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Purification and characterization of exo-β-D-glucosaminidase from commercial lipase

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ABSTRACT

A chitosanolytic enzyme without lipolytic activity was purified to apparent homogeneity from a commercial lipase preparation by using a combination of DEAE-Sepharose CL-6B exchange chromatography, Phenyl-Sepharose CL-4B hydrophobic interaction chromatography, and Sephacryl S-200 gel filtration chromatography, and the purified enzyme was characterized. The molecular mass of the homodimeric protein was about 130 kDa. The optimum action temperature and pH of the enzyme on chitosan were 60 °C and 4.6–4.8, respectively, and the enzyme was stable at temperatures lower than 60 °C and at pH 4–9. The purified enzyme exhibited the highest activity toward chitosan which was 73–82% deacetylated. TLC analysis shows that the purified enzyme released glucosamine residues successively from the substrates and final hydrolysis products of both chitosan tetramer and pentamer were glucosamine, indicating that the enzyme exhibited exo-β-D-glucosaminidase activity.

1. Introduction

Chitosan, a polysaccharide of β-1,4 linked *N*-acetyl-glucosamine and glucosamine, has been found in the cell walls of fungi. Most commercial chitosans are produced from chitin, which is the second most abundant polysaccharide in nature next to cellulose. Recently, chitosan oligosaccharides (COS) attract increasing attention because of their outstanding biological activities, such as antimicrobial activities, antiviral activities, antitumor activities, antioxidant, and radical scavenging activities, fat lowering effects, and immunostimulant effects, etc., and their ready solubility in water and lower viscosity than chitosan facilitate their utilization in functional food and medicine (Kim & Rajapakse, 2005).

The production of COS can rely on chemical methods, physical methods and enzymatic hydrolysis, among which, enzymatic hydrolysis receives more attention for its mild reaction conditions. Chitosanase (EC 3.2.1.132), the specific enzyme for chitosan hydrolysis, has been found in a wide range of organisms, including bacteria (Park et al., 1999; Pelletier & Sygusch, 1990), fungi (Chen, Xia, & Yu, 2005; Zhang et al., 2000), and plants (Ouakfaoui & Asselin, 1992). Most of these chitosanases were characterized as endo-type and they split β-1,4 glycosidic linkages in chitosan in a random way to form chitosan oligosaccharides. Exo-β-D-glucosaminidase (GlcNase) is the exo-type specific enzyme for chitosan hydrolysis. GlcNase releases glucosamine successively from the non-reducing ends of COS and it is needed to hydrolyze chitosan and COS completely to monomers, glucosamines. Recently, GlcNase was found to participate in the chitinolytic pathway in vivo (Tanaka, Fukui, Fujiwara, Atomi, & Imanaka, 2004). Insofar as we are aware, this enzyme has been purified and characterized from *Nocardia orientalis* (Nanjo, Katsumi, & Sakai, 1990), *Trichoderma reesei* (Nogawa et al., 1998), *Amycolatopsis orientalis* (Cote et al., 2006), *Penicillium funiculosum* (Matsumura, Yao, & Toshima, 1999), *Thermococcus kodakaraensis* (Tanaka et al., 2004), and *Aspergillus* spp. (Ji, Yang, & Hur, 2003; Jung, Kuk, Kim, Jung, & Park, 2006; Kim, Shon, & Lee, 1998; Zhang et al., 2000), but reports on the gene structure, physiological role and protein structure of GlcNase are still rather limited.

However, these specific enzymes are not available for bulk quantity due to their high cost of production till now. Nowadays, many non-specific commercial hydrolytic enzymes including pectinases (Kittur, Kumar, Gowda, & Tharanathan, 2003), cellulases (Qin et al., 2004), hemicellulases (Qin et al., 2006), proteases (Kumar & Tharanathan, 2004; Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002), and lipases (Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Sashiwa et al., 2003) have been found to be effective in chitosan degradation. These commercial enzymes have been used in production of COS because of their relatively lower price than chitosanase. However, little is known about the mechanism of non-specific enzymatic process, and even then controversial

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views exist. On the one hand, Kittur et al. (2003) reported that a multiple functional pectinase could be used to hydrolyze chitosan, and Liu and Xia (2006) found a bifunctional enzyme in a commercial cellulase; but on the other hand, some authors have purified chitosanases or/and chitinases from several commercial proteases (Chiang, Chang, Chang, & Sung, 2005; Fu, Wu, Chang, & Sung, 2003; Hung et al., 2002). Several authors investigated the effects of lipases on chitosan and chitin degradation (Muzzarelli et al., 1995; Sashiwa et al., 2003), but lipases these authors used were in crude form an it has not been determined whether these lipases were able to split both carboxyl ester bonds in acylglycerol and β-1,4-glycosidic linkages in chitosan. In a previous study, we found that a commercial lipase preparation from *Aspergillus oryzae* could hydrolyze chitosan effectively (unpublished data). In this present work, we purified a chitosanolytic enzyme free of lipolytic activity from this lipase and this purified enzyme was then characterized as a GlcNase.

2. Materials and methods

2.1. Materials

Chitosans from crab with various degrees of deacetylation (DD) were purchased from Shandong Haidebei Co. Ltd., China. Lipase from *A. oryzae* was purchased from Novozymes China. Low molecular weight calibration kit, DEAE-Sepharose CL-6B, Phenyl-Sepharose CL-4B, and Sephacryl S-200 were all purchased from Amersham (Sweden). $(GlcN)_{2\text{-}6}$ (Chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose) were purchased from Seikagaku Co. (Japan), and D-glucosamine was purchased from sigma. Buffer salts and other chemicals were of the reagent grade.

2.2. Enzyme purification

The crude lipase preparation was dissolved in the appropriate volume of the starting Tris–HCl buffer (20 mM, pH 8.0), then, after the precipitate was removed by refrigerated centrifugation (10,000*g*, 15 min, Beckman, USA), the supernatant was concentrated by ultrafiltration with a cutoff of 10 kDa, while salt was removed simultaneously. The concentrated enzyme solution was then filtrated using a 0.45 μm membrane filter (Shanghai Yadong Co., Ltd., China). The filtrate was first passed through an anion exchange (25 × 200 mm) column packed with DEAE-Sepharose CL-6B and pre-equilibrated with the starting Tris–HCl buffer (20 mM, pH 8.0). After washed thoroughly with the starting buffer, the column was then eluted with a linear gradient formed by two Tris–HCl buffer solutions containing 0 and 0.4 M NaCl at a flow rate of 0.5 mL/min. Five milliliters of eluent was collected for every fraction, and fractions with both lipase activity and chitosanase activity were pooled. Saturated ammonium sulfate solution was added to the collected active fractions to a concentration of 1.5 M. Then the enzyme solution was loaded onto a Phenyl-Sepharose CL-4B column previously equilibrated with the 20 mM Tris–HCl buffer containing 1.0 M ammonium sulfate. After the column was washed with the same buffer, a linear gradient elution with the buffer containing 1.0–0 M of ammonium sulfate was applied, and then the column was eluted with 30% ethanol (V/V). The flow rate was 0.5 mL/min, and fractions of 5 mL each were collected, and the fractions possessing chitosanolytic activity were pooled and concentrated by ultrafiltration. Afterwards, the concentrated enzyme solution was put through a gel filtration column packed with Sephacryl S-200 and pre-equilibrated with Tris–HCl buffer (20 mM, pH 8.0) containing 0.3 M NaCl. The column was eluted with the same buffer at a flow rate of 15 mL/h, and fractions of 2.0 mL each were collected and active fractions were pooled.

2.3. Characterization of purified enzyme

2.3.1. Effect of temperature on enzyme activity and stability

The optimum temperature of the purified enzyme was determined by incubating the enzyme in 1% chitosan at temperatures ranging from 20 °C to 80 °C for 30 min, at pH 5.0. After incubation, the residual activity of the enzyme was immediately measured. For determination of the heat stability, the enzyme was pre-incubated at different temperatures (40–100 °C) without the addition of the substrate and portions of the enzyme were withdrawn at appropriate intervals. The residual activities were measured.

2.3.2. Effect of pH on the activity and stability of the enzyme

The determination of optimum pH of the enzyme activity was performed by incubating the enzyme in 1% of chitosan in 0.2 M acetic acid–sodium acetate buffers (pH 3–6) at 50 °C for 30 min. For the determination of pH stability, the enzyme was kept in buffers at various pH values for 24 h at 40 °C. The pH values of the enzyme solutions were then adjusted to 5.2 and the remaining activities were determined.

2.3.3. Effect of metal ions

The purified enzyme was incubated with 1 mM and 5 mM of various metal ions (Hg^{2+}, Co^{2+}, Sn^{2+}, Pb^{2+}, Ca^{2+}, Zn^{2+}, K^{2+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+}, and except for Pb^{2+} and Cd^{2+} which were sulfated, other ions were all chloridized) for 30 min, and the residual activities were then measured. The relative activity was expressed as the percentage ratio of the activity of the enzyme incubated with metal ions to that of the untreated enzyme.

2.3.4. Michaelis constant and rate constant determination

The Michaelis constant (K_m) and the maximal velocity (V_{max}) of the purified enzyme were determined as the substrate concentrations ranged from 0.2 mg/mL to 5 mg/mL. The K_m and V_{max} of the enzyme were calculated from a double reciprocal plot according to Lineweaver and Burk (1934).

2.4. Analytical methods

2.4.1. Determination of protein

The protein concentration was measured using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard substance. During the purification of the enzyme, the protein concentration in every fraction of eluent from column chromatographies was determined by measuring the absorbance spectrophotometrically at 280 nm.

2.4.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The purified enzyme was resolved by SDS–PAGE using 10% acrylamide to check its purity and to determine its molecular weight according to the method of Laemmli (1970). Rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.3 kDa), bovine carbonic anhydrase (30.1 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa) served as SDS–PAGE standard makers. The protein bands on the gel after electrophoresis were visualized by silver staining.

2.4.3. Determination of chitosanase activity

Chitosanase activity was determined by measuring the increase in reducing sugar produced from chitosan hydrolysis. The incubation mixture in a total volume of 2 mL contained 1.5 mL of 1% chitosan in 0.2 M acetate buffer (pH 5.0) and 0.5 mL of enzyme solution. The incubation was carried out in a thermostatic water bath at 50 °C. After 30 min of reaction, 3.0 mL of alkaline ferricyanide reagent was added, and the reducing sugar produced was measured colorimetrically at 420 nm using the method described

by Imoto and Yagishita (1971). One unit of enzyme was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute at 50 °C. During the purification of the enzyme, the ΔA_{420} was also used to denote the enzyme activity.

2.4.4. Determination of lipase activity

All reactions were carried out at 40 °C for 15 min. Determination of lipase activity was done by the titrimetric method (Dharmsthiti & Luchai, 1999) with slight modification. The assay mixture contained 4 mL of olive oil, 5 mL of 20 mM Tris–HCl (pH 8.0), and 1 mL of the enzyme solution. The reaction was stopped by the addition of 10 mL of 95% ethanol. Fatty acids released were measured by titration with 10 mM NaOH standardized with potassium hydrogen phthalate (KHP). One unit of lipase activity was defined as 1.0 μmol of fatty acid liberated per minute at 40 °C.

2.4.5. Determination of deacetylation degree (DD)

The deacetylation degree of chitosan was determined according to Liu and Xia (2006): chitosan (0.3 g) was dissolved in a known excess of 0.1 M hydrochloric acid (20 mL). From the titration of this solution with a 0.1 M NaOH solution, a curve with two inflexion points was obtained. The difference between the volumes at these two points corresponded to the acid consumed for protonation of amine groups and allowed the determination of DD of the chitosan. The titration was performed with a DELTA-320-S pH meter.

2.4.6. Thin layer chromatography (TLC) of hydrolysis products

The purified enzyme was incubated with chitosan oligomers in 0.2 M acetic acid–sodium acetate buffer (pH 5.0) at 37 °C for 0–48 h. At appropriate reaction intervals, portions of the reaction mixture were withdrawn and boiled for 10 min to stop the enzymatic reaction. The hydrolysis products were 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 oligosaccharides on the TLC plate were stained by spraying 0.1% ninhydrin dissolved in ethanol and visualized by baking the plate in an oven at 100 °C for 10 min.

3. Results and discussion

3.1. Enzyme purification

A hydrolase with chitosanolytic activity but without lipolytic activity was purified from a commercial lipase preparation by using a combination of DEAE-Sepharose CL-6B anion exchange chromatography, hydrophobic interaction chromatography on a Phenyl-Sepharose CL-4B column, and gel filtration on a Sephacryl S-200 column. As shown in Fig. 1, a protein (Peak A) containing only lipase activity, a protein (Peak B) showing both lipase activity and chitosanase activity, and a protein component (Peak C) with neither of the two activities were eluted from the ion exchange column. Then hydrophobic interaction chromatography of the collected protein Peak B from ion exchange chromatography resulted in two major peaks: a protein (Peak II) with only lipolytic activity and a protein (Peak III) with only chitosanolytic activity, as shown in Fig. 2. The former was discarded, and the latter with chitosanase activity was further purified on a Sephacryl S-200 column. Two proteins were eluted from the gel filtration column (Fig. 3) and Peak II exhibiting chitosanolytic activity was collected for the examination of molecular mass and purity.

The purification results are summarized in Table 1. And through these steps, the obtained enzyme was purified 33.8-fold with a recovery of 12.5%.

3.2. Purity and molecular mass of the purified enzyme

After purification by the steps as described above, the active component was examined for its purity and molecular mass by SDS–PAGE. As shown in Fig. 4, a single protein band was seen on the SDS–PAGE gel, indicating that the enzyme had been purified to apparent homogeneity by the chromatography steps. The molecular weight of the enzyme estimated by SDS–PAGE was about 71 kDa according to the standard proteins. However, the molecular mass estimated by gel filtration on the Sephacryl S-200 column was about 130 kDa (Fig. 3). Therefore, the purified enzyme was a homodimeric protein.

According to the literature available at the present time, the molecular weight of GlcNase was much higher than that of chitosanase. The molecular weight of most chitosanases was in the

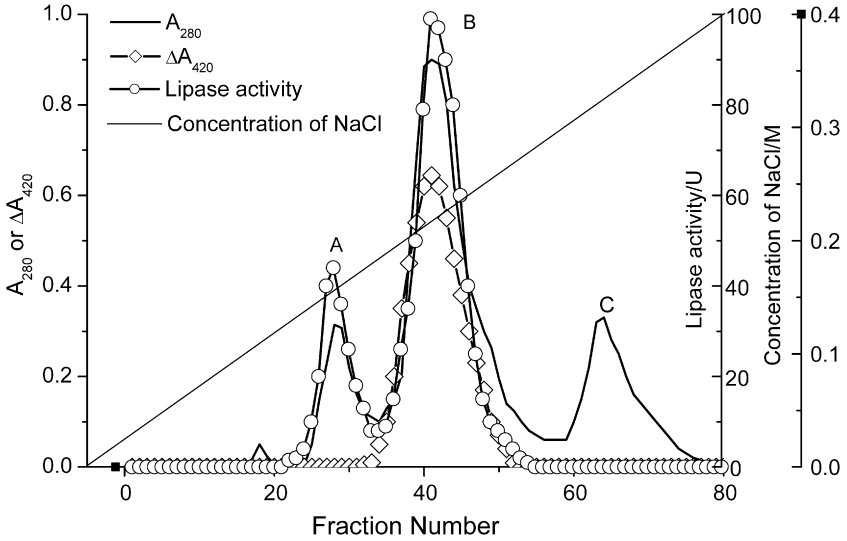

Fig. 1. Column chromatography of the chitosanolytic enzyme on DEAE-Sepharose CL-6B. The column was equilibrated with 20 mM Tris–HCl buffer (pH 8.0). About 20 mL of the concentrated crude enzyme was applied. The column was first washed with the equilibrium buffer and then eluted with 400 mL of a linear gradient of 0–0.4 M Tris–HCl buffer at a flow rate of 60 mL/h. Fractions of 5 mL were collected.

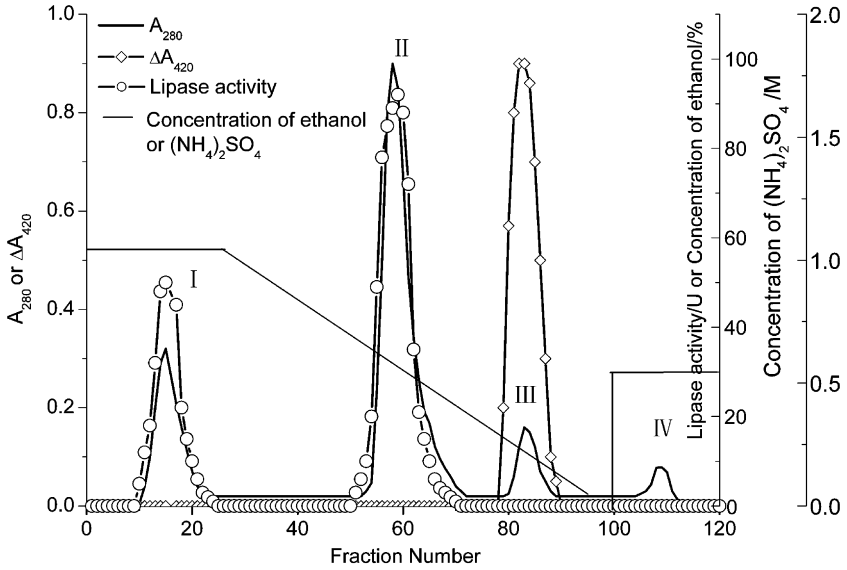

Fig. 2. Hydrophobic chromatography of the enzyme on Phenyl-Sepharose CL-4B. The column was equilibrated with Tris–HCl buffer (pH8.0) containing 1.0 M ammonium sulfate. Chitosanolytic fractions from DEAE-Sepharose CL-6B column were applied. The column was washed with 100 mL of the equilibrium buffer and then eluted with 400 mL of a linear gradient of 1–0 M Tris–HCl buffer at a flow rate of 60 mL/h. Fractions of 5 mL were collected.

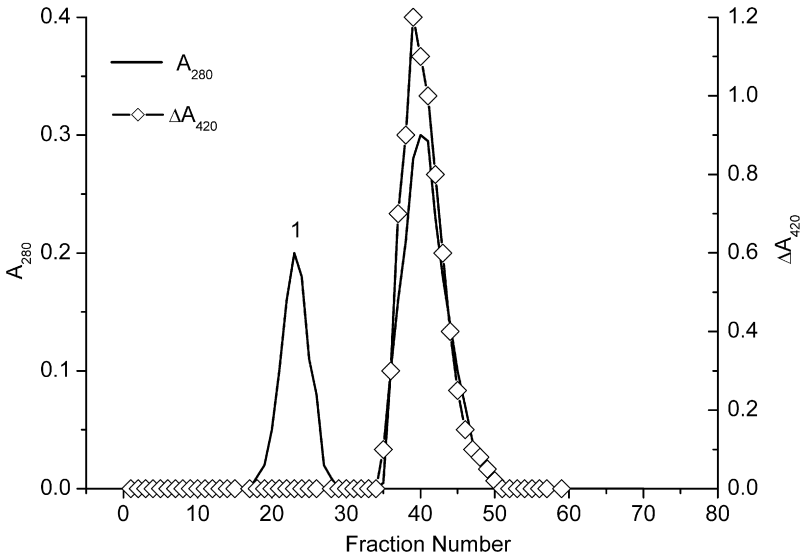

Fig. 3. Gel filtration of the enzyme on Sephacryl S-200. The column was equilibrated with 20 mM Tris–HCl buffer (pH 8.0). Two milliliters of the concentrated enzyme collections from Phenyl-Sepharose CL-4B column was applied. The column was then washed with the equilibrium buffer at a flow rate of 15 mL/h. Fractions of 2.0 mL were collected.

Table 1
Purification of the chitosanolytic protein from lipase

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Crude enzyme solution	48.3	5.8	0.12	100	1.0
Ultrafiltration	30.8	5.5	0.18	94.8	1.5
DEAE-Sepharose CL-6B	7.5	4.8	0.64	82.8	5.3
Phenyl-Sepharose CL-4B	0.72	1.3	1.81	68.8	15.1
Sephacryl S-200	0.18	0.73	4.06	12.5	33.8

range of 10–50 kDa (Somashekar & Joseph, 1996), while the molecular mass of GlcNase ranged from 90 kDa to 135 kDa with the exception of that from *A. flavus* (45 kDa) (Ji et al., 2003). The GlcNase purified in this paper had a molecular weight of 130 kDa, which just fell into the range of 90–135 kDa. Besides, this enzyme is a protein composed of two subunits of the same molecular weight, and this is similar to GlcNase of *T. kodakaraensis*, which is also a homodimeric protein (Tanaka et al., 2004).

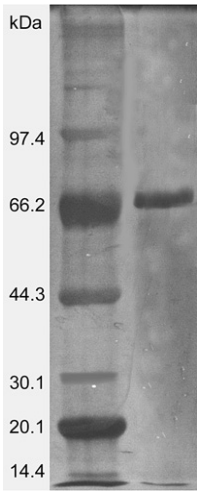

Fig. 4. SDS–PAGE analysis of the purified enzyme. SDS–PAGE was performed in 10% polyacrylamide gel and visualized by silver staining. Lane 1 indicates the purified enzyme; Lane S, standard proteins markers: rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.3 kDa), bovine carbonic anhydrase (30.1 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa).

3.3. Substrate specificity

The activities of the purified GlcNase toward various substrates are listed in Table 2. Although all chitosans with different DD used in the experiment were observed to be degraded by the enzyme, the enzyme demonstrated different levels of activity on different chitosans. As shown in Table 2, the enzyme exhibited higher activity toward chitosans with DD of 73% and 82%, while it showed lower activity toward those with DD of 64% and 90%. In addition, it can be seen from Table 2 that, when chitosans with the same DD were used as the substrates, chitosans with lower molecular mass were more susceptible to be hydrolyzed by the enzyme than those with higher DP (degree of polymerization).

3.4. Effect of temperature on the enzyme

The optimum temperature for activity of the purified enzyme was determined by incubating the enzyme in 1% chitosan at various temperatures ranging from 20 °C to 80 °C for 30 min, at pH 5.0. As shown in Fig. 5A, the purified enzyme showed the highest reducing sugar-producing activity at a temperature of 60 °C. And at temperatures below 60 °C, the enzyme activity increased slowly as the temperature increased, while at temperatures above 60 °C, the enzyme activity decreased sharply with the increase of temperature. The heat stability of the enzyme is illustrated in Fig. 5B. The enzyme did not lose its activity after incubation at 40 °C and 50 °C for 1 h. However, when the enzyme was kept at 60 °C, the enzyme activity began to decline after incubated for 15 min, and after 1 h of incubation, 80% of the activity was inhibited. When incubated at 70 °C, 80 °C, and 100 °C, the enzyme was thoroughly inactivated at 45 min, 15 min, and 5 min, respectively.

Table 2
Substrate specificity of the purified enzyme

Substrate (DD/%)	$M_\eta \times 10^5$	Relative activity (%)
Chitosan (64%)	27.5	78.2
Chitosan (73%)	15.1	89.8
Chitosan (73%)	7.9	91.7
Chitosan (81%)	2.8	99.4
Chitosan (81%)	9.9	83.9
Chitosan (82%)	5.2	100
Chitosan (90%)	3.5	77.1
Chitosan (90%)	5.0	55.9

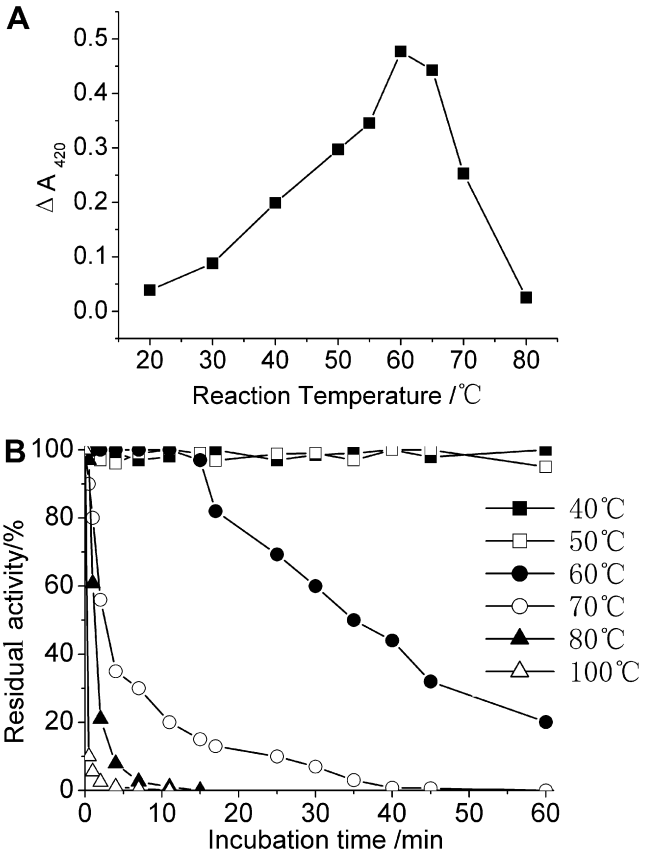

Fig. 5. Effect of temperature on enzyme activity (A) and heat stability of the purified enzyme (B).

3.5. Effect of pH on the purified enzyme

Fig. 6 shows the effect of pH on the chitosanolytic activity of the purified enzyme. At pH 4.6–4.8, the purified enzyme showed the highest activity toward chitosan (DD of 82%) (Fig. 6A). As shown in Fig. 6B, the enzyme did not lose its activity after incubation in buffers at pH 4.5–9.5 for 24 h, at 40 °C, while as pH was below 4 or above 9, an obvious decrease in the activity of purified enzyme was observed.

3.6. Michaelis constant and maximal velocity

The effect of substrate concentration on the hydrolysis rate of chitosan degradation was determined. The Michaelis constant (K_m) and maximum velocity (V_{max}) calculated according to Lineweaver–Burk plots were 3.588 mg/mL and 0.065 U/mL enzyme solution, respectively.

3.7. Effects of metal ions on the enzyme

The effects of metal ions on the enzyme are presented in Table 3. The chitosanolytic activity of the purified enzyme was inhibited significantly by Fe^{3+}, Pb^{2+}, Hg^{2+}, and Sn^{2+}, among which, Hg^{2+} showed the most serious inactivating effect, because it inhibited 78.8% of enzyme activity at a concentration of 5.0 mM. On the other hand, Ni^{2+}, Co^{2+}, and Mn^{2+} activated the enzyme to a large extent; while in the presence of Ca^{2+}, K^+, Mg^{2+}, and Cd^{2+}, no obvious effect on the enzyme activity was observed.

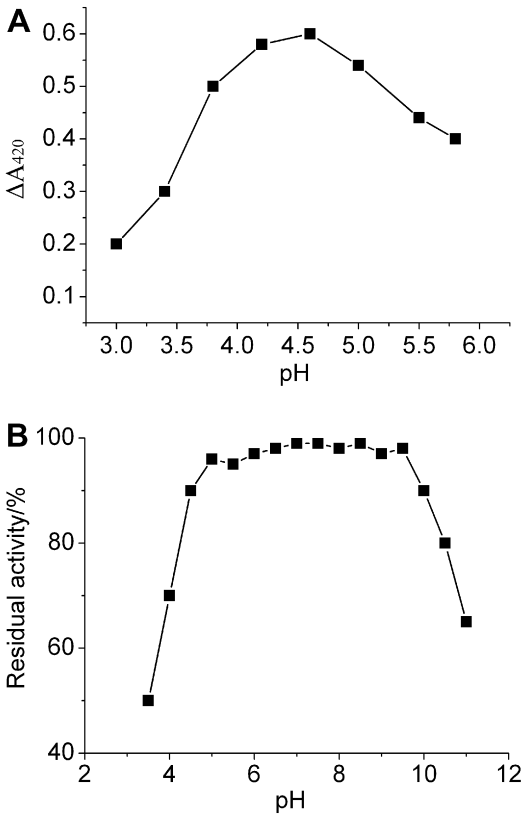

Fig. 6. Effect of pH on enzyme activity (A) and pH stability of the purified enzyme (B).

Table 3
Effects of metal ions on the activity of the purified enzyme

Reagent	Concentration (mM)	Relative activity (%)
None	0	100
$CaCl_2$	5	91.5
KCl	5	84.9
$MgCl_2$	5	102
$FeCl_3$	5	72.6
$ZnCl_2$	10	90.9
$NiCl_2$	10	127.7
$Pb(NO_3)_2$	10	67.31
$CdSO_4$	5	99.8
$CoCl_2$	10	131.2
$HgCl_2$	5	21.2
$SnCl_2$	5	80.1
$MnCl_2$	5	135.0

3.8. TLC analysis of COS hydrolysis products by the purified enzyme

The hydrolytic mode of the purified enzyme on chitosan tetramer $(GlcN)_4$ and chitosan pentamer $(GlcN)_5$ was investigated by the method of TLC. The results turned out that the enzyme could hydrolyze chitosan oligomers with DP ranging from 2 to 6 (data not shown), and the hydrolysis products of $(GlcN)_4$ and $(GlcN)_5$ by the purified enzyme were shown in Fig. 7. As shown in Fig. 7A, at the early stage of hydrolysis of $(GlcN)_4$, small quantities of GlcN and $(GlcN)_3$ were produced; with the increase of the reaction time, $(GlcN)_2$ was also generated; and in the end, the substrate $(GlcN)_4$ and the intermediate products, i.e., $(GlcN)_3$ and $(GlcN)_2$, disappeared, while GlcN was the sole end product.

For the hydrolysis of $(GlcN)_5$, a similar results were observed (Fig. 7B). Firstly, the substrate was hydrolyzed to glucosamine and $(GlcN)_4$, then $(GlcN)_4$ was hydrolyzed to GlcN and $(GlcN)_3$, and then $(GlcN)_3$ was hydrolyzed to GlcN and $(GlcN)_2$, and the final product was also glucosamine.

These results suggested that this purified enzyme released glucosamine successively from the non-reducing ends of chitosan oligomers, and this is just the characteristic action of a GlcNase. In addition, no oligomers with DP higher than that of the substrates were produced during the course of hydrolysis (Fig. 7), indicating that this purified GlcNase possessed no transglycosylase activity. Although several GlcNases with transglycosylase activity had been reported (Cote et al., 2006; Matsumura et al., 1999; Jung et al., 2006; Kim et al., 1998), GlcNases from *N. Orientalis* (Nanjo et al., 1990) and *Tr. Reesei* (Nogawa et al., 1998) did not exhibit this kind of activity.

In this study, we purified a chitosanolytic enzyme free of lipase activity from a commercial lipase preparation, which showed hydrolytic activity toward chitosan in its crude form. This result shows that the chitosanolytic activity of this commercial enzyme was a result of the presence of the chitosanolytic enzyme, because this purified enzyme was the only chitosanolytic component of the crude enzyme and no other chitosanolytic activity was detected during the purification process (Figs. 1–3). Therefore, the lipase, the essential component of the commercial lipase preparation, was not a bifunctional enzyme with both lipolytic activity and chitosanolytic activity.

This result is similar to those of previous studies on several proteases (Chiang et al., 2005; Fu et al., 2003; Hung et al., 2002). The production of commercial enzymes includes the purification process of the enzyme. For enzymes for commercial use, the purification process may be rather rough and proteins and enzymes with similar properties to the objective enzyme may be included in the preparation. Therefore, there is the possibility that the some chitosanolytic enzymes may be present in the crude lipase. According to the previous literature mentioned above and results of this present work, among the commercial enzymes which exhibit both their labeled activity and chitosanolytic activity, we speculate that carbohydrases such as pectinases and cellulases are more likely to be bifunctional enzymes than lipases and proteases, because the structures of the substrates of these carbohydrases are similar to each other. In fact, the genesis, evolution and structural features of multifunctional glucanases have been reviewed by Kumar and Deobagkar (1996). In viewing of these respects, for biological production of COS in the long term, instead of non-specific enzymatic hydrolysis, the authors suggest that the best way may be to increase the yield and activity and reduce cost of chitosanases by high throughout screening and gene modification of chitosanase-producing microorganisms.

The purified chitosanolytic enzyme was then characterized as an exo-β-D-glucosaminidase (GlcNase), which possesses strong chitosanolytic activity toward chitosan and chitosan oligosaccharides. We studied some properties and action mode of this GlcNase on COS, but further investigations on its structure (for example, the determination of its isoelectric point and analysis of its terminal amino acid sequence) remain to be done. Glucosamine, the 2-amino-2-deoxy-D-glucose, is an amino monosaccharide and is an essential component of chitosan. Because of the high concentration of glucosamine in joint tissues, the hypothesis that glucosamine would provide symptomatic relief for osteoarthritis has been developed for more than 30 years, many clinical trials have tested this hypothesis and now glucosamine is widely used to relieve symptoms from osteoarthritis (Anderson, Nicolosi, & Borzelleca, 2005). Glucosamine is produced from chitin by chemical methods at the present time. Owing to its activity of releasing glucosamine from chitosan, GlcNase, along with the use of chitosanase, may have a potential industrial utilization in the production of glucosamine in a safer, gentler, and more environmental friendly way. But

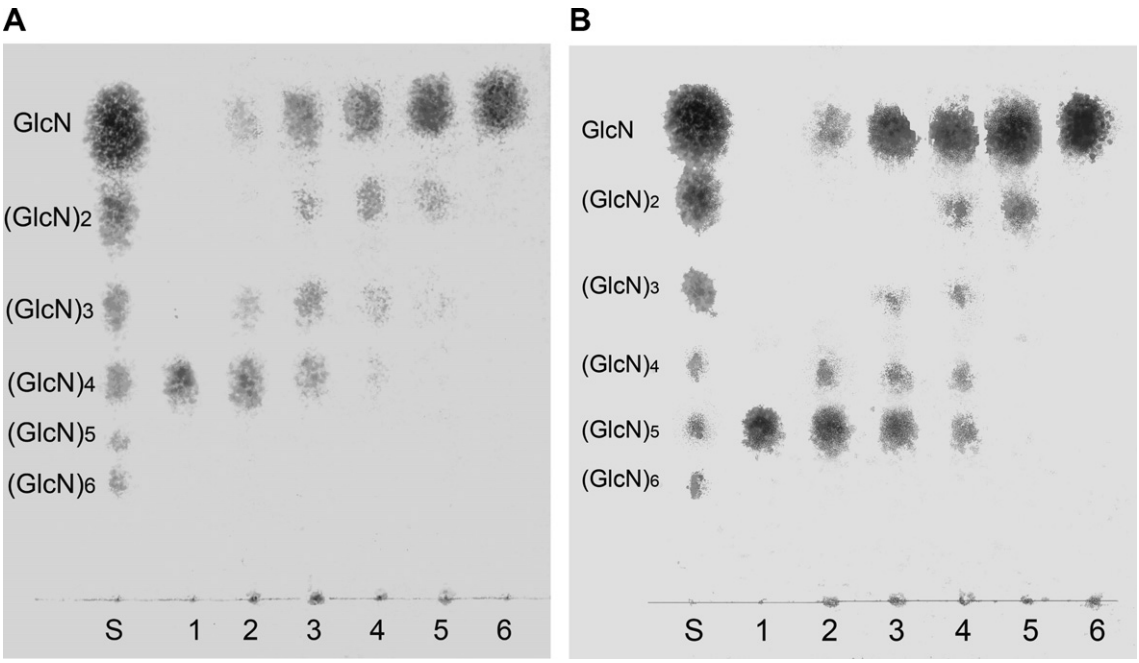

Fig. 7. TLC analysis of hydrolysis products by the purified enzyme. (A) Enzymatic hydrolysis of $(GlcN)_4$, Lane S denotes the standard chitosan oligomers. Lanes 1–6 denote products of $(GlcN)_4$ hydrolysis at 0 h, 0.5 h, 1 h, 3 h, 6 h, and 12 h, respectively. (B) Enzymatic hydrolysis of $(GlcN)_5$, Lane S denotes chitosan oligomers: glucosamine, chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose. Lanes 1–6 denote products of $(GlcN)_5$ hydrolysis at 0 h, 0.5 h, 1 h, 3 h, 6 h, and 12 h, respectively.

more importantly, the physiological role in vivo, gene structure, and evolutionary origin of GlcNase should be further investigated.

In conclusion, a chitosanolytic enzyme was purified to homogeneity from a commercial lipase preparation using a series of chromatographic separations and purifications, and this enzyme was then characterized as an exo-β-D-glucosaminidase which releases GlcN residues successively from its substrates. In addition, various enzymatic properties of the GlcNase were investigated. These results indicate that the chitosanolytic activity the commercial lipase exhibited was fulfilled by GlcNase present in the lipase.

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