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Full Length Research Paper

A chitosanase purified from the snail of *Achatina fulica*

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A chitosanase from the snail of *Achatina fulica* was purified 18.27-fold with 0.68% recovery of protein and 12.53% recovery of enzymatic activity by phenyl-sepharose column chromatography, diethylaminoethanol (DEAE)-sepharose column chromatography and Sephacryl S-300 gel filtration. The molecular mass of chitosanase was 72 kDa determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the isoelectric point (pI) of purified chitosanase was 5.45 estimated by isoeletrofocusing electrophoresis. The K_m measured for the chitosanase was 0.154 μM, with an apparent V_{max} of 0.005 μM/min. The current work for the first time obtains a chitosanase from *A. fulica* might be potentially served as a useful enzyme for hydrolyzing chitosan. It is purified as an important initial material for mass spectrometric sequencing, gene cloning and protein expression of this chitosanase.

Key words: *Achatina fulica*, chitosanase, purification, characterization.

INTRODUCTION

Chitosan, a linear copolymer composed by β-(1→4)-2-acetamido-D-glucosamine (GlcNAc), produced from chitin through the N-deacetylation with varied degree of acetylation, is nontoxic and biodegradable. Composed by GlcNAc, chitosan and chitin have similar molecular structures; the differences are that chitin is 50 to 100% acetylated while chitosan is 0 to 50% acetylated (Yoon et al., 2001; Johnsen et al., 2010; El-Sherbiny, 2011). Chitosan and chitin can be converted into highly water-soluble oligosaccharides by showing anti-tumor, anti-microbial and immunopotentiating biological activities (Zakrzewska et al., 2005; Son et al., 2003; Cheng et al., 2006). They exhibit broad applications in biomedical, pharmaceutical, agricultural and biotechnological fields (Casal et al., 2006; Samdancioglu et al., 2006; Ishihara et al., 2006). The oligosaccharides were mainly produced by chemical methods from polymeric chitosan, however, low yields, high cost and environmental pollution accompany the processes (Gao et al., 2008; Wang et al., 2008). Enzymatic oligosaccharides production has advantages over chemical degradation with less environmental pollution, low cost and high reproducibility. Chitosanase hydrolysis is powerful for oligosaccharide production, which has attracted tremendous attention (Gao et al., 2008; Wang and Yeh, 2008; Wang et al., 2008; Liu and Xia, 2006).

Chitosanase (EC 3.2.1.123), a glycosyl hydrolase, specially hydrolyzes β-(1→4)-glycosidic bond of chitosan to produce oligosaccharides. Chitosanases are divided into endo- and exo- chitosanases, and classified into three classes according to their specificities for hydrolysis of the β-glycosidic linkages. Chitosanases exist in many organisms and have exhibited potential utilization in large-scale production of oligosaccharides (Shimosaka et al., 2000; Fu et al., 2003).

There are a variety of polysaccharide degradation enzymes in snails (El-Rigal Nagy et al., 2011; Halima et al., 2008; Al-Daihan, 2008). Most chitosanases are found in snail. However, no study was reported on chitosanase purification from *Achatina fulica.* In the current work, we purified a novel chitosanase from *A. fulica* that efficiently degrades chitosan with biotechnological and medical

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Abbreviations: DEAE, Diethylaminoethanol; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **pI,** isoelectric point.

application values and characterized its molecular characteristics, which provides an initial material for gene cloning and protein expression of this chitosanase.

MATERIALS AND METHODS

Snail crude enzyme was purchased from Shanghai Sibas Bio-tech Company (China). It is a mixed enzyme preparation from the crop and digestive tract of the snail. Powdered chitosan (90% deacetylated) was from Jinan Haidebei Marine Bioengineering Company (China). DEAE-Sepharose Fast Flow [diethylaminoethanol (DEAE) –Sepharose], Sephacryl S-300, Phenyl Sepharose Fast Flow (low sub) and Bio-gel P-10 were from Amersham Biosciences (USA). p- hydroxybenzoic acid hydrazide H-9882 (PAHBAH) was from Sigma Company (USA). D(+)-glucosamine was from WAKO (Japan). Ampholine for isoelectric focusing (IEF) was from Amersham Biosciences (USA). Bovine serum albumin was from Sino-American Biotechnology Company (China). All other chemicals were of analytical grade from commercial sources.

Purification of chitosanase

Phenyl sepharose column chromatography

One gram crude enzyme was dissolved in 50 ml 20 mM phosphate buffer (pH 7.0) containing 1.5 M ammonium sulfate as the crude enzyme extract solution. 10 ml enzyme solution was loaded onto a Phenyl Sepharose Fast Flow (low sub) column (1.8 × 18.1 cm) equilibrated well with 20 mM phosphate buffer (pH 7.0) containing 1.5 M ammonium sulfate. A decreased linear gradient elution of ammonium sulfate ranged from 1.5 to 2.0 M was then performed in the same buffer. The elution flow rate was controlled at 0.5 ml/min and the fractions of 4 ml/tube were collected. The active fractions were pooled and concentrated by polyethylene glycol (PEG) 20000, then dialyzed against 10 mM Tris-HCl (pH 8.5) at 4°C.

DEAE-Sepharose column chromatography

The dialyzed and concentrated solution was applied to a DEAE-Sepharose column (1.8 × 18.1 cm) already equilibrated with 10 mM Tris- HCl (pH 8.5). Being washed well, the column was eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer at a flow rate of 0.2 ml/min. Fractions of 2.5 ml/tube were collected, the peak showing higher chitosanase activity was pooled and concentrated by PEG 20000.

Sephacryl S-300 chromatography

The resultant enzyme solution was loaded onto a Sephacryl S-300 gel filtration column (1.0 × 57.0 cm) already equilibrated well with 10 mM phosphate buffer (pH 6.9), then the column was eluted with same buffer at a flow rate of 0.5 ml/min and fractions of 2 ml/tube were collected. The active fractions were pooled, concentrated by ultrafiltration, and stored at -20°C.

Protein concentration determination of chitosanase

Protein concentration of chitosanase was determined by the method of Bradford using bovine serum albumin as the standard protein. The column chromatography elution was monitored by the absorbance of the fractions at 280 nm.

Activity measurement of chitosanase

The enzymatic activity of chitosanase was measured by PAHBAH assay using chitosan with 90% degree of deacetylation (Lever et al., 1984). Briefly, the reaction mixtures contain 150 µL of 0.5% soluble chitosan prepared in 1% acetic acid (HAc), 100 µL of enzyme solution, 150 µL of 5% PAHBAH prepared in 0.5 M NaOH and 600 µL of 0.5 M NaOH with a final volume of 1 ml. The mixtures were incubated at 40°C for 4 h with shaking, then, the reaction was stopped by heating at 100°C for 15 min and the mixture was centrifuged at 4000 rpm for 10 min. The enzyme activity of each fraction in the chromatography was measured by the absorbance of the reducing sugars liberated during the hydrolysis of chitosan produced at 405 nm. One enzymatic activity unit (U) is defined as the amount of enzyme required to produce 1 µmol reducing sugar (glucose base) in 120 min under the conditions described earlier. D-glucosamine was used as a standard. Triplicate experiments were performed for each analysis.

Purity and molecular mass determination of chitosanase

The purity and molecular mass of chitosanase was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Asryants et al., 1985). Protein bands were visualized by normal silver staining (use glutaric dialdehyde as sensitizer). The gel was first fixed in 50% CH_3OH, 12% HAc and 38% H_2O for 15 min, then washed with H_2O for 3 x 10 min. Being sensitized in 0.02% sodium thiosulfate (0.04 g $Na_2S_2O_3$, 200 ml H_2O) for 2 min, the gel was washed again with H_2O for 3 x 5 min and stained in 0.2% silver nitrate solution (0.2 g $AgNO_3$, 100 ml H_2O, 0.05% formaldehyde) for 15 min. The gel was then washed with H_2O for 3 x 1 min and developed in 6% (W/V) sodium carbonate solution containing 0.05% (V/V) formaldehyde. The staining was terminated by 5% HAc (V/V). Protein standards for silver staining were from TIANGEN Biotech Company (Beijing, China).

Enzymatic properties

Isoelectric point (pI)

The pI of chitosanase was measured using 5% polyacrylamide slab gel containing 2% carrier ampholyte of pH 5 to 7. Proteins on the gel were stained with silver staining. The pI was estimated by comparing the migration distance of protein on the gel.

Kinetic parameters

The V_{max} and K_m of purified chitinase were determined using chitosan (90% deacetylated) as substrate and calculated from a Lineweaver-Burk plot. Enzyme activity was measured at 40°C in 0.5 M NaOH by measuring the release of reducing sugars as described earlier.

Hydrolysis products

The hydrolysis products of GlcNAc by chitosanase were loaded onto on a Bio-Gel P-10 column (1 × 54.5 cm) being equilibrated with 0.5% HAc and eluted with the same buffer, the amount of reducing sugar was measured by PAHBAH assay as described earlier.

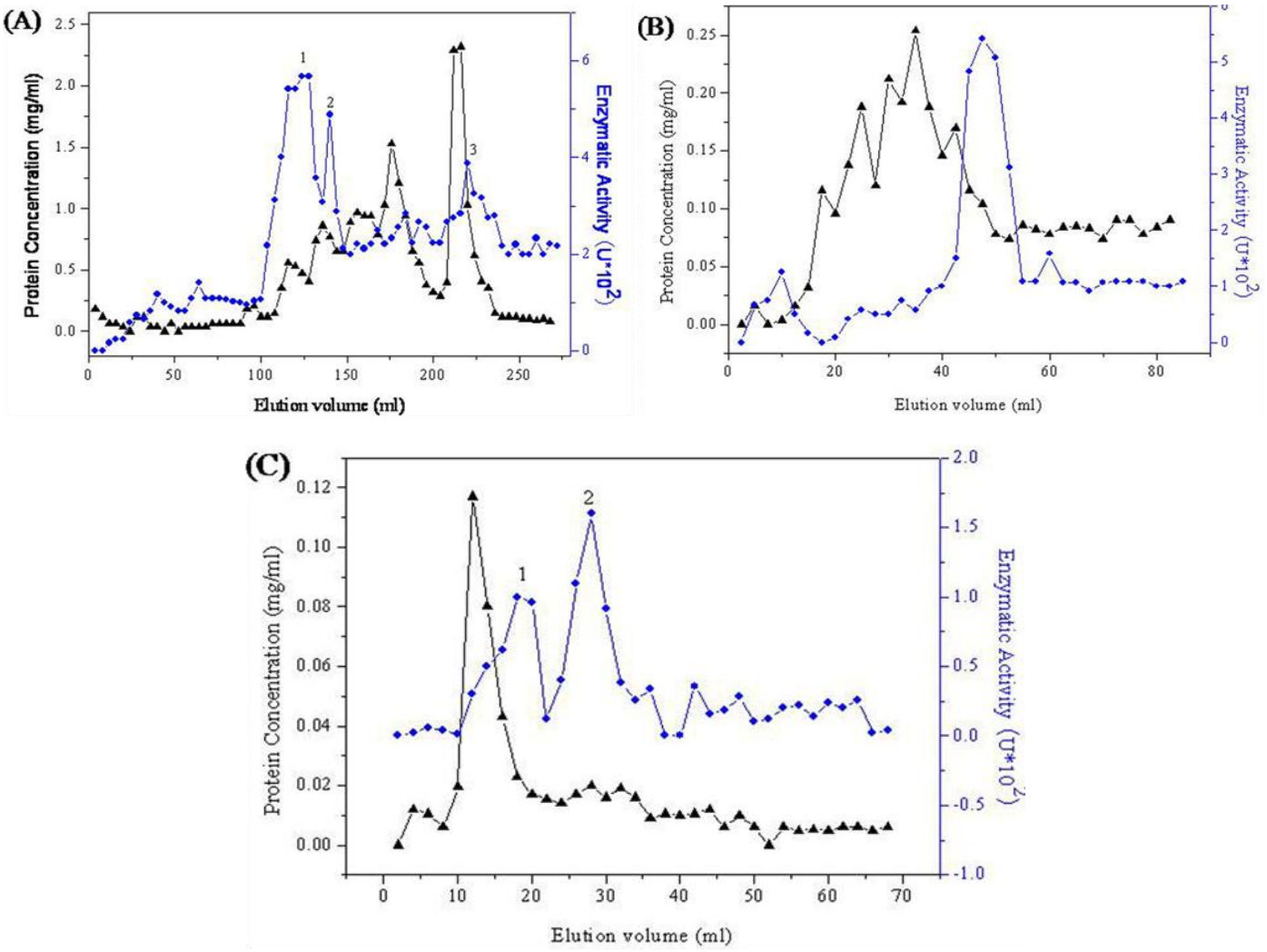

Figure 1. Purification process of chitosanase *A. fulica*. (A) Phenyl Sepharose column chromatography (1.8 × 18.1 cm). The column was equilibrated with 20 mM phosphate buffer (1.5 M ammonium sulfate, pH 7.0) and eluted employing a linear gradient (1.5 to 2.0 M) of ammonium sulfate. Flow rate was 0.5 ml/min and fractions of 4 ml/tube were collected. (B) DEAE-Sepharose column purification (1.8 × 18.1 cm). Column was eluted with a linear gradient of 0 to 0.5 M NaCl at a flow rate of 0.2 ml/min and fractions of 2.5 ml/tube were collected. (C) Sephacryl S-300 chromatography (1.0 × 57.0 cm). The column was equilibrated with 1 mM phosphate buffer (pH 6.9) and eluted with the same buffer at a flow rate of 0.5 ml/min and fractions of 2 ml/tube were collected. –▲–, Protein elution; –●–, chitosanase activity distribution.

RESULTS AND DISCUSSION

Enzyme purification

All isolation processes were carried out at 4°C. *A. fulica* chitosanase was purified to homogeneity by Phenyl-Sepharose column chromatography, DEAE-Sepharose column chromatography and Sephacryl S-300 gel filtration.

As shown in Figure 1A, three protein peaks 1, 2 and 3 with enzymatic activity were obtained from Phenyl-Sepharose column chromatography purification. The active fractions in peak 1 were collected and dialyzed against 10 mM Tris-HCl (pH 8.5), concentrated to 1 ml and purified using DEAE-Sepharose column chromatography. The enzyme active fractions (Figure 1B) were collected and separated using Sephacryl S-300 gel filtration (Figure 1C). The active fractions of peak 2 were collected, pooled and concentrated for further characterizations.

The purification results are shown in Table 1. *A. fulica* chitosanase with chitosan hydrolytic specific activity was purified 18.27-fold with 0.68% recovery of protein and 12.53% recovery of enzymatic activity.

Enzyme purity, molecular mass and isoelectric point

The purified *A. fulica* chitosanase showed a single band with molecular mass anout 72 kDa (Figure 2). It showed

Table 1. Summary of the purification of chitosanase from snail *A. fulica*.

Purification step	Total protein (mg)	Protein recovery (%)	Specific activity (U/mg)*	Activity recovery (%)	Purification (fold)
crude enzyme	26.29	100.0	1.82	100.0	1.00
Phenyl-Sepharose	14.72	55.99	5.78	177.8	3.17
DEAE-Sepharose	0.204	0.77	77.94	33.22	42.72
Sephacryl S-300	0.18	0.68	33.33	12.53	18.27

*One enzymatic activity unit (U) is defined as the amount of enzyme required to produce 1 μmol of reducing sugar per 2 h.

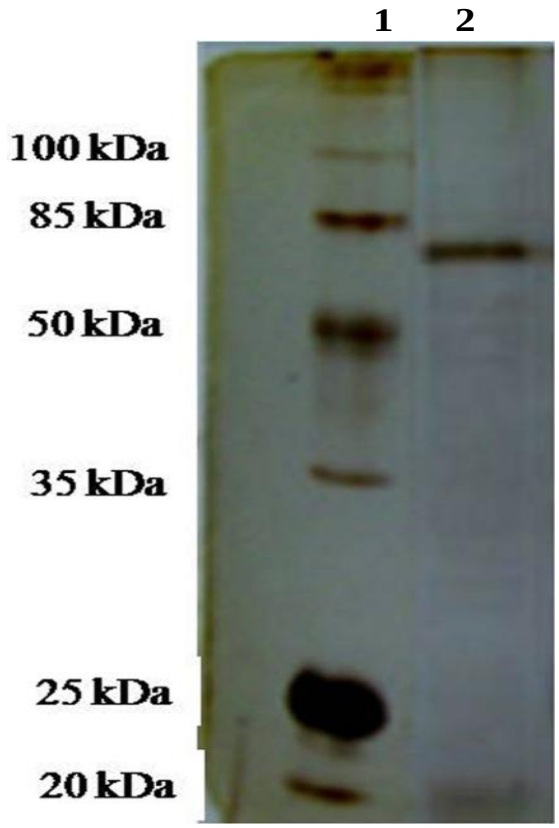

Figure 2. Sliver staining of chitosanase. SDS-PAGE was performed by using 4% stack gel and 12% separation gel. 1, standard proteins used were β-galactosidase (100 kDa), bovine serum albumin (85 kDa), ovalbumin (50 kDa), carbonic anhydrase (35 kDa), β-lactoglobulin (25 kDa) and lysozyme (20 kDa); 2, band of chitosanase.

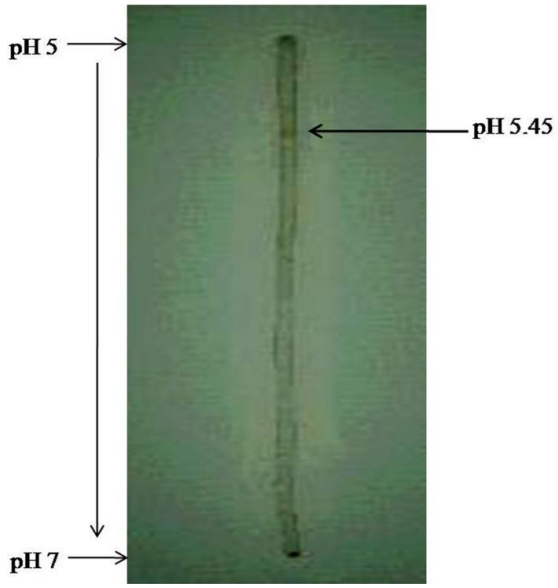

Figure 3. The isoelectric point (pI) of chitosanase. It was measured using isoelectric focusing on 5% polyacrylamide gel slab containing 2% carrier ampholyte of pH 5 to 7.

a pI of 5.45 estimated by isoeletrofocusing electrophoresis (Figure 3).

The enzymes including cellulase, pepsin, lipase A and chitosanase can hydrolyze chitosan into chito-oligosaccharides (Milala et al., 2009; Ghalehkandi et al., 2011). Chitosanase is probably the most efficient one due to its specific activity and the controllable hydrolysates. Accumulated studies showed that the chitosanases differ in their pIs, substrate specificities, requirements for N-acetylglucosamine residues and residues adjacent to the cleavage site on the chitosan chain. No study has been reported on the purification and characterization of snail chitosanase. *A. fulica* chitosanase was the first homogenous chitosanase enzyme reported so far. The molecular masses of chitosanases from *Janthinobacterium, Streptomyces cyaneogriseus, Streptomyces* N174, *Bacillus cereus* D-11, *Trichoderma viride, Burkholderia gladioli* CHB101, *Microbacterium* sp. OU01, *Bacillus subtilis* TKU007, *Serratia marcescens* TKU011 and *Bacillus* sp. CK4 cells were ranged from 20 to 86 kDa (Johnsen et al., 2010; El-sherbiny, 2011; Boucher et al., 1992; Fu et al., 2003; Gao et al., 2008; Liu and Xia, 2006; Shimosaka et al., 2000; Jung et al., 2006; Sun et al., 2006; Wang and Yeh, 2008; Wang et al., 2008; Yoon et al., 2001; Liu and Bao, 2009). A. *fulica* chitosanase shows a relative high molecular mass among the chitosanases reported so far. No much study

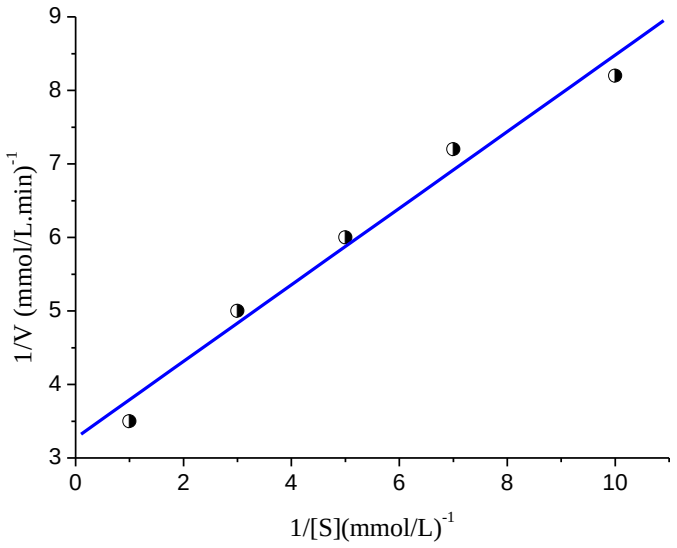

Figure 4. Kinetic parameter determination from Lineweaver–Burk plot. Enzyme activities were measured at 40°C by incubating chitosan with chitosanase in 0.5 M NaOH.

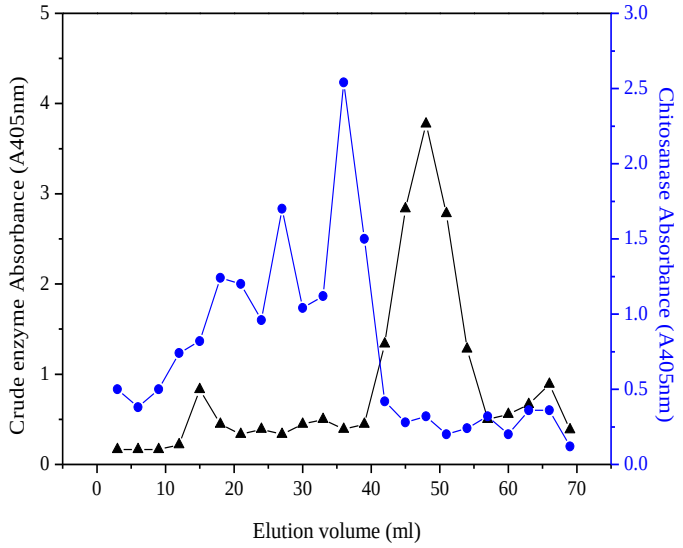

Figure 5. Hydrolysis products analysis of chitosanase. The hydrolysis products of GlcNAc by snail crude enzyme (–▲–) and purified chitosanase (–●–) were analyzed by Bio-Gel P-10 column (1 × 54.5 cm) being equilibrated with 0.5% acetic acid and eluted with the same buffer.

has been made on pIs determinations of chitosanases. The pIs of three chitosanases from crude porcine pepsin, PSC-I, PSC-II and PSC-III, were 4.9, 4.6, and 4.4, respectively (Fu et al., 2003). Our data indicates that *A. fulica* chitosanase showed an acidic pI of 5.45. The relative optimal pHs for previous reported chitosanases were pH 5 to 7 for *Janthinobacterium chitosanase* (Johnsen et al., 2010), pH 3 to 8 for *S. cyaneogriseus* chitosanase (El-sherbiny, 2011), pH 4 to 6 for *Streptomyces* N174 chitosanase (Boucher et al., 1992), pH 5.0, 5.5 and 4.4 for PSC-I, PSC-II, and PSC-III, respectively (Fu et al., 2003), pH 6.0 for *B. cereus* D-11 chitosanase (Gao et al., 2008), pH 5.2 for *T. viride* chitosanase (Liu and Xia, 2006), pH 6.2 and 6.6 for two *Microbacterium* sp. OU01 chitosanases (Sun et al., 2006), pH 4 to 9 for *B. subtilis* TKU007 chitosanase and pH 4 to 8 for *S. marcescens* TKU011 chitosanase (Wang and Yeh, 2008; Wang et al., 2008) and pH 6.5 for *Bacillus sp.* CK4 chitosanase (Yoon et al., 2001). The enzymatic activity of *A. fulica* chitosanase was performed in 0.5 M NaOH, it should be pretty stable in relative basic aqueous solutions and might be used more widely.

Enzyme kinetic parameters

The purified enzyme was incubated with various concentrations of chitosan (90% deacetylated) at 40°C for 4 h and a Lineweaver-Burk plot was constructed (Figure 4), the K_m and V_{max} were calculated from the linear plot. The enzyme showed a K_m of 1.54 mmol/L and a V_{max} of 0.3 mmol/L·min for chitosan hydrolysis. The Kms for chitosan hydrolysis of PSC-I, PSC-II and PSC-III were 5.2 mg/ml (0.13 mmol/L), 4.0 mg/ml (0.1 mmol/L) and 5.6 mg/ml (1.4 mmol/L), respectively (Fu et al., 2003), 7.5 mg/ml (0.18 mmol/L) for *B. cereus* D-11 chitosanase (Gao et al., 2008), and 10 mg/ml (0.15 mmol/L) for *T. viride* chitosanase (Liu and Xia, 2006). Comparing to the afore-mentioned chitosanases, *A. fulica* chitosanase showed a relative higher K_m.

Hydrolysis products produced by purified enzyme

Figure 5 shows the hydrolytic products of chitosan cleaved by *A. fulica* chitosanase. Two main peaks were observed in the chromatography between the inner and void volume for the chitosan product hydrolyzed by crude snail enzyme. While, three peaks were detected for the chitosan hydrolyzed by *A. fulica* chitosanase (Figure 5). As the void and the volume of Bio- gel P-10 were 15 and 45 ml, the purified chitosanase most probably catalyzes the endo-cleavage of chitosan. Most chitosanases cleave the bond between two GlcNAc residues in chitin polymers and hydrolyze partially acetylated chitosan by cleaving the bond between GlcNAc and GlcN residues (Shimosaka et al., 2000). As for other chitosanases, the main products detected were dimmer and trimer of D-glucosamine with traces of free D-glucosamine or higher oligomers. The tetramer should be the shortest oligomer still recognized as a substrate by the chitosanase (Boucher et al., 1992).

Conclusion

A. fulica chitosanase has a molecular mass of 72 kDa

and a pI of 5.45. It shows specific hydrolytic activity against chitosan by producing oligosaccharides. The apparent K_m and V_{max} for chitosanase toward chitosan in 0.5 M NaOH at 40°C are 1.54 mmol/L and 0.3 mmol/L.min, respectively. The current work indicates *A. fulica* might be suitable for large scale chitosanase production, and the purification and molecular characteristic study of *A. fulica* chitosanase might provide certain contribution in the research field of chitosanases. It is purified as an important potential initial material basis for mass spectrometric sequencing and chemical gene synthesis. It may make it possible to clone and express this valuable chitosanase.

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