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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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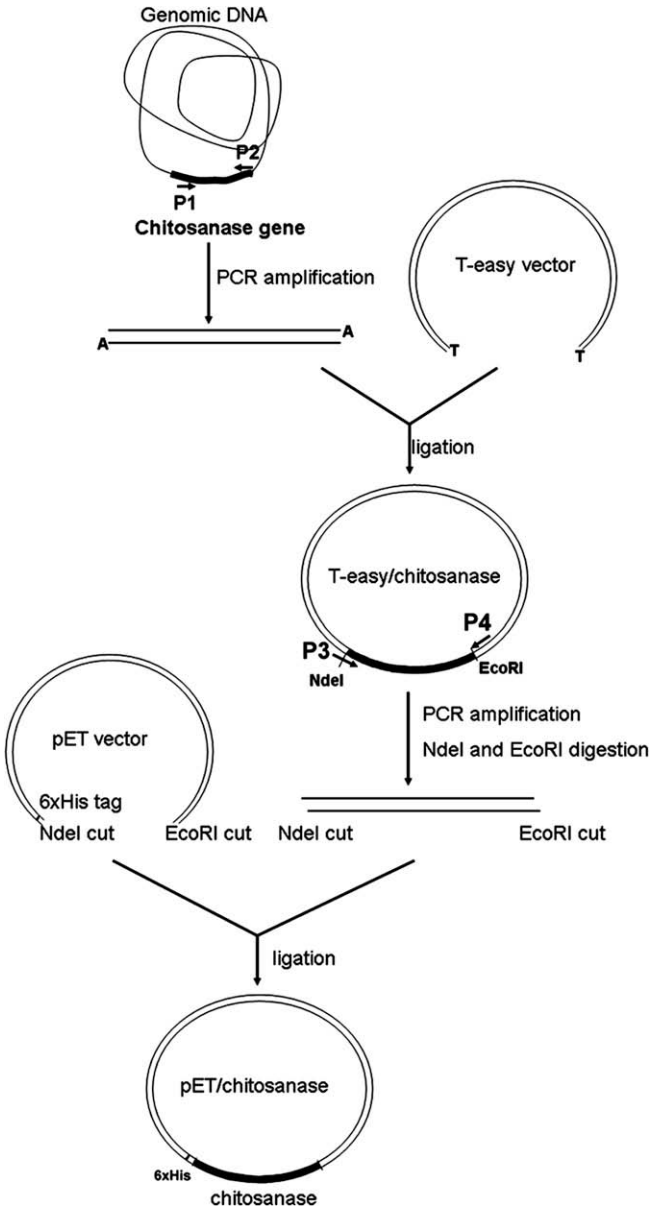

Figure 1. The procedure used to construct the chitosanase expression vector.

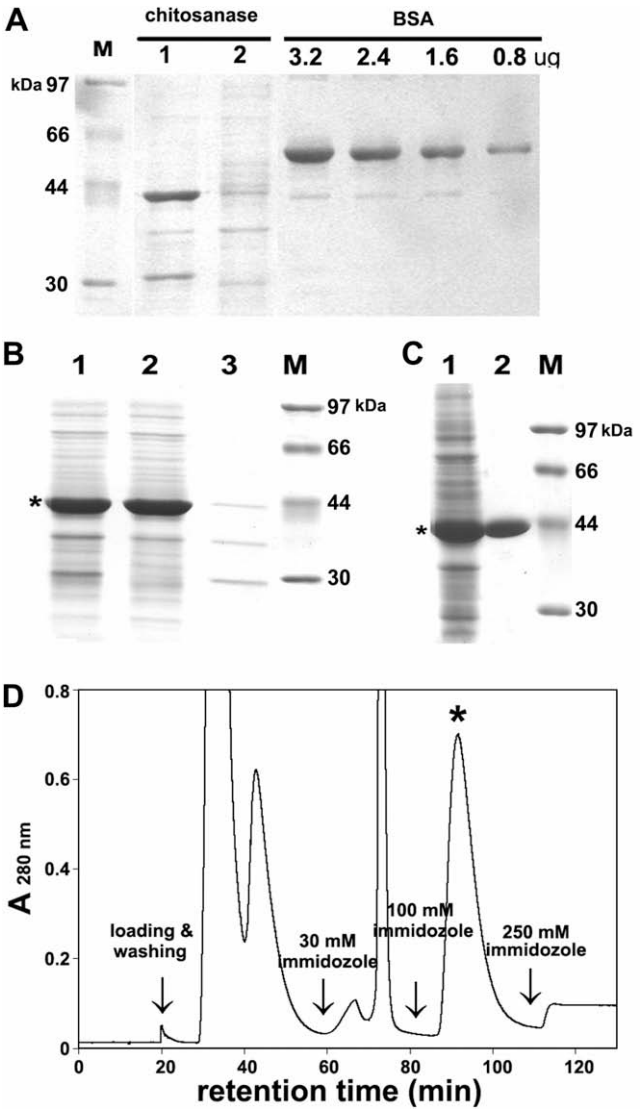

Figure 2. (A) Quantifying the chitosanase expression level by SDS–PAGE and by densitometry. The culture broth (containing *E. coli* cells) before IPTG induction (labeled as 2) and after IPTG induction (labeled as 1) was mixed with equal volume of SDS–PAGE loading buffer. After boiling, 8 μL of the mixture (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