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Enzymatic preparation of chitooligosaccharides by commercial lipase

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ABSTRACT

The effect of a commercial lipase on chitosan degradation was investigated. When four chitosans with various degrees of deacetylation were used as substrates, the lipase showed higher optimal pH toward chitosan with higher DD (degree of deacetylation). The optimal temperature of the lipase was 55 °C for all chitosans. The enzyme exhibited higher activity to chitosans which were 82.8% and 73.2% deacetylated. Kinetics experiments show that chitosans with DD of 82.8% and 73.2% which resulted in lower K_m values had stronger affinity for the lipase. The chitosan hydrolysis carried out at 37 °C produced larger quantity of COS (chitooligosaccharides) than that at 55 °C when the reaction time was longer than 6 h, and COS yield of 24 h hydrolysis at 37 °C was 93.8%. Products analysis results demonstrate that the enzyme produced glucosamine and chitooligosaccharides with DP (degree of polymerization) of 2–6 and above, and it acted on chitosan in both exo- and endo-hydrolytic manner.

1. Introduction

Chitosan, a linear polysaccharide composed of 2-acetamido-2-deoxy-β-D-glucose (*N*-acetylglucosamine, GlcNAc) and 2-amino-2-deoxy-β-D-glucose (D-glucosamine, GlcN) with various contents of these two monosaccharides, is a partial deacetylated derivative of chitin. Chitin is the second most abundant polysaccharide on earth (after cellulose) and is largely available in the exoskeletons of invertebrates and the cells of fungi (Shahidi, Arachchi, & Jeon, 1999). Although chitosan has many attractive properties and has been used in many fields, such as food, agriculture, and medicine industry, its solubility only in dilute acid and high viscosity restrict its application to a certain extent.

Recently, chitooligosaccharides (COS) have received much attention because of their outstanding biological properties, such as antimicrobial effect, anticancer effect, antioxidant effect, and immunostimulant effect, etc. (Kim & Rajapakse, 2005). COS can be made from chitosan by chemical method, physical method and enzyme hydrolysis, among which, the enzyme hydrolysis receives more attention for its mild reaction conditions. However, due to their high cost for production, chitosanase and chitinase for specific degradation of chitosan and chitin are not available in bulk quantity till now. In view of these respects, researchers turned their eyes to non-specific commercial enzymes a decade years ago (Pantaleone, Yalpani, & Scollar, 1992), because these commercial enzymes, especially food grade enzymes, have been used in food industries for decades and are safe and relatively inexpensive. Although it is not clear why these commercial enzymes degrade chitosan, however, pectinase, cellulase, hemicellulase, papain, pepsin, and lipase have been found to catalyze chitosan hydrolysis effectively (Kittur, Kumar, Gowda, & Tharanathan, 2003; Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Pantaleone et al., 1992; Qin et al., 2004; Yalpani & Pantaleone, 1994).

However, compared with pectinases, cellulases, and proteases, studies of chitosanolysis by lipases have rarely been reported up to now (Muzzarelli et al., 1995; Sashiwa et al., 2003; Shin, Lee, & Lee, 2001). Moreover, to our knowledge, there has been no report on qualitative analysis of chitosan oligomers resulted from the chitosan hydrolysis aided by lipase. In this study, we aimed to study the effect of a commercial lipase on chitosan hydrolysis and qualitatively analyze COS products by using thin layer chromatography (TLC) method.

2. Materials and methods

2.1. Materials

Lipase, purchased from Novozymes corp. (China), was used to hydrolyze chitosan. Chitosan produced from shrimp wastes with various DD (degree of deacetylation) was provided by Haidebei corp. Ltd., China.

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2.2. Determination degree of deacetylation

The method on the basis of potentiometry described by Lin, Jiang, and Zhang (1992) was followed. Dried chitosan sample of 0.5 g were accurately weighed and thoroughly dissolved in 0.1 M HCl. The solution was then titrated with 0.1 M NaOH. The degree of deacetylation was calculated according to the following formulas:

$$NH_2\% = \frac{(C_1V_1 - C_2V_2) \times 0.016}{G(100 - W)} \times 100\%,$$

where C_1 is the concentration of HCl (M); C_2, the concentration of NaOH (M); V_1, the volume of HCl (ml); V_2, the consumed volume of NaOH (ml); G, the weight of chitosan sample; W, the water concentration of chitosan sample (%); and 0.016 is the weight of NH_2 equal to 1 ml 0.1 M HCl (g).

$$\text{Degree of deacetylation}(\%) = \frac{NH_2\%}{9.94\%} \times 100\%,$$

where 9.94% is the theoretical NH_2 percentage.

2.3. Determination of the molecular weight of chitosan

The viscosity average molecular weight ($\overline{M}v$) determination of chitosan was performed by using an Ubbelohde capillary viscosimeter ($\phi = 0.5$ mm) at 30 °C in triplicate (Mao et al., 2004). $\overline{M}v$ was calculated according to the classical Mark–Houwink equation:

$$[\eta] = K\overline{M}_v^a,$$

where $[\eta]$ is the intrinsic viscosity, and K and a are constants for given polymer – solvent system and temperature. In this experiment, K and a values provided by Knaul, Kasaai, Bui, and Creber (1998) were used.

2.4. Hydrolysis of chitosan

Chitosan was dissolved in acetic acid/sodium acetate buffer (0.2 M) at various pH to a concentration of 0.5% (W/V) and was incubated in water bath at different temperatures for 5 min, and the hydrolysis was initiated by the addition of the crude enzyme (0.5%, W/V). At different reaction intervals, proper volume of reaction mixture was withdrawn and enzymatic hydrolysis was terminated by boiling the mixture for 10 min. Then the viscosity and the amount of reducing sugar of the mixture were measured. The yield of hydrolysis was defined as the percentage of the weight of released reducing sugar to that of original chitosan in the mixture.

2.5. Determination of chitosanolytic activity

About 1.5 ml of chitosan solution in 0.2 M acetic acid/sodium acetate buffer at a certain pH was pre-incubated in thermostatic water bath for 5 min at a certain temperature, then 0.5 ml of enzyme solutions were added and mixed with the substrate thoroughly. The mixture was incubated at the same temperature for 30 min and then the amount of reducing sugar released during the reaction period was measured according to the 3,5-dinitrosalicylic (DNS) reagent method (Miller, 1959), using GlcN as the standard compound. The enzyme activity was valued by the quantity of the reducing sugar released during the reaction, and one unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute.

2.6. Kinetics study of the enzyme on chitosan

For each chitosan, solutions at concentrations of 0.2–2.0 mg/ml were prepared, at its optimal pH for hydrolysis by lipase. The Michaelis constant (K_m) and the maximal velocity (V_{max}) of the lipase on chitosan hydrolysis were calculated from a double reciprocal plot according to Lineweaver and Burk equation (Lineweaver & Burk, 1934).

2.7. Thin layer chromatography (TLC) analysis of hydrolysis products

The lipase (0.5%, W/V) was incubated with chitosan (0.5%, M/V) in 0.2 M acetic acid-sodium acetate buffer (pH 5.0) at 37 °C for 0–24 h. At appropriate reaction times, portions of the reaction mixture were withdrawn and boiled for 10 min to stop the enzymatic reaction. Absolute ethanol was added to the mixture to a concentration of 70%, and the insoluble high molecular chitosan was discarded by centrifugation. The supernatant was concentrated by a rotary vacuum evaporator and then subjected to TLC on a Silica gel plate and developed in a solvent system composed of *n*-propanol–water–ammonia water (70:30:1, v/v/v) (Liu & Xia, 2006). The TLC plate was stained by spraying 0.1% ninhydrin dissolved in ethanol and the COS on the plate were visualized by keeping the plate in oven at 100 °C for 10 min.

3. Results and discussion

3.1. Characteristics of chitosan

Chitosan samples used in this article are listed in Table 1. Four chitosans were used and DD of them were 64.4%, 73.2%, 82.8% and 90.5%, and for convenience they were named as CHS1, CHS2, CHS3, and CHS4, respectively. Moreover, except CHS1 whose $\overline{M}v$ was 2.75×10^6, the molecular weights of the other three chitosans were all around 5×10^5, which would eliminate the effect of molecular weight to a certain extent in this study.

3.2. Effect of pH and DD on chitosan hydrolysis

The pH affects not only the dissociation behavior of the substrate but also the space structure and dissociation state of the active group in the enzyme, therefore, pH is among the most significant factors affecting the enzyme-catalyzed reaction. On the other hand, DA (degree of acetylation) and distribution of *N*-acetyl groups significantly affected the properties of chitosan solution (Anthonsen, Varum, & Smidsrod, 1993). Fig. 1A shows the effect of pH on the hydrolysis of the chitosans with various DD values by lipase. At pH 4.2, 4.4, 4.8, and 5.0, CHS1, CHS2, CHS3, and CHS4 showed the greatest tendency toward lipase respectively, that is, with the increase of the DD of chitosan, the optimal pH of the lipase increased.

In addition, Fig. 1A also shows that the initial rate of lipase activity on chitosan with DD of 82.8% was much higher than the other three chitosans. In a previous report (Shin-ya, Lee, Hinode, & Kajiuchi, 2001), effects of DA on chitosan hydrolysis by pectinase were investigated, and it was found that DA strongly affected the hydrolytic characteristics of the enzyme. The authors reported that initial hydrolysis rate increased with increase in DA up to 0.3 and the rate did not change in the region of DA over 0.3, which is different from results of the present work.

3.3. Effect of temperature and DD on chitosan hydrolysis

The effects of temperature on an enzyme-catalyzed action are mainly embodied in two respects: one is the increase of the reac-

Table 1
Chitosan samples used in this experiment

Chitosan samples	DD (%)[a]	$[\eta]$ (ml/g)	$\overline{M}v \times 10^{-5}$
CHS1	64.4 ± 0.95	1690	27.5
CHS2	73.2 ± 0.87	291	5.7
CHS3	82.8 ± 1.02	437	5.2
CHS4	90.5 ± 1.24	108	5.0

[a] Mean ± SD, $n = 3$.

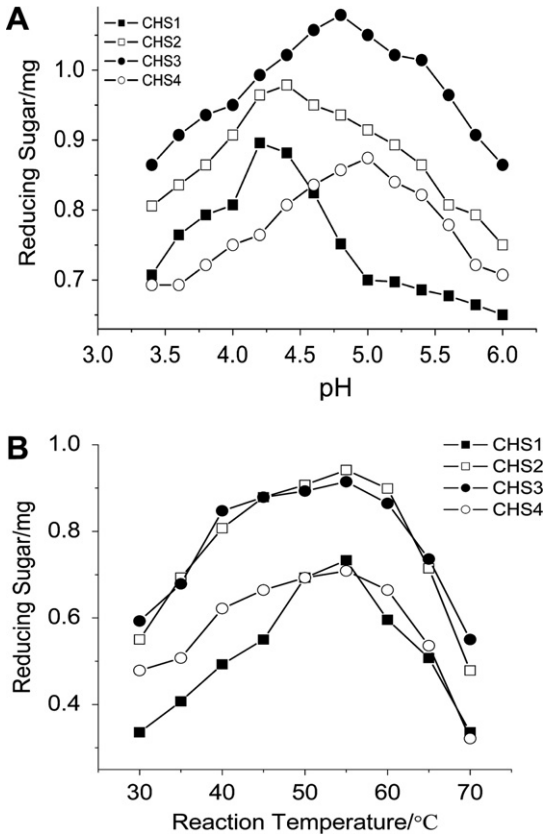

Fig. 1. (A) Effects of pH and DD on the lipase activity on chitosan hydrolysis at 55 °C; (B) Effects of temperature and DD on the lipase activity on chitosan hydrolysis. For CHS1, CHS2, CHS3, and CHS4, experiments were performed at pH 4.2, 4.4, 4.8, and 5.0, respectively.

tion rate because a higher temperature accelerates molecular collisions between the enzyme and the substrate; the other is the inactivation of the enzyme for a higher temperature denatures the enzyme. Effects of temperature on the lipase-aided chitosan hydrolysis are illustrated in Fig. 1B. For the four chitosans, activities were determined at their own optimal pH for hydrolysis by the lipase. Fig. 1B shows that the optimal temperatures of the lipase toward the four chitosans were all around 55 °C. This result suggests that DD had no obvious effect on optimal temperature of the chitosan hydrolysis, which is different from that on pH. In addition, enzyme activity increased gradually with the increase of temperature, while at temperatures higher than the optimal temperature, enzyme activity decreased sharply with the increase of temperature. This result coincides with the typical temperature dependency of an enzyme reaction. On the other hand, at the optimal temperature 55 °C, the enzyme activities on CHS2 and CHS3 were stronger than those on chitosans CHS1 and CHS4.

3.4. Kinetics of the chitosan hydrolysis

K_m, Michaelis–Menten constant, is considered to be a measure of affinity between the enzyme and the substrate, therefore, K_m value is usually used to find the favorite substrate of an enzyme. DD of substrate would lead to a change in the affinity between enzyme and substrate. Therefore, initial hydrolysis rates were measured with varying initial substrate concentrations from 0.2 to 2 mg/ml, using chitosans with different DD as substrates. For each chitosan, experiments were carried out at its optimal hydrolysis temperature and pH by the lipase. The curves drawn according to Lineweave–Burk equation suggest that the lipase action on all chitosans obeyed the Michaelis–Menten kinetics (data not shown). The kinetic parameters, K_m and V_{max}, were calculated according to Lineweave–Burk equation and the results are listed in Table 2. As shown in Table 2, hydrolysis of CHS2 and CHS3 by the lipase resulted in lower K_m values (0.893 and 0.810 mg/ml, respectively) and higher V_{max}/K_m ratios (0.027 and 0.028, respectively). These results indicate that CHS2 and CHS3, compared with CHS1 and CHS4, possessed stronger affinity for the enzyme and faster degradation rate, which justified results above. Moreover, these results also suggest that the presence of GlcNAc residues in the chitosan molecules is important for the enzyme to exhibit chitosanolytic activity and the enzyme recognized not only the GlcN residues, but also the GlcNAc residues in the substrate.

Table 2
Kinetic parameters of chitosan hydrolysis by lipase obtained at 55 °C and at pH 4.2, 4.4, 4.8, and 5.0 for CHS1, CHS2, CHS3, and CHS4, respectively

Chitosan	K_m (mg/ml)	V_{max} (μmol/min)	V_{max}/K_m
CHS1	1.18	0.018	0.015
CHS2	0.893	0.024	0.027
CHS3	0.810	0.023	0.028
CHS4	1.13	0.020	0.018

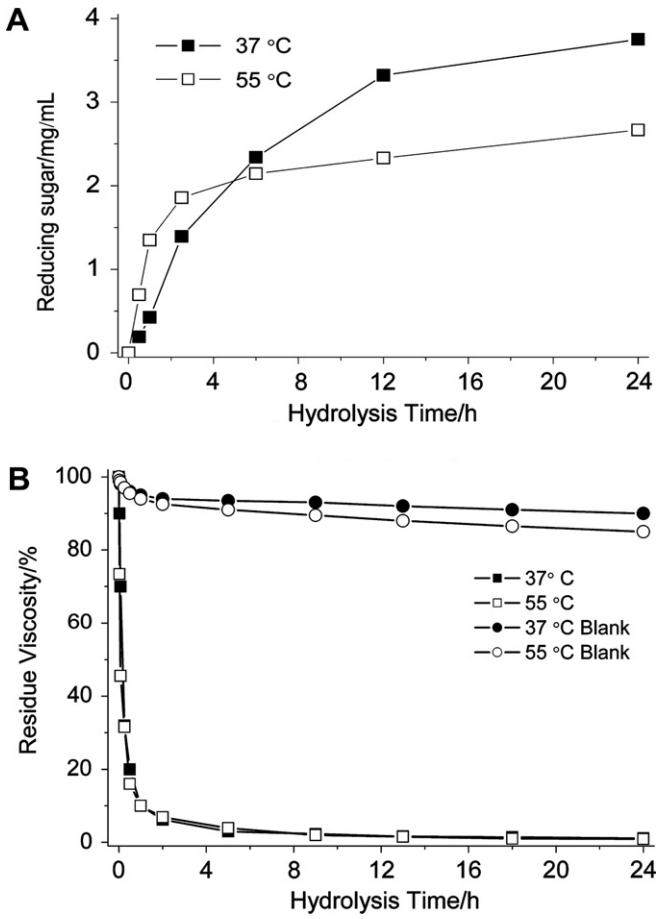

Fig. 2. Time course of chitosan hydrolysis by lipase, CHS3 was used as the substrate and hydrolysis were carried out at pH 4.8. (A) Time course of amounts of reducing sugar produced by chitosan hydrolysis; (B) Time course of viscosity of reaction mixture caused by chitosan hydrolysis, "Blank" denotes chitosan hydrolysis in the same conditions except that the lipase was omitted.

3.5. Time course of chitosan hydrolysis

Time course of hydrolysis of chitosan with the aid of lipase is described in Fig. 2, in terms of changes in viscosity and content of reducing sugar. In this experiment, CHS3 that was 82.8% deacetylated was used for the enzymatic hydrolysis. The reducing sugar produced during hydrolysis was determined according to the DNS reagent method, using glucosamine as a standard substance, and reducing sugar produced in blank samples was subtracted.

As can be seen in Fig. 2A, compared with reaction at 37 °C, hydrolysis at 55 °C produced more reducing sugar during the first several hours of reaction. However, after about 6 h, COS produced at 37 °C exceeded that at 55 °C, and this trend sustained till the reaction reached a relative balance. As for the viscosity decrease of the reaction mixture (Fig. 2B), there was a sharp decrease in viscosity of the mixture during the early hydrolysis stage at both temperatures, however, there was a very weakly marked difference in viscosity.

The yield of COS in the hydrolysis process in terms of reducing sugar is summarized in Table 3. When hydrolysis was carried out at 37 °C, the yield of 24 h hydrolysis was 93.8%, while that at 55 °C was only 67.9%. This result suggests that for the chitosan hydrolysis in large scale, 37 °C is a more suitable temperature than 55 °C, although the latter is the optimal temperature for lipase in chitosan hydrolysis. This is due to that the enzyme was ready to denature at its optimal temperature when reaction time was prolonged.

3.6. TLC analysis of hydrolysis products

The products, mainly chitosan oligomers, produced during the enzymatic hydrolysis were analyzed by TLC (Fig. 3). CHS3 was used as the substrate in this experiment since it was the favorite substrate of the lipase. Fig. 3A shows the products generated during the chitosan hydrolysis at different action times. At the beginning of hydrolysis (0 h), no oligosaccharide was found; then small amount of glucosamine appeared; and then COS with different DP (degree of polymerization, up to six and above) were generated; but with the increase of the reaction time, the amount of oligomers with DP higher than two decreased gradually; and at last, only chitosan monomer, glucosamine, was left. This result suggested that the lipase could hydrolyze chitosan oligomers to monomers.

Enzymatic hydrolysis products of different chitosans with various DD were also analyzed by TLC, and the results are shown in Fig. 3B. The reaction mixtures were all withdrawn for analysis at reaction time 6 h because the longer reaction time would cause the hydrolysis of the oligomers to glucosamine. Fig. 3B shows that the four chitosans hydrolysis produced the same series of products, viz., glucosamine and chitosan oligomers with DP from 2 to 6 and above. This means that chitosans with different degrees of deacetylation did not result in different products.

The result that the lipase hydrolyzed chitosan to an end product of GlcN tells that the enzyme acted on chitosan in an exo-type mode. On the other hand, the result that the viscosity decreased sharply during the hydrolysis process (Fig. 2B) indicates that the enzyme also acted on chitosan in an endosplitting manner. At the early stage of hydrolysis, the lipase cleaved glycosidic bonds in both endo- and exo-mode, and GlcN was first produced, and COS with higher DP were then produced. During this time, viscosity of the mixture decreased dramatically. Then the lipase hydrolyzed the oligomers mainly in an exo-mode, therefore, the oligomers gradually disappeared, the amount of GlcN increased, and the viscosity of the action mixture almost remained the same. This result is useful in the direction of production of COS. If the objective is to obtain oligomers with higher DP, the reaction time should be strictly controlled in order to avoid the production of large quantity of monomers; on the other hand, this lipase can be used to prepare the chitosan monomer, glucosamine, which is widely used to relieve arthritic complaints (Anderson, Nicolosi, & Borzelleca, 2005).

Concerning the mechanism of the unspecific activity of commercial enzymes toward chitosan, there exist two different views. Some researchers believe that the unspecific enzyme could catalyze two different reactions owing to the different active sites in the enzyme molecule or one bifunctional active site (Kittur et al., 2003; Liu & Xia, 2006); while other researchers think that the unspecific activities were due to some chitinases or chitosanases accompanying with the commercial enzymes (Chiang, Chang, Chang, & Sung, 2005; Fu, Wu, Chang, & Sung, 2003; Hung, Chang, Sung, & Chang, 2002). In this paper, although the commercial lipase was effective in producing COS, it is of theoretical importance to make clear the mechanism of the unspecific hydrolysis of chitosan by the commercial lipase in the following work.

Table 3
Yield (%) of chitosan hydrolysis by lipase

Temperature (°C)	Hydrolysis time (h)				
	1	2.5	6	12	24
37	11.3	35.0	58.2	83.7	93.8
55	22.5	46.3	55.1	60.6	67.9

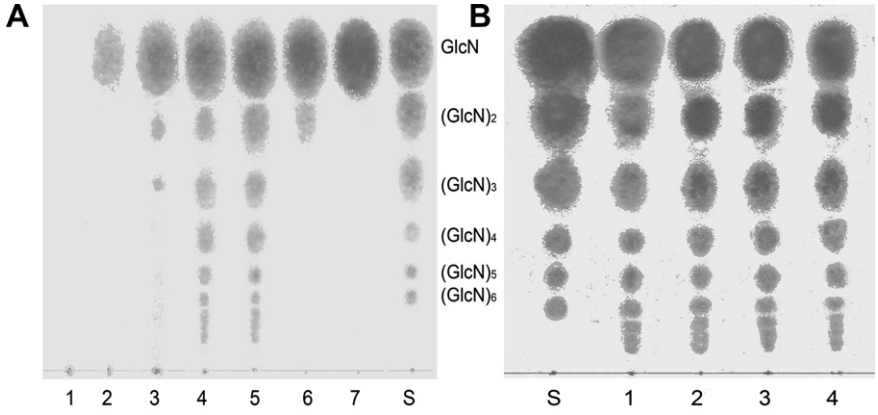

Fig. 3. (A) COS produced during chitosan (CH3) hydrolysis: Lanes 1–7 denote hydrolysis products of chitosan by lipase at 0, 0.5, 1, 2.5, 6, 12, and 24 h, respectively. Lane S denotes COS standard; (B) TLC of hydrolysis products of different chitosans by lipase: Lanes 1–4 denote hydrolysis products of CHS1, CHS2, CHS3, and CHS4 at 6 h, respectively; Lane S denotes COS standard.

4. Conclusions

In conclusion, the commercially available lipase was effective in preparation of COS by hydrolyzing chitosan. The enzyme showed higher activity and stronger affinity to chitosans with moderate DD (73.2% and 82.8%). The optimal temperature was 55 °C and the lipase showed higher optimal pH toward chitosan with higher DD. The yield of chitosan hydrolysis by the lipase at 37 °C for 24 h was 93.8%, in terms of reducing sugar produced. The enzyme acted on chitosan in both endo- and exo-mode, and at different reaction times, different kinds of COS were produced. The lipase may be of potential application in commercial production of COS.

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