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Purification of chitosan by using sol–gel immobilized pepsin deproteinization

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ABSTRACT

The residual proteins in commercially available chitosan products potentially induce immunological responses, thus compromising their clinical usage. Conventional deproteinization processes use diluted base and heat. However this heterogeneous hydrolysis is inefficient. In the present study, pepsin was selected and immobilized with tetramethoxysilane (TMOS) and 3-aminopropyltriethoxysilane (APTES). The immobilized pepsin was utilized in an alternative approach for the purification of chitosan. Optimum deproteinization was carried out at pH 4.5 and 45 °C. Amino acid analysis proved a removal of 53.8–80.4% protein in chitosan after 160 min incubation, which was more efficient than conventional sodium hydroxide deproteinization. When chitosan was deproteinizated by immobilized pepsin, its molecular weight decreased, but in a much milder manner than the free pepsin. The study revealed that immobilized pepsin was an efficient method for deproteinizing chitosan.

1. Introduction

Chitosan, the N-deacetylated derivate of chitin, is a heteropolysaccharide consisting of linear β-1,4-linked glucosamine (GlcN) and N-acetyl-glucosamine (GlcNAc) units (Franca, Lins, Freitas, & Straatsm, 2008). Both chitin and chitosan, either in their pure or modified forms, are used in a wide range of applications, such as in food, biotechnology, material science, pharmaceuticals, and recently in gene therapy (Muzzarelli, 2009; Prashanth & Tharanathan, 2007).

For the production of medical grade chitosan, an indispensible step is to remove protein residue from chitosan (Zhang et al., 2010), particularly tropomyosin the potent allergen from crustacean flesh, as they induce immunological responses (Muzzarelli, 2010). Under certain circumstances, the inflammatory response can have deleterious effects, resulting in significant tissue damage or even death (Goldsby, 2006, Chapter 16). Many studies also have reported that residual proteins are bound by covalent bonds to chitin, forming stable complexes (Iconomidou, Willis, & Hamodrakas, 2005; Kasaai, 2008; Kurita, 2006; No, Meyers, & Lee, 1989; Vincent & Wegst, 2004). Besides covalent bonds, hydrogen-bonding and hydrophobic interactions might also be involved (Percot, Viton, & Domard, 2003).

Conventional processes for chitin and chitosan production from crustacean shells involve deproteinization of the crustacean shells with a dilute base (e.g. sodium hydroxide) and heat (Khor, 2001, Chapter 5; No et al., 1989). However, deproteinization with dilute base is a heterogeneous reaction, so interactions between chitin/chitosan and the protein can prevent the protein hydrolyzing during alkaline treatment (Chaussard & Domard, 2004; Percot et al., 2003a). In addition, basic deproteinization inevitably induces the degradation of chitosan. For example, the molecular weight (MW) of chitosan decreased from 813 kDa to 549 kDa after a 30-min deproteinization in 3% NaOH at 15 psi/121 °C (No, Lee, Park, & Meyers, 2003). NaOH deproteinization is also time-and-energy-consuming and produces voluminous waste base.

Enzymatic hydrolysis is an effective method to reduce the protein molecular weight, peptide size, and consequently the allergenicity (Chandra, 1997; Hays & Wood, 2005), thus it has already become a standard procedure for the production of hypoallergenic hydrolyzates (Monaci, Tregoat, Hengel, & Anklam, 2006). However, its application to remove protein in chitin and chitosan was limited since the enzyme usage might induce protein contamination. Moreover, proteases such as lysozyme (Vårum, Holme, Izume, Stokke, & Smidsrød, 1996), papain (Terbojevich, Cosani, & Muzzarelli, 1996), bromelain (Hung, Chang, & Sung, 2002), and pepsin (Kumar, Varadaraj, & Tharanathan, 2007; Roncal, Oviedo, Armentia, Fernández, & Villarán, 2007) was proved to retain chitosanase activity, further restrained the application of enzymatic deproteinization in chitosan purification.

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Fortunately, immobilized enzymes can be easily separated from the final product, thereby minimizing or eliminating protein contamination of the product (Duran, Rosa, D'Annibale, & Gianfreda, 2002; Sheldon, 2007). Furthermore, the substrate selectivity of immobilized enzymes can also be manipulated by tailoring the immobilization condition (Fernandez-Lorente, Palomo, Cabrera, Guisán, & Fernández-Lafuente, 2007; Marin-Zamora, Rojas-Melgarejo, Garcia-Canovas, & García-Ruiz, 2007; Park, Yoon, & Kim, 2010). Through immobilization, enzymes can also be reused and enzyme stability can also be enhanced (Brady & Jordaan, 2009; Hanefeld, Gardossi, & Magner, 2009; Sheldon, 2007).

In the present study, pepsin was immobilized and utilized for chitosan purification. Hybrid silane, tetramethoxysilane (TMOS) and 3-aminopropyltriethoxysilane (APTES) were applied to entrap pepsin. The deproteinization by immobilized pepsin was studied. Purification of chitosan was performed with the immobilized product. Meanwhile, amino acid analysis was carried out to evaluate the protein removal efficiency.

2. Experimental

2.1. Materials

Commercially available pepsin (porcine, EC 3.4.23.1, 1:3000) was obtained from Amresco (Solon, United States). Tetramethoxysilane (TMOS, >98.0%), 3-aminopropyltriethoxysilane (APTES, >98.0%) and polyvinyl alcohol (PVA, MW 22,000) were from Shanghai Jingchun Industry (Shanghai, China). Glucosamine hydrochloride (>99.0%) and amino acids (>98.0%) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Hemoglobin (porcine) was obtained from Sigma (St. Louis, United States), and chitosan (Degree of deacetylation 84.2%, MW 404 kDa) was from Jiangsu Shuanglin Marin Biological Pharmaceutical Ltd. (Nantong, China). Water was prepared using Milli-Q system. Other chemical reagents were analytical grade.

2.2. Preparation of immobilized pepsin

TMOS (18 mmol) and APTES (2 mmol) were mixed and vortexed, 1.0 mL of 1 mmol/L HCl was added to catalyze the hydrolysis of silane (Silva & Airoldi, 1997). Pepsin (100 mg) was dissolved in phosphate buffer (0.1 M, pH 4.0) and mixed with aqueous PVA. The mixture was then added into the hydrolyzed silane solution, vortexed and kept for 48 h to complete the gelation process. The gel was ground, washed with phosphate buffer to remove loosely entrapped enzyme and then lyophilized. All the processes were performed at 4 °C to avoid the denaturation and degradation of pepsin. The immobilized product was crushed into powder and kept at 4 °C till use.

2.3. Characterization of immobilized pepsin

2.3.1. Pepsin immobilization efficiency

The amount of enzyme that leaked into the solution, after washing as described above, was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. The immobilization efficiency (*IE*) was determined following Eq. (1) where E_{free} and E_{total} are the amount of enzyme in the solution and the total initial amount of enzyme, respectively.

$$IE = \frac{E_{total} - E_{free}}{E_{total}} \times 100\% \quad (1)$$

2.3.2. Protease activity

The protease activity was determined by the amount of tyrosine hydrolyzed from denatured hemoglobin solution by the enzyme after incubation for 10 min at 40 °C (Poojari, Palsule, Clarson, & Gross, 2009).

Four milliliters of denatured hemoglobin solution (10 g/L), proper amount of immobilized pepsin (containing about 1.0 mg pepsin), and 1.0 mL of acetate buffer was mixed and incubated at 40 °C for 10 min. For the method of free pepsin, 1.0 mL of 0.1 mg/mL pepsin solution with acetate buffer as a solvent was used in replace of immobilized pepsin and acetate buffer. The reaction was stopped by adding 5.0 mL trichloroacetic acid (TFA) solution; and samples were then centrifuged and filtered. The amount of tyrosine was determined by the absorbance of the filtrate at 280 nm with a tyrosine solution as a standard. One unit (U) was counted as the amount of pepsin that produced 1 μmol tyrosine per minute.

2.3.3. Protein selectivity

As pepsin showed chitosanase activity and capability of hydrolyze chitosan, protein selectivity was used to evaluate the immobilized enzyme, along with protease activity. The protein selectivity was calculated as the ratio of protease activity to chitosanase activity. Chitosanase activity was determined by the amount of GlcN hydrolyzed from chitosan. Each reaction mixture contained 2.0 mL 5.0 mg/mL chitosan, 0.2 mL enzyme solution for free enzyme, or proper amount of immobilized pepsin (containing about 1.0 mg pepsin) and 0.2 mL acetate buffer (pH 4.0) for immobilized products. After incubation at 40 °C for 60 min, the reaction was stopped by heating in boiling water for 10 min. The amount of reducing sugar was determined by dinitrosalicylic acid method (Miller, 1959) with glucosamine hydrochloride as a standard. One unit of chitosanase was defined as the amount of enzyme that liberated 1 μmol of GlcN per minute.

2.4. Deproteinization of chitosan by immobilized pepsin

Immobilized pepsin was mixed with preheated 1% chitosan solution (in pH 4.5 acetate buffer) and incubated in plastic syringe in a water bath at 45 °C for 160 min. The suspension was filtered and the supernatant was neutralized with NaOH to precipitate chitosan. The chitosan was then filtered and lyophilized. In comparison, NaOH deproteinization was performed with 1 M NaOH at 45 °C for 160 min.

2.5. Determination of the MW of chitosan

The intrinsic viscosity of chitosan was determined with an Ubbelohde capillary viscometer in 0.2 M HAc/0.1 M NaAc solution, and the viscosity-average molecular weight was calculated by the Mark–Houwink equation (Wang, Bo, Li, & Qin, 1991) with k and α of 1.424×10^{-3} and 0.96 respectively.

2.6. Determination of protein content of chitosan

The protein content of chitosan was determined by elemental analysis and amino acid analysis (AAA). In elemental analysis, the percentage of proteins was calculated from the nitrogen content with the following equation (Chang & Tsai, 1997; Cira, Huerta, Hall, & Shirai, 2002; Percot, Viton, & Domard, 2003):

$$\%P = (\%N - N_T) \times 6.25 \quad (2)$$

where 6.25 corresponds to the theoretical percentage of nitrogen in proteins; %P represents the percentage of proteins remaining in the chitosan sample; %N represents the percentage of nitrogen measured by elemental analysis; N_T represents the theoretical nitrogen content of chitosan sample. It was calculated based on the degree of deacetylation of chitosan and percentage of nitrogen for fully acetylated chitin and fully deacetylated chitosan (6.89 and 8.69,

Table 1
Gradient program for the amino acid analysis of chitosan.

Time (min)	A (%)	B (%)	Flow rate (mL/min)
0	100	0	1
40	50	50	1
45	10	90	1
55	10	90	1
56	100	0	1.2
65	100	0	1.2

respectively). The nitrogen content was measured by elemental analysis (Vario EL III, Elementar Co.).

Amino acid analysis (AAA) was also used for the quantification of protein in chitosan. The protein content was calculated from the total amino acid weight in chitosan which was determined via amino acid analysis (AAA) with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl, >99.0%) derivation and high performance liquid chromatography (HPLC) determination (Haynes, Sheumack, Greig, Kibby, & Redmond, 1991, Haynes, Sheumack, Kibby, & Redmond, 1991). Five milligrams of sample and 2 mL of 6 M hydrochloric acid were mixed in a hydrolysis tube. The tube was degassed under vacuum, flushed with N_2, sealed and kept at 110 °C for 24 h to break peptide bonds and hydrolyze the protein into amino acids. The hydrolyzate was diluted to 5 mL and 200 μL of the resulting solution was vacuum dried. The residue was then dissolved with 200 μL borate buffer (pH 9.5) and derived with 300 μL FMOC-Cl solution. Standard amino acids were derived in the same manner.

The separation and determination of derived amino acids were carried out in HPLC system with a binary Shimadzu 10ATvp pump and a Shimadzu RF-10A_{XL} fluorescence detector. Chromatographic analysis was performed with a Bischoff Prontosil Eurobond C18 column (4.0 mm × 250 mm, 5 μm), with gradient elution (Table 1) of 5 mM ammonium acetate buffer (pH 6.7): acetonitrile (80:20) as mobile phase A and 5 mM ammonium acetate buffer (pH 6.7):acetonitrile (20:80) as mobile phase B. Fluorescence detection was performed at λ_{ex} 266 nm and λ_{em} 313 nm, and the data were recorded by an N2000 workstation (Zhejiang University, China).

2.7. Statistical analysis

All experiments were carried out in twice or triplicate, and average values or means ± standard deviations are reported. Means of the main effects were separated by Duncan's test with the SPSS11.0 software package.

3. Results and discussion

3.1. Selection of enzyme

Some commercial available enzymes, including pepsin, bromelain (1000 U/mg, Green Chemical, China), trypsin (3000 U/mg, Shanghai Jingchun, China), and chymotrypsin (800 U/mg, Shanghai Jingchun, China), were studied to select proper enzyme for further immobilization and deproteinization. Their protease activity was determined in pH 4.5 acetate buffer. Chitosanase activities were also determined and the protein selectivity (protease activity/chitosanase activity) was studied because the deproteinization should induce minimum hydrolysis of chitosan.

As shown in Fig. 1, all the four enzymes possess both protease activity and chitosanase activity. Furthermore, the protease activity and chitosanase activity showed similar trend, with bromelain the highest and chymotrypsin the lowest. Chymotrypsin and trypsin were not applicable because their protease activities were very low. Meanwhile, the protein selectivity of pepsin was significantly higher than that of bromelain, for the chitosanase activity

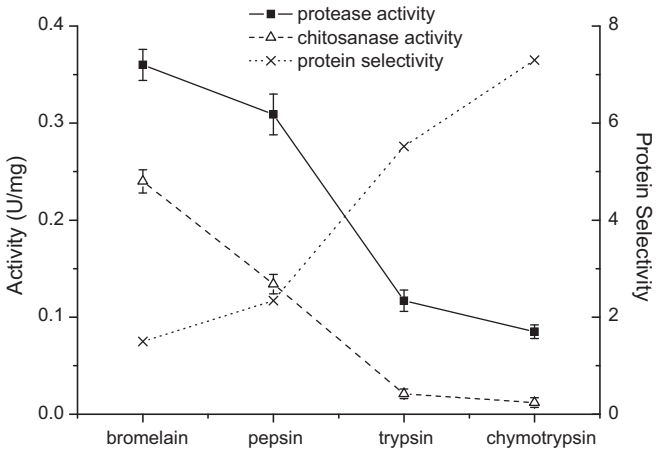

Fig. 1. The protease activity, chitosanase activity, and protein selectivity of different enzymes.

of bromelain was higher than that of other enzymes. As pepsin possessed relatively high protease activity and high protein selectivity, it was used in further immobilization and deproteinization research.

3.2. Pepsin immobilization efficiency

In the sol–gel method, TMOS acted as a tetrafunctional monomer and a network-forming agent (Noureddini, Gao, Joshi, & Wagner, 2002). The organic–inorganic hybrid matrices formed by different silane precursors were non-shrinkable (Chen & Lin, 2003). Results showed that the immobilization efficiency decreased at APTES above 60% (TMOS:APTES, 40:60) (Table 2). At 60% APTES or higher, the gelation was too fast and insufficient for the pepsin solution and silane to mix well. Rapid gel formation is thus believed to be at least partially responsible for the decrease in immobilization efficiency.

3.3. Optimization of the relative amount of APTES for immobilization

In the present study, protease activity and protein selectivity were used to evaluate the immobilized pepsin. As shown in Table 2, the protease activity and protein selectivity varied significantly with the relative amount of APTES. Similar change of activity and selectivity was reported after immobilization of different enzymes (Gupta & Chaudhury, 2007; Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007; Park et al., 2010).

Highest protease activity was observed at 40% APTES, which was even higher than free pepsin. Meanwhile, protease activity at 10%

Table 2
Immobilization efficiency, activity and selectivity of various forms of sol–gel immobilized pepsin ($n = 3$).

Silane ratio TMOS:APTES	Immobilization efficiency % ± SD	Relative protease activity[a] % ± SD	Protein selectivity ±SD
100:0	98.5 ± 1.1a	16.8 ± 6.9a	0.96 ± 0.17a
95:5	95.8 ± 1.1a	16.5 ± 6.4a	0.68 ± 0.09a
90:10	96.6 ± 0.8a	125.3 ± 14.3b	5.74 ± 0.58b
80:20	95.4 ± 1.0a	78.2 ± 8.0c	2.51 ± 0.26c
60:40	94.7 ± 1.3a	131.0 ± 12.7b	3.98 ± 0.30d
40:60	77.7 ± 5.0b	51.4 ± 8.6d	1.57 ± 0.24e
20:80	76.1 ± 5.1b	49.5 ± 9.4d	1.75 ± 0.10e

Means with different letters within a column indicate significant differences ($p < 0.05$).

[a] Use free pepsin as 100%.

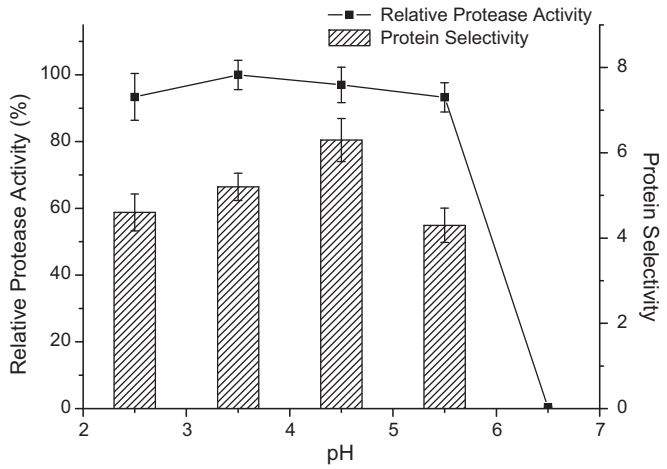

Fig. 2. Effect of pH on the relative protease activity and protein selectivity of immobilized pepsin at 40 °C ($n = 3$).

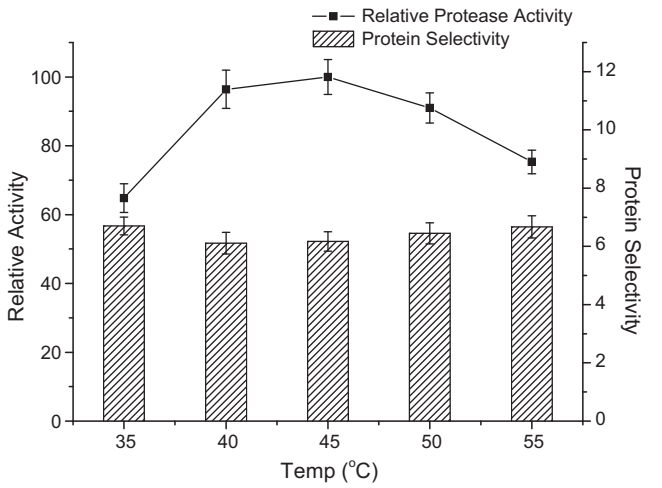

Fig. 3. Effect of temperature on the relative protease activity and protein selectivity of immobilized pepsin at pH 4.5 ($n = 3$).

APTES and 40% APTES showed no significant difference ($p > 0.05$). Maximum protein selectivity was also observed at 10% APTES, 2.7 times higher than that observed in free pepsin. Therefore, 10% APTES immobilized pepsin was used in the subsequent tests.

The increase of protease activity after immobilization was consistent with Altun and Cetinus (2007), who reported that immobilized pepsin possessed higher activity than free enzyme at pH 3–7. The underlying mechanism could be that the immobilization enhanced the enzyme stability, e.g. preventing denaturation and autolysis, in operation condition (Kumar et al., 2007). The electrostatic effect and hydrophobic effects of the matrix, which were induced by the addition of organic silane such as APTES, may also be responsible for increasing the activity and selectivity of the enzyme after immobilization (Gupta & Chaudhury, 2007).

3.4. Selection of pH for the deproteinization of chitosan

The optimum of pH for deproteinization was evaluated by investigating the protein selectivity and protease activity of immobilized enzyme. The protein selectivity was studied with the pH 2.5–5.5. Higher pH was not studied because chitosan precipitates at neutral pH and the protease activities of both free and immobilized pepsin were negligible at pH 6.5.

The protease activities showed no significant difference ($p > 0.05$) at pH 2.5–4.5. Meanwhile, maximum protein selectivity of immobilized pepsin occurred at pH 4.5, as shown in Fig. 2. Therefore, the deproteinization of chitosan was performed at pH 4.5.

3.5. Selection of temperature for the deproteinization of chitosan

The temperature for the deproteinization of chitosan was studied at 35–55 °C in phosphate buffer (pH 4.5). Higher temperature was not studied because the thermal degradation of chitosan became remarkable at elevated temperature.

Generally, the immobilization of enzymes contributes to increasing their thermostability and extending their biotechnology potential, since the diffusion rate was increased and substrate viscosity was lowered at elevated temperatures (Kharrat, Ali, Marzouk, Gargour, & Karra-Châabouni, 2011). The optimum temperature for protease activity for immobilized pepsin was 45 °C, 5 °C higher than that for free pepsin (Fig. 3). Beyond the optimum temperature, the decline of activity of immobilized pepsin was milder than free pepsin. This could be due to the matrix protected pepsin against thermal denaturation.

As the protein selectivity of immobilized pepsin showed no significant variation ($p > 0.05$) within the temperature range studied (Fig. 3), and maximum protease activity was found at 45 °C, deproteinization was processed at 45 °C.

3.6. Protein removal efficiency of immobilized pepsin

Elemental analysis and AAA showed that the protein content of initial chitosan samples varied from 3451 to 7316 ppm (Table 3). The results were similar with the data reported by Qian and Glanville (2005) and higher than Palace and Phoebe's report (1997). Meanwhile, the results of different methods varied significantly, possibly due to both methods have some deficiency. For example, elemental analysis is insensitive (Chang & Tsai, 1997; Cira et al., 2002); amino acid analysis is a sensitive method, but tryptophan (Trp) and cysteine (Cys) were destroyed in the acid hydrolysis step. Furthermore, isoleucine (Ile), leucine (Leu), and phenylalanine (Phe) cannot be detected since peaks related to them could not be separated from the peaks corresponding to GlcN, the hydrolyzate of chitosan (Fig. 4).

Bradford method was also studied, but the results were significantly higher than both elemental analysis and AAA (data not

Table 3
Protein removal efficiency of immobilized pepsin.

Chitosan sample	Initial protein content (ppm ± SD)[a]		Deproteinization efficiency (% ± SD)[a,b]		Yield (%)	
	Elemental analysis	AAA	Immobilized pepsin	NaOH	Immobilized pepsin	NaOH
Chitosan (Nantong)	5517 ± 884	6374 ± 334	80.4 ± 6.5	12.3 ± 0.7	69.8	71.3
Chitosan (Zhejiang)	6886 ± 1326	7316 ± 120	53.8 ± 3.7	21.2 ± 1.3	69.9	73.6
Chitosan (Sigma)	3451 ± 442	4521 ± 253	65.3 ± 4.8	19.2 ± 1.7	79.6	84.8

[a] The values are the average of two experiments.
[b] Determined by AAA method.

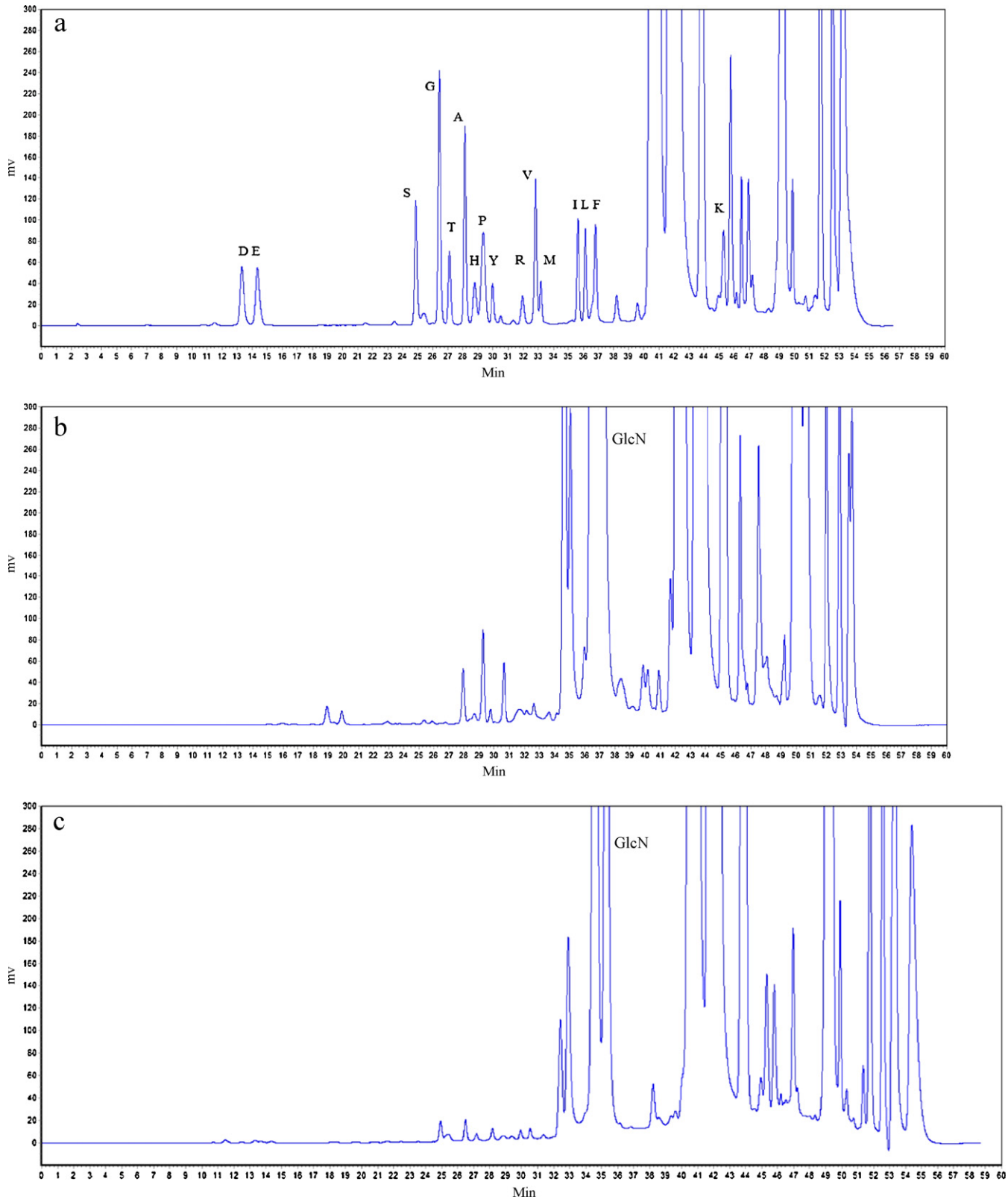

Fig. 4. Chromatogram of (a) standard amino acids (D: aspartic acid, E: glutamic acid, S: serine, G: glycine, T: threonine, A: alaine, H: histidine, P: proline, Y: tyrosine, R: arginine, V: valine, M: methionine, I: isoleucine, L: leucine, F: phenylalanine, K: lysine). Chromatogram of (b) chitosan. Chromatogram of (c) chitosan deproteined with immobilized pepsin.

shown) for initial chitosan samples. A recent report (Carlsson, Borde, Wölfel, Åkerman, & Larsson, 2011) indicated that chitosan interacted directly with the anionic coomassie dye and thus perturbed the Bradford method. Bovine serum albumin (BSA) was also considered not an ideal reference for the Bradford method when determinating protein in chitosan (Percot et al., 2003a). Therefore, Bradford method was not applied in estimating the protein removal efficiency.

Table 4
Amino acids in chitosan (Nantong) deproteinized by immobilized pepsin (ppm, the values are the average of two experiments with an error below 10% for every amino acid).

Amino acids	Time of deproteinization (min)				
	0	40	100	160	280
Asp	85.7	51.4	37.9	39.6	18.6
Glu	60.8	35.9	24.4	20.4	–[a]
Ser	200.4	114.9	118.6	79.0	75.0
Gly	321.6	292.4	226.3	258.7	266.6
Thr	51.1	51.6	41.7	13.6	21.5
Ala	216.3	188.1	131.3	95.8	42.6
His	166.9	113.2	57.2	29.2	24.7
Pro	83.8	146.0.	68.6	24.2	15.0
Tyr	148.6	115.7	59.4	12.4	24.9
Arg	90.5	53.7	49.8	61.9	55.9
Val	3264.8	956.4	500.3	391.4	417.1
Met	1615.0	1313.7	366.4	221.7	257.3
Lys	68.3	57.7	–	–	–
Ile	ND[b]	ND	ND	ND	ND
Leu	ND	ND	ND	ND	ND
Phe	ND	ND	ND	ND	ND
Total	6373.8	3490.7	1681.9	1247.9	1228.9

[a] Lower than the limit of quantification.
[b] Not determined.

As elemental analysis was insensitive and not precision, the deproteinization efficiency was determined by AAA. Results showed 53.8–80.4% protein removed after the treatment with immobilized pepsin, more effective than NaOH deproteinization (Table 3). Meanwhile, the yield of chitosan for immobilized pepsin deproteinization was just slightly lower than NaOH deproteinization.

Chitosan (Nantong) was deproteinized by immobilized pepsin and its protein content was determined at different time interval. The results of aAAA showed that the protein content decreased with the increase of incubation time up to 160 min; longer incubation time did not result in significant decreases in protein content (Table 4). Furthermore, tyrosine (Tyr) and Met were most effectively eliminated after 160 min of deproteinization. This might because pepsin was efficient in cleaving bonds involving aromatic residues, including those of Tyr, Phe, Trp, Leu and Met (Lee & Shively, 1990). Valine was the most prevalent amino acid, so it can be effectively removed in the form of small peptides, although the peptide bond of Val could not be hydrolyzed by pepsin. The glycine (Gly) content decreased only 19.6% after 160 min hydrolysis, which was partly due to the protein covalently linked with chitin was rich in Gly (Addadi & Weiner, 1992) and pepsin cannot hydrolyze Gly effectively.

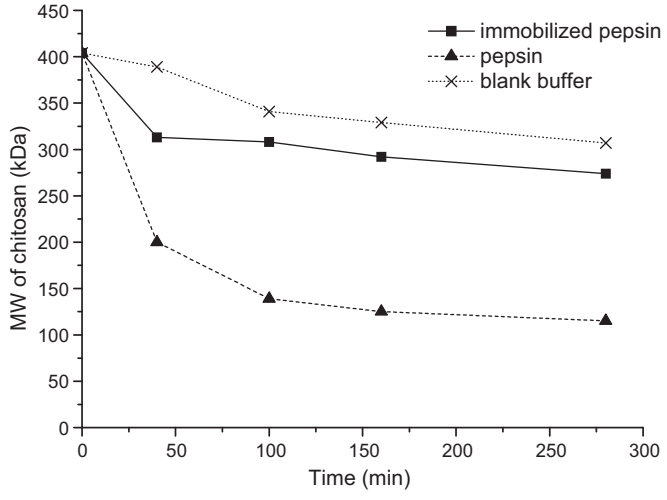

Fig. 5. MW of chitosan after different time of incubation with (■) immobilized pepsin, (▲) pepsin, and (×) blank buffer ($n = 3$, with RSD of every sample below 2.4%).

3.7. Degradation of chitosan in immobilized pepsin

As the MW of chitosan is an important factor for its medical application, the decrease of chitosan MW during the deproteinization of chitosan by immobilized pepsin was investigated.

As shown in Fig. 5, the decrease of MW mainly occurred in the initial 40 min and was insignificant after 160 min. After 280 min incubation, the MW of chitosan decreased 32.2% after the treatment by immobilized pepsin, slightly lower than treated with blank buffer solution (24.0% decreased). In contrast, the MW significantly decreased 71.5% after the treatment by free pepsin. This was consistent with our data showing lowered chitosanase activity after immobilization of pepsin, which dramatically restrained the hydrolysis of chitosan.

4. Conclusions

The study revealed that purification of chitosan by immobilized pepsin entrapped with a hybrid silane (TMOS and APTES) was more efficient than traditional NaOH deproteinization. Amino acid analysis showed that the removal of Tyr, Val and Met was most efficient. The enzyme deproteinization was energy efficient and environmental friendly. Only slightly degradation of chitosan was observed after immobilized pepsin deproteinization.

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