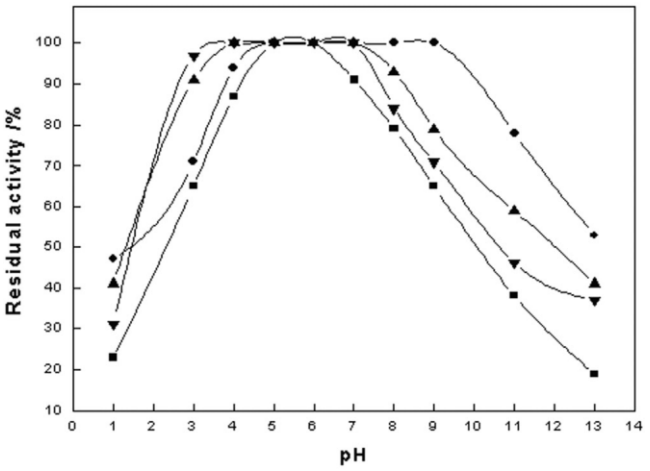

Figure 5 pH stability of free (■) free and immobilized neutral proteases on (●) CS, (▲) CMCS, and (▼) NSCS hydrogel beads. The preincubation time was 2 h. Enzyme activity was determined at optimum reaction conditions.

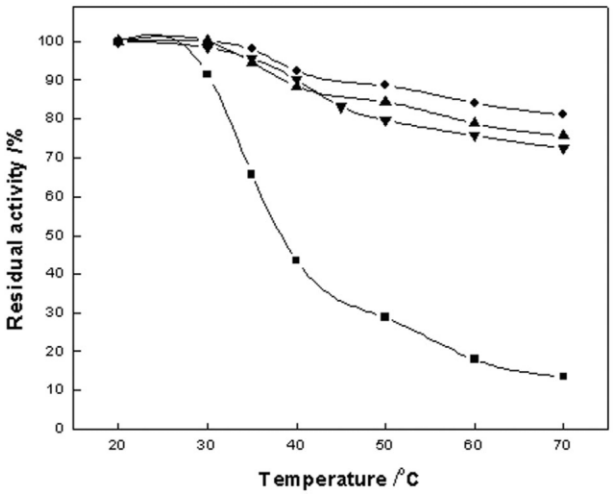

Figure 6 Thermal stability at various temperature of (■) free and immobilized neutral proteases on (●) CS, (▲) CMCS, and (▼) NSCS. The preincubation time was 2 h. Enzyme activity was determined at optimum reaction conditions.

When the performance of immobilized biocatalysts intended for preparative or industrial use are compared, characterization of their operational stabilities is very important. The operational stability of the immobilized neutral protease in this study was evaluated in a repeated batch process. The immobilized enzyme (equal to 5 mg of enzyme protein) was added to 10 mL of a 1% CS solution in a water bath at a corresponding optimum reaction temperature for 4 h. Figure 8 shows the effect of repeated use on the activity of the immobilized enzyme. The neutral protease immobilized on CS, CMCS, and NSCS hydrogel beads retained residual specific activities of 70.4, 78.2, and 82.5%, respectively, after 10 reuses.

CONCLUSIONS

Neutral protease can be of great value for the preparation of LMWC and chito-oligomers. In this study, neutral protease was immobilized on GA-pretreated CS, CMCS, and NSCS hydrogel beads. Our experiments showed that the immobilized neutral protease on NSCS beads exhibited the highest enzyme activity and the immobilized enzyme on CS beads exhibited the highest enzyme stability. The results show that different enzyme supports led to different microenvironments of enzyme, and the immobilized enzymes showed different enzyme activity. The operational and storage stability of all three kinds of chitosaneous supports presented in this article may indicate the applicability of immobilized neutral protease for the continuous degradation of CS and preparation of LMWC and chito-oligomers.

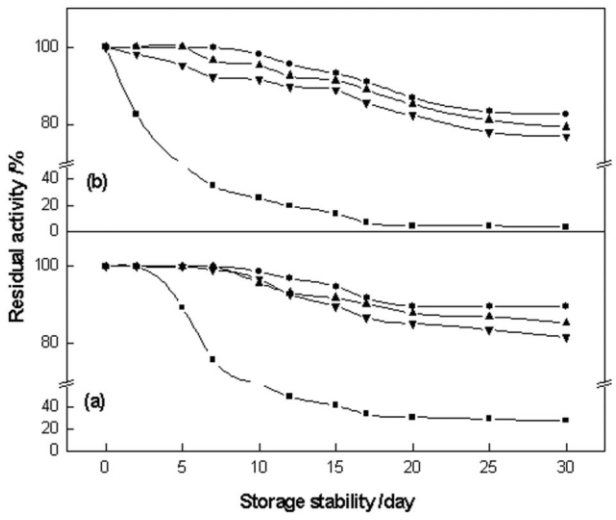

Figure 7 Storage stability at (a) 5 and (b) 25°C of (■) free and immobilized neutral proteases on (●) CS, (▲) CMCS, and (▼) NSCS hydrogel beads. Enzyme activity was determined at optimum reaction conditions.

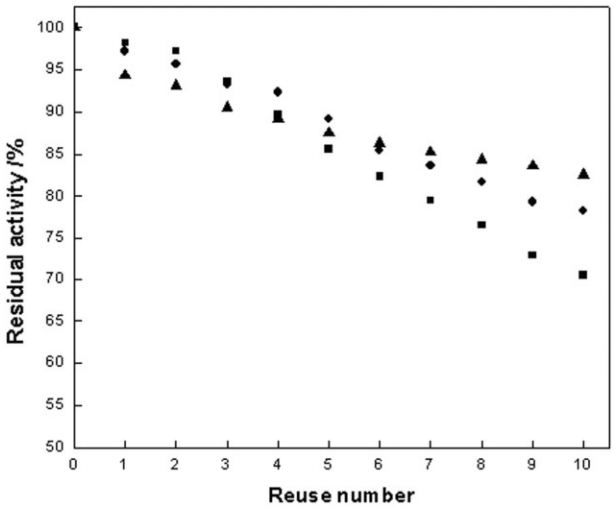

Figure 8 Operational stability of immobilized neutral protease on (■) CS, (●) CMCS, and (▲) NSCS hydrogel beads. Enzyme activity was determined at optimum reaction conditions.

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