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# Recombinant expression of a chitosanase and its application in chitosan oligosaccharide production

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ABSTRACT

Recently, considerable attention has been focused on chitosan oligosaccharides (COSs) due to their various biological activities. COSs can be prepared by enzymatic degradation of chitosan, which is the deacetylation product of chitin, one of the most abundant biopolymers in nature. In the current study, we recombinantly expressed a chitosanase and used it for COS preparation. A bacillus-derived GH8 family chitosanase with a 6×His tag fused at its N-terminal was expressed in the *Escherichia coli* strain BL21(DE3) as a soluble and active form. Its expression level could be as high as 500 mg/L. Enzymatic activity could reach approximately 140,000 U/L under our assay conditions. The recombinant chitosanase could be purified essentially to homogeneity by immobilized metal-ion affinity chromatography. The enzyme could efficiently convert chitosan into monomer-free COS: 1 g of enzyme could hydrolyze about 100 kg of chitosan. Our present work has provided a cheap chitosanase for large-scale COS production in industry.



Chitin is one of the most abundant biopolymers in nature. It is mainly composed of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) units that are linked together by β-(1→4)-glycosidic bonds.[1–3] Chitin can be extracted from the shells of crabs and shrimps in industry. However, its insolubility in common solvents limits its utilization. After treated by alkaline solution, chitin is converted to chitosan due to partial deacetylation. Chitosan can be used in food, pharmaceutical, textile, waste water treatment, and other industries.[4–6] Chitosan can also be degraded into chitosan oligosaccharides (COSs) by chemical or enzymatic methods. Enzymatic COS production has advantages over chemical degradation, such as high COS yield and less environmental pollution.[7–13] COSs are highly water soluble and have various biological activities,[14–27] such as inhibiting growth of bacteria and fungi, exerting anti-tumor activity, acting as immunopotentiating effectors, elucidating pathogenesis-related proteins in higher plants, and as specific inhibitors of family 18 chitinase.

Chitosanase (EC 3.2.1.123) can hydrolyze the β-(1→4)-glycosidic bonds of chitosan,[28–36] and therefore can be used for COS preparation. In previous work, we isolated a Gram-positive Bacillus strain that can secrete a chitosanase that belongs to the GH8 family, but its expression level is quite low.[37] To improve its expression level, the chitosanase was recombinantly expressed in *Escherichia coli* cells in our current work. Using a two-step approach, we amplified and cloned the chitosanase gene into an expression vector pET (Fig. 1). First, the DNA fragment encoding the mature peptide of the chitosanase was amplified from the Bacillus genomic DNA and subsequently cloned into a T-vector. Second, the chitosanase gene was subcloned from the T-vector into the expression vector. The expression construct was designated as pET/chitosanase. A 6×His tag that would facilitate enzyme purification was fused at the N-terminal of the recombinant chitosanase. Compared with our previously published sequence,[37] two single nucleotide substitutions were found. One occurred at position 472: an A to G replacement changed its encoding amino acid from Thr to Ala (the 158th residue in the mature peptide). The other occurred at position 867: a C to T replacement that did not change its encoding amino acid (Gly289 in the mature peptide). The recombinant enzyme is still active as shown below.

The chitosanase was first expressed in the *E. coli* strain BL21(DE3) in small scale. After optimizing the culture conditions, the expression level of the chitosanase could reach approximately 500 mg/L as analyzed by SDS–PAGE and quantified by densitometry (Fig. 2A). After the *E. coli* cells were lysed by sonication, all of the chitosanase was present in the supernatant as shown in Figure 2B, suggesting that the recombinant enzyme is soluble. Enzymatic activity assay showed the expression level of the recombinant chitosanase could reach approximately 140,000 U/L under our assay conditions. The chitosanase was purified by immobilized

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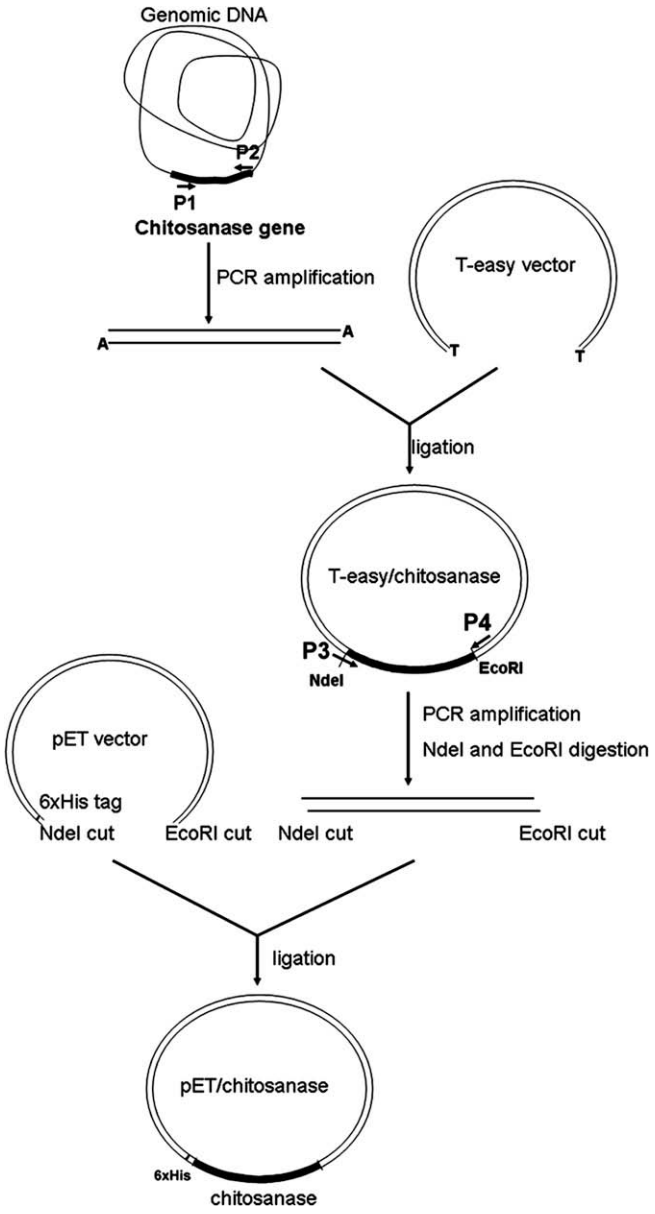


**Figure 1.** The procedure used to construct the chitosanase expression vector.

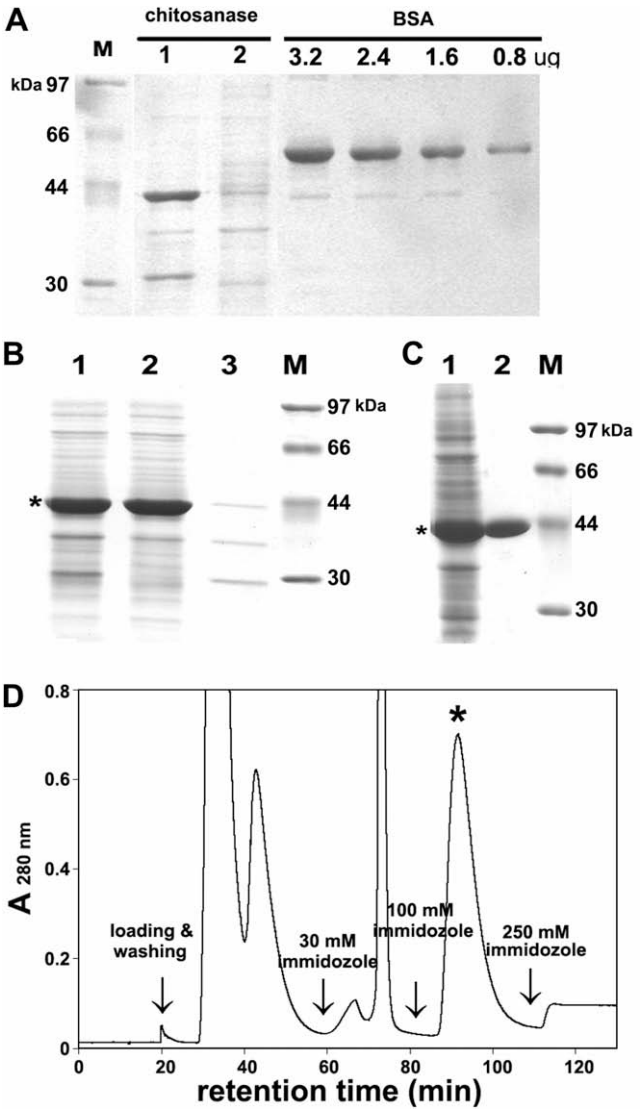


**Figure 2.** (A) Quantifying the chitosanase expression level by SDS–PAGE and by densitometry. The culture broth (containing *E. coli* cells) before IPTG induction (labeled as 2) and after IPTG induction (labeled as 1) was mixed with equal volume of SDS–PAGE loading buffer. After boiling, 8 μL of the mixture (equal to 4 μL of culture broth) was loaded onto a 10% SDS-gel. Different amounts of bovine serum albumin (BSA) were loaded as control. The gel was stained by Coomaissie Brilliant Blue R250 after electrophoresis, and chitosanase bands and BSA bands were quantified by densitometry using software Scion image. (B) SDS–PAGE analysis after sonication. The *E. coli* cells expressing the chitosanase were lysed by sonication. The total cell lysate (lane 1), the supernatant (lane 2), and the pellet (lane 3) were loaded onto a 10% SDS-gel, respectively. The amount loaded onto each lane was equal to 7 μL of the culture broth. (C) SDS–PAGE analysis after immobilized metal-ion affinity chromatography. Lane 1, before purification; lane 2, after purification. The chitosanase band is indicated by a star. (D) Purification of the chitosanase by immobilized metal-ion affinity chromatography. The supernatant of total cell lysate (from 50 mL culture broth) was loaded onto a $Ni^{2+}$ column (1 cm × 4 cm) and eluted by lysate buffer (20 phosphate buffer, pH 7.5, 0.5 M NaCl), 30 mM, 100 mM, and 250 mM imidazole solution (in lysate buffer), respectively. The flow rate was 2 mL/min. The chitosanase peak is indicated by a star.

metal-ion affinity chromatography (Fig. 2D). Activity analysis showed the peak eluted by 100 mM imidazole had chitosanase activity, while other peaks had no detectable activity. Activity assay also showed the chitosanase recovery could be over 95%. SDS–PAGE analysis showed that the purified chitosanase was homogeneous (Fig. 2C). The specific activity of the recombinant chitosanase was approximately 270–290 U/mg (or 270–290 μmol/mg enzyme/min) under our assay conditions. In large-scale culture (5 L), the chitosanse expression level is similar to that of the small-scale culture.

We analyzed the thermal stability and the temperature–velocity relationship of the recombinant chitosanase. At higher temperatures, the enzyme showed a moderately higher hydrolysis velocity (Fig. 3A) but with a much shorter half-life (Fig. 3B): its half-life was only several minutes at 60 °C. At lower temperatures, the enzyme was quite stable (its half-life was extended to several hours at 50 and 40 °C; no detectable activity loss at room temperature for several days) and was still quite active. So, the COS preparation was carried out at 50 °C or at 40 °C.

The chitosan solubility in the activity assay buffer (0.1 M sodium acetate, pH 4.5) was not high enough for COS preparation. So, 5% aqueous acetic acid was used as solvent to dissolve chitosan. Chitosan powder was first mixed with water containing the appropriate amount of chitosanase, then pure acetic acid was added to a final concentration of 5%. For small-scale COS preparation, 10 g of chitosan powder (in 100 mL 5% aqueous acetic acid) was hydrolyzed at 50 °C. As shown in Table 1, long-chain chitosan (average

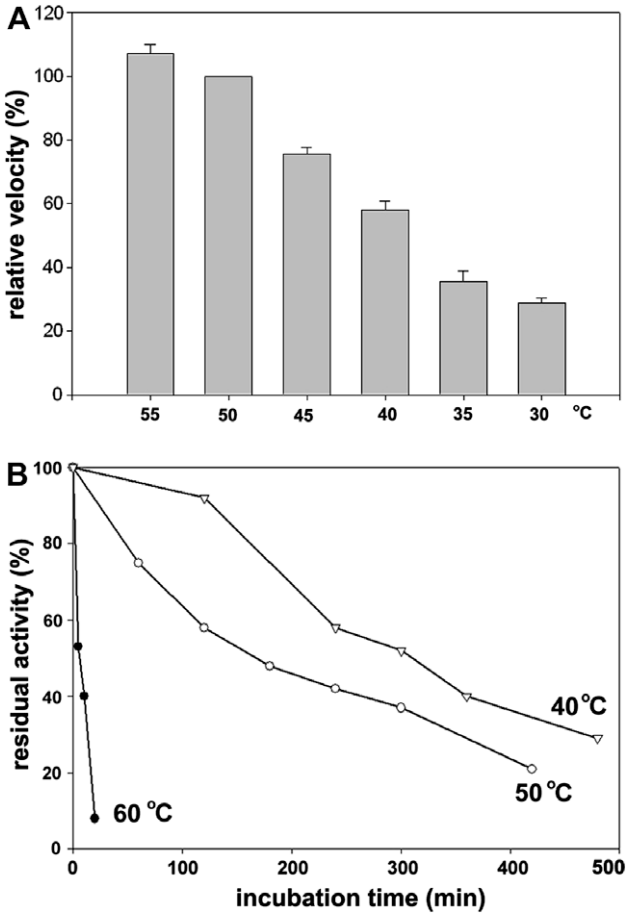


**Figure 3.** Enzymatic properties of the recombinant chitosanase. (A) Effect of temperature on the reaction velocity. The activity assay was carried out at different temperatures using 0.5 g/L chitosan (dissolved in 0.1 M sodium acetate, pH 4.5) as substrate. (B) Thermal stability of the recombinant chitosanase. The recombinant chitosanase was pre-incubated in 0.1 M sodium acetate (pH 4.5) for different times at a certain temperature, then the remaining activity of the pre-incubated chitosanase was assayed at 50 °C using 0.5 g/L of chitosan (dissolved in 0.1 M sodium acetate, pH 4.5) as substrate.

chain length is about 400 residues) was gradually converted to short-chain COS: after 6 h hydrolysis, the average chain length was shortened to 5.1 residues, a suitable value for a COS product. The COS composition was also analyzed by thin-layer chromatography (TLC, Fig. 4A): the COS mixture contains chitobiose, chitotriose, chitotetraose, chitopentaose, chitohexaose, and other larger components, but without glucosamine (monomer). Mass spectrometry analysis confirmed the TLC result: chitosan oligos from GlcN2 to GlcN9 were detected using mass spectrometry. For large-scale COS preparation, 30 kg of chitosan powder (in 100 L of 5% aqueous acetic acid) was hydrolyzed at 40 °C. As shown in Figure 4C, TLC analysis showed that the COS product (the average chain length was 5.5) was obtained after 15 h hydrolysis at 40 °C. Lower hydrolysis temperature is better for the appearance of the COS product: the product hydrolyzed at 50 °C is yellow, while the product hydrolyzed at 40 °C is almost white.

**Table 1**
Monitoring enzymatic chitosan hydrolysis by average chain length measurement. Chitosan powder (10 g) was mixed with 95 mL of water containing 30 U of recombinant chitosanase, then 5 mL of pure acetic acid was added and well mixed

| Hydrolysis time (h) | 2 | 3 | 4 | 6 | 7 | 10 | 24 |
|---|---|---|---|---|---|---|---|
| Average chain length | 13.3 | 9.2 | 7.3 | 5.1 | 4.4 | 3.8 | 3.1 |

The enzymatic hydrolysis was carried out at 50 °C. At different reaction times, aliquots of the reaction mixture were removed, and the amount of reducing sugars was measured. The average chain length (*N*) was calculated as follows: *N* = amount of total sugar/amount of reducing sugar. The average chain length of chitosan is about 400 residues.

Chitin and chitosan are abundant biomaterials, COS production is a promising direction for chitin/chitosan utilization in that COS has various biological activities and can be widely used in food, pharmaceutics, and agriculture. The enzymatic COS production needs a cheap enzyme source. In our present work, the expression level of the recombinant chitosanase could reach 500 mg/L. The enzymatic activity could reach approximately 140,000 U/L under our assay conditions. When assayed at 50 °C using 10 g/L chitosan (dissolved in 0.1 M sodium acetate, pH 5.0) as substrate that has been used by Choi et al.,[33] the enzymatic activity could reach approximately 240,000 U/L. To our knowledge, our expression level is the highest so far. The host *E. coli* cells grow quickly in cheap medium: the whole culture process is completed within one day. Furthermore, purification of the recombinant chitosanase is quite simple: the enzyme could be purified to homogeneity in one step with high recovery. So the recombinant chitosanase could be obtained in large quantity cheaply and quickly. The recombinant enzyme worked efficiently: 1 g of enzyme could hydrolyze about 100 kg of chitosan into monomer-free COS in several hours. So our present work provided a cheap chitosanase source for large-scale COS production.

In the Gram-positive Bacillus strain, the chitosanase is secreted into the medium due to a signal peptide at its N-terminus.[37] For recombinant expression, the signal peptide was removed and the mature enzyme was expressed in the cytosol of *E. coli* cells. In general, *E. coli* cells cannot secrete heterologous proteins with high efficiency. So expression in the cytosol is a suitable approach for high-level expression. The mature chitosanase has no disulfide bonds, so it is suitable for expression in the cytosol where a reductive redox potential does not favor formation of disulfide bonds.

## 1. Materials and methods

### 1.1. Materials

D-glucosamine hydrochloride, chitosan (deacetylation over 90%), and $K_3Fe(CN)_6$ were from Sinopharm Chemical Reagent Co. Ltd. Silica gel thin-layer chromatography (TLC) plates were from E. Merck. The pGEM T-easy vector was purchased from Promega. The pET vector was purchased from Stratagene and modified in our laboratory. The COS standard was a generous gift from professor Yu-Guang Du (Dalian Institute of Chemical Physics, Chinese Academy of Sciences). The PCR primers were chemically synthesized at Invitrogen. Their nucleotide sequences were listed as follows: P1, 5′-GCT GCT GCA AAG GAA ATG AAA-3′; P2, 5′-TTA ATT ATC GTA TCC TTC ATA AAT T-3′; P3, 5′-AAAAAAA CAT ATG GCT GCT GCA AAG GAA ATG AAA-3′; P4, 5′-GGC CGC GAA TTC ACT AGT G-3′.

### 1.2. Construction of the chitosanase expression vector

The Gram-positive Bacillus strain that secretes a chitosanase was previously isolated in our laboratory.[37] The genomic DNA of the Bacillus strain was prepared according to standard procedure. Subsequently, the DNA fragment encoding the mature chitosanase was amplified by PCR using the genomic DNA as template and using P1 and P2 as primers. The amplified DNA fragment was ligated into a pGEM T-easy vector and sequenced. Thereafter, the chitosanase gene was PCR amplified from the T-vector using P3 and P4 as primers. The amplified DNA fragment was digested by

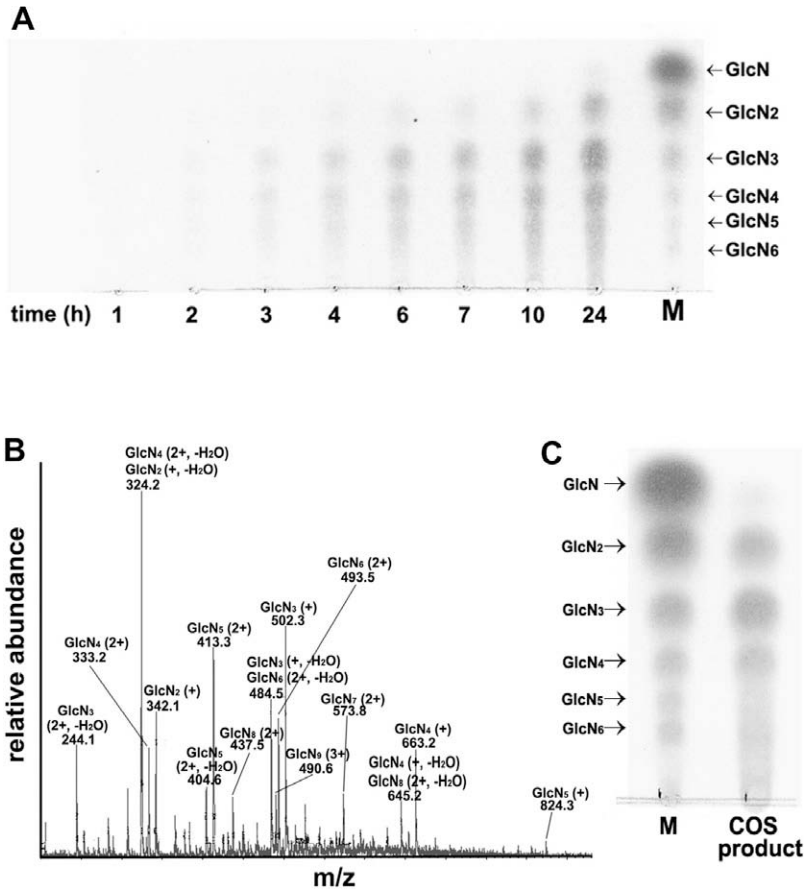


**Figure 4.** (A) Monitoring enzymatic chitosan hydrolysis by TLC. Chitosan (10 g) was mixed with 95 mL of water containing 30 U of recombinant chitosanase, then 5 mL of pure acetic acid was added and mixed well. The enzymatic hydrolysis was carried out at 50 °C. At different reaction times, aliquots of the reaction mixture were removed and immediately stored at −20 °C. Subsequently, the samples were spotted onto a silica gel TLC plate (50–100 μg COS per spot), and the plate was developed with the solvent of *n*-propanol and 30% ammonia (2:1, vol/vol). The developed plate was heated on a hotplate until the ammonia smell was gone, then it was sprayed by 0.1% ninhydrin solution (dissolved in *n*-butanol–satd water) and heated on the hotplate until red sugar spots were visualized. (B) Analyzing the enzymatically prepared COS by electrospray ionization mass spectrometry. The hydrolysis product in A at 6 h was analyzed by the electrospray mass spectrometry. (C) TLC of COS powder prepared on large scale. Chitosan powder (30 kg) was mixed with 95 L of water containing 120,000 U of recombinant chitosanase, then 5 L of pure acetic acid was added and mixed well. The enzymatic hydrolysis was carried out at 40 °C, and the hydrolysis was monitored by average chain length measurement. After 15 h hydrolysis (average chain length reached 5.5), the hydrolysis mixture was dried through spray drying and COS powder was obtained.

restriction enzymes NdeI and EcoRI, and subsequently ligated into a pET vector pretreated with the same enzymes. The sequence of the chitosanase in the pET vector was confirmed by DNA sequencing.

### 1.3. Expression and purification of the recombinant chitosanase

The pET/chitosanase construct was transformed into the *E. coli* strain BL21(DE3). For small-scale culture, the transformed single colony was first cultured in 3 mL of liquid LB medium plus 100 mg/L of ampicillin at 37 °C overnight with vigorous shaking (250 rpm). Next morning the overnight culture broth (3 mL) was transferred into 100 mL of fresh liquid LB medium plus 100 mg/L of ampicillin and continuously cultured at 37 °C for 5–6 h with vigorous shaking (250 rpm). Then 10 mL of the concentrated medium was added, and cells were continuously cultured for 3–4 h. Afterwards, the isopropyl β-D-thiogalactoside stock solution (IPTG, 1 mM at final concentration), 10 mL concentrated medium, and the ampicillin stock solution (50 mg/L at final concentration) were added, and the cells were continuously cultured at 37 °C for about 15 h with gentle shaking (150 rpm). For large-scale culture, the culture volume was increased to 5 L (500 mL in each 2 L flask) according to above conditions. The *E. coli* cells were collected by centrifugation (5000*g*, 5 min), re-suspended in lysate buffer (20 mM phosphate, pH 7.5, 0.5 M NaCl), and lysed by sonication. After centrifugation (8000*g*, 10 min), the supernatant was loaded onto an immobilized metal-ion affinity column ($Ni^{2+}$ column), and eluted by stepwise increase in imidazole concentration in the lysate buffer. One liter of concentrated medium contains 100 g tryptone, 50 g yeast extract, and 5 g NaCl.

### 1.4. Chitosanase activity assay

The chitosanase activity was measured as the production of reducing sugars according to the method of Kazuyoshi.[38] The substrate chitosan was dissolved in 0.1 M sodium acetate buffer (pH 4.5) at a final concentration of 0.5 g/L. During the activity assay, an appropriate amount of chitosanase (0.005–0.01 U in 5–10 μL) was added into 0.6 mL of pre-warmed substrate solution. The reaction was carried out at 50 °C for 15 min, then 0.8 mL of $K_3Fe(CN)_3$ solution (dissolved in 0.5 M $Na_2CO_3$ at the final concentration of 0.5 g/L) was added, mixed, and immediately incubated in boiling water for 5 min. After centrifugation (10,000*g*, 2 min), the absorbance of the supernatant at 420 nm was measured. Based on the glucosamine standard curve, the amount of reducing sugars liberated by the chitosanase could be calculated. One enzymatic unit (U) was defined as the amount of chitosanase

needed to liberate 1 μmol of reducing sugars in 1 min under our assay conditions.

### 1.5. Effect of temperature on reaction velocity of the recombinant chitosanase

An appropriate amount (0.005–0.01 U in 5–10 μL) of chitosanase was added into 0.6 mL of chitosan substrate solution (0.5 g/L, dissolved in 0.1 M NaOAc, pH 4.5) that had been pre-warmed at a certain temperature. The reaction was carried out at that temperature for 15 min, then 0.8 mL of $K_3Fe(CN)_3$ solution (dissolved in 0.5 M $Na_2CO_3$ at a final concentration of 0.5 g/L) was added, mixed, and immediately incubated in boiling water for 5 min. After centrifugation (10,000*g*, 2 min), the 420 nm absorbance of the supernatant was measured.

### 1.6. Thermal stability of the recombinant chitosanase

The recombinant chitosanase was pre-incubated in 0.1 M sodium acetate buffer (pH 4.5) for different times at a certain temperature. Subsequently an appropriate amount of the pre-incubated enzyme was added into 0.6 mL of chitosan solution (pre-warmed at 50 °C). The activity assay was carried out at 50 °C for 15 min.

### 1.7. COS preparation using the recombinant chitosanase

For small-scale COS preparation, 10 g of chitosan powder was mixed with 95 mL of water that is pre-mixed with the recombinant chitosanase (3 U/g chitosan). Then 5 mL of pure HOAc was added and mixed well. The mixture was incubated at 50 °C and gradually converted to viscous liquid due to chitosan hydrolysis. At different reaction times, aliquots of the reaction mixture were removed, and the amount of reducing sugars was measured according to the method of Kazuyoshi.[38] The average COS chain length (*N*) was calculated as follows: *N* = amount of total sugars/amount of reducing sugars. The hydrolysis mixture was also analyzed by silica gel TLC and by electrospray ionization mass spectrometry. For large-scale COS preparation, 30 kg of chitosan powder was mixed with 95 L of water that was pre-mixed with the recombinant chitosanase (4000 U/kg chitosan). Then 5 L of pure acetic acid was added and mixed well. The mixture was incubated at 40 °C. At different reaction times, aliquots of the reaction mixture were removed, and the amount of reducing sugars was measured according to the method of Kazuyoshi.[38] After optimal average chain length was reached, the temperature of the hydrolysis mixture was lowered to 10 °C, and the hydrolysis mixture was converted to COS powder through spray drying. The composition of the COS powder was analyzed by silica gel TLC.

## References


1. Roberts, G. A. F. *Chitin Chemistry*; Macmillan Press: Basingstoke, United Kingdom, 1992.
2. Tharanathan, R. N.; Kittur, F. S. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 61–87.
3. Austin, P. R.; Brine, C. J.; Castle, J. E.; Zikakis, J. P. *Science* **1981**, *212*, 749–753.
4. Domard, A.; Roberts, G. A. F.; Vårum, K. M. *Advances in Chitin Science*; Jacques André Publisher: Lyon, France, 1997.
5. Knorr, D. *Food Technol.* **1991**, *45*, 114–122.
6. Muzzarelli, R. A. A. *Carbohydr. Polym.* **1996**, *29*, 309–316.
7. Fukamizo, T. *Curr. Protein Pept. Sci.* **2000**, *1*, 105–124.
8. Kumar, B. A.; Varadaraj, M. C.; Tharanathan, R. N. *Biomacromolecules* **2007**, *8*, 566–572.
9. Zhang, H.; Du, Y.; Yu, X.; Mitsutomi, M.; Aiba, S. *Carbohydr. Res.* **1999**, *320*, 257–260.
10. Roncal, T.; Oviedo, A.; López de Armentia, I.; Fernández, L.; Villarán, M. C. *Carbohydr. Res.* **2007**, *342*, 2750–2756.
11. Vishu Kumar, A. B.; Varadaraj, M. C.; Gowda, L. R.; Tharanathan, R. N. *Biochem. J.* **2005**, *391*, 167–175.
12. Sørbotten, A.; Horn, S. J.; Eijsink, V. G.; Vårum, K. M. *FEBS J.* **2005**, *272*, 538–549.
13. Kuroiwa, T.; Ichikawa, S.; Sato, S.; Mukataka, S. *Biotechnol. Bioeng.* **2003**, *84*, 121–127.
14. Palma-Guerrero, J.; Jansson, H. B.; Salinas, J.; Lopez-Llorca, L. V. *J. Appl. Microbiol.* **2008**, *104*, 541–553.
15. Cho, E. J.; Rahman, M. A.; Kim, S. W.; Baek, Y. M.; Hwang, H. J.; Oh, J. Y.; Hwang, H. S.; Lee, S. H.; Yun, J. W. *J. Microbiol. Biotechnol.* **2008**, *18*, 80–87.
16. Rahman, A.; Kumar, S. G.; Kim, S. W.; Hwang, H. J.; Baek, Y. M.; Lee, S. H.; Hwang, H. S.; Shon, Y. H.; Nam, K. S.; Yun, J. W. *Proteomics* **2008**, *8*, 569–581.
17. Nam, K. S.; Kim, M. K.; Shon, Y. H. *J. Microbiol. Biotechnol.* **2007**, *17*, 1546–1549.
18. Yoon, H. J.; Moon, M. E.; Park, H. S.; Im, S. Y.; Kim, Y. H. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 954–959.
19. Moon, J. S.; Kim, H. K.; Koo, H. C.; Joo, Y. S.; Nam, H. M.; Park, Y. H.; Kang, M. I. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 989–998.
20. Huang, R.; Mendis, E.; Rajapakse, N.; Kim, S. K. *Life Sci.* **2006**, *78*, 2399–2408.
21. Lee, H. W.; Park, Y. S.; Choi, J. W.; Yi, S. Y.; Shin, W. S. *Biol. Pharm. Bull.* **2003**, *26*, 1100–1103.
22. Jeon, Y. J.; Park, P. J.; Kim, S. K. *Carbohydr. Polym.* **2001**, *44*, 1–76.
23. Hadwiger, L.; Ogawa, T.; Kuyama, H. *Mol. Plant Microbe Interact.* **1994**, *7*, 531–533.
24. Tokora, A.; Gatewaki, N.; Suzuki, K.; Mikami, T.; Suzuki, S.; Suzuki, M. *Chem. Pharm. Bull.* **1988**, *36*, 784–790.
25. Kauss, H.; Jeblick, W.; Domard, A. *Planta* **1989**, *178*, 385–392.
26. Walker-Simmons, M.; Hadwiger, L.; Ryan, C. A. *Biochem. Biophys. Res. Commum.* **1983**, *110*, 194–199.
27. Cederkvist, F. H.; Parmer, M. P.; Vårum, K. M.; Eijsink, V. G. H.; Sørlie, M. *Carbohydr. Polym.* **2008**, *74*, 41–49.
28. Lee, H. S.; Jang, J. S.; Choi, S. K.; Lee, D. W.; Kim, E. J.; Jung, H. C.; Pan, J. G. *FEMS Microbiol. Lett.* **2007**, *277*, 133–141.
29. Zhou, W.; Yuan, H.; Wang, J.; Yao, J. *Lett. Appl. Microbiol.* **2008**, *46*, 49–54.
30. Su, C.; Wang, D.; Yao, L.; Yu, Z. *Food Chem.* **2006**, *54*, 4208–4214.
31. Lee, Y. S.; Yoo, J. S.; Chung, S. Y.; Lee, Y. C.; Cho, Y. S.; Choi, Y. L. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 113–121.
32. Cheng, C. Y.; Chang, C. H.; Wu, Y. J.; Li, Y. K. *J. Biol. Chem.* **2006**, *281*, 3137–3144.
33. Choi, Y. J.; Kim, E. J.; Piao, Z.; Yun, Y. C.; Shin, Y. C. *Appl. Environ. Microbiol.* **2004**, *70*, 4522–4531.
34. Kim, P. I.; Kang, T. H.; Chung, K. J.; Kim, I. S.; Chung, K. C. *FEMS Microbiol. Lett.* **2004**, *240*, 31–39.
35. Adachi, W.; Sakihama, Y.; Shimizu, S.; Sunami, T.; Fukazawa, T.; Suzuki, M.; Yatsunami, R.; Nakamura, S.; Takénaka, A. *J. Mol. Biol.* **2004**, *343*, 785–795.
36. Fukamizo, T.; Brzezinski, R. *Cell Biol.* **1997**, *75*, 687–696.
37. Xu, R.; Guo, Z. Y.; Chi, C. W. *Pharm. Biotechnol.* **2008**, *15*, 94–99 (in Chinese).
38. Kazuyoshi, Y. *Agric. Biol. Chem.* **1971**, *35*, 1154–1156.