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Enzymatic Production of High Molecular Weight Chitooligosaccharides Using Recombinant Chitosanase from *Bacillus thuringiensis* BMB171

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The chitosanase gene (btbchito) of *Bacillus thuringiensis* BMB171 was cloned and heterologously expressed in the yeast *Pichia pastoris*. After purification, about 300 mg of recombinant chitosanase was obtained from the 1-1 culture medium with a specific activity of 240 units/mg. Results determined by the combined use of thin layer chromatography (TLC) and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) showed that the chitooligosaccharides (COSs) obtained by chitosan (N-deacetylated by 70%, 80%, and 90%) hydrolysis by rBTBCHITO were comprised of oligomers, with degrees of polymerization (DP) mainly ranging from trimers to heptamers; high molecular weight chitopentaose, chitohexaose, and chitoheptaose were also produced. Hydrolysis products was also deduced using MS since the COSs (n) are complex oligosaccharides with various acetyl groups from one to two, so the non-acetyl COSs (GlcN)n and COSs with more acetyls (> 2) were not detected. The employment of this method in the production of high molecular weight COSs may be useful for various industrial and biological applications, and the activity of chitosanase has great significance in research and other applications.

Keywords: *B. thuringiensis* BMB171, chitosanase, recombinant, high molecular weight chitooligosaccharides

Introduction

COSs have aroused considerable interest due to their biological properties, such as their antitumor [1, 2] and antibacterial activities [3, 4], their activating immune-enhancing [5] and anti-inflammatory effects [6], and their ability to stimulate the growth of *Lactobacillus* [7]. It has been reported that several functions of COSs are obviously dependent upon their polymerization, whether the pentamers, hexamers, and heptamers are especially active. For example, chitopentaose and chitohexose can promote the mRNA expression of IL-1, TNF-α, IFN-γ and complement III receptor (CR3), as well as increase the secretion of those cytokines [8, 9]. In vitro, chitohexaose can stimulate macrophages to release IL-1β and TNF-α [10]. chitohexaose can inhibit endotoxin, such as LPS, induced production of the inflammatory molecules such as TNF-α, IL-1β, and IL-6, it has an inhibitory influence on CAM angiogenesis, and it induces the proliferation of *Candida albicans* [11–13]. Chitoheptaose has a significant effect on the promotion of the growth and photosynthesis of wheat seedlings [14]. Therefore, it is important to study the hydrolysis of chitosan to produce COSs of high molecular weights.

Enzymes catalyzed chitosan depolymerization is a very promising approach for the production of COSs. Chitosanase (EC 3.2.1.132), chitosan-hydrolyzing enzyme, has been found in various organisms, including bacteria, fungi, viruses, and plants. Of these, prokaryotic chitosanases from Bacillus species, such as *B. subtilis* [15,

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16], *B. cereus* [17–19], *B. licheniformis* [20], and *B. circulans* [21], their enzymatic activity, catalytic mechanisms, as well as protein structures of active site have been well studied. Although the chitosanases produced by *B. thuringiensis* were investigated in several studies [22, 23], more information on the chitosanases needs to be accumulated in order to provide a detailed understanding of their functions.

This study describes the recombinant expression of chitosanase from *B. thuringiensis* BMB171 and the catalytic features of chitosan for COS preparation. Enzymatic characterization would be of great value in understanding the catalytic mechanisms and applying the enzymatic hydrolysates. The DP of final enzymolysis products by rBTBCHITO was different from other chitosanolytic enzymes, having high molecular weight COSs of pentamer, hexamer and heptamer, which can be used for further application on biological functions.

Materials and Methods

Materials

Powdered chitosan was bought from Sigma-Aldrich Corporation (USA), used to determine enzyme activity and produce COSs. COS markers of (GlcN)2-6 were purchased from Seikagaku Corporation (Japan). The separation of mono-oligosaccharide was carried out using the Silica Gel G (Merck, Germany).

Strains and Plasmids

B. thuringiensis BMB171, provided by Professor Ming-Sun of Huazhong Agriculture University, was used as chitosanase gene producer. The plasmid pUC119 and pHBM905A, constructed on the basis of pPIC9K, were used as cloning vector and methanol-inducible heterologous expression, respectively. *E. coli* XL10-Gold was used as the host for DNA transformation, *P. pastoris* GS115 was used for the production of heterologous protein.

Gene Cloning of Chitosanase

Two primers, B171-F (*gtca*caccatcatcatcatcatatgaatggaaaaagaaatatttttacatgta, the underlined portion contains a *Cpo*I restriction site and 6×His) and B171-R (*ggcca*ttaattatcgtatccttcataaattgcatc, containing a *Not*I restriction site), were synthetized to amplify the mature peptide of BTBCHITO. PCR products were treated for 20 min at 12℃ with T4 DNA polymerase in the presence of dTTP to generated cohesive ends of *Cpo*I and *Not*I, then they were cloned into pHBM905A and pretreated with *Cpo*I and *Not*I. The recombinant plasmid, designated as pHBM905A-btbchito, was constructed according to a previously described method [24]. The pHBM905A-btbchito was linearized with restriction enzyme *Sal*I and electrotransformed into GS115. The His^+ positive transformants were grown on Minimal Dextrose (MD) plate medium, and colonies were screened by PCR and enzyme activity assay.

Expression and Purification of Recombinant Chitosanase

Chitosanase-producing recombinant yeast was cultured and induced according to the manual of Pichia Expression Kits (Invitrogen, USA). After induction, the precipitation was removed by centrifugation (4,200 ×*g* for 15 min at 4℃), the supernatant was saturated with ammonium sulfate up to 75%, and kept at 4℃ for overnight, the precipitate was collected, then dissolved in buffer with 50 mM Tris-HCl at pH 6.0. The recombinant BTBCHITO was purified by Ni-NTA agarose affinity chromatography according to a previous report [25]. The Bradford method was used for protein concentration measuring [26].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Recombinant protein expression was estimated by SDS-PAGE analysis, the concentrations of stacking gel and separating gel were 5% (v/v) and 12% (v/v), respectively. Coomassie Brilliant Blue G-250 was used for visualizing protein bands. Prestained protein marker (SM0431, Fermentas, USA) was used as molecular weight standards.

Chitosanase Activity

Chitosanase activity was assayed using our previous method [27]. The definition of unit was the amount of chitosanase required to produce 1 mmol equivalent of reducing sugars per min at 37℃, glucosamine was used as a standard.

Chitosan Hydrolysis

5% (m/v) chitosan with different DDA (70%, 80% and

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90%) were dissolved in water, respectively. The chitosanase was added into the solution (3 U/g chitosan), then the pH was adjusted to 5.6 by adding acetic acid. The mixtures were incubated overnight at 45℃.

Hydrolysis Products Analysis

Hydrolysis products of chitosan with different DDA were analyzed by TLC and MS. For TLC analysis, the solvent system was composed of ethanol-ethyl acetate-28% ammonia-water (5:5: 0.3: 4, v/v), and oligosaccharides were visualized by heating at 110℃ for 10 min. The MS analysis was determined according to previous method [27]. External mass calibration was performed with peptide calibration standard (Bruker Daltonics).

Results and Discussion

Recombinant Expression and Purification of Chitosanase

The Recombinant *P. pastoris* strain with chitosanase activity was selected for induction expression. 96 h after methanol induction, the supernatant was collected by centrifugation and the proteins were purified. An N-terminal 6×His-tag had been added to the target product to facilitate its later purification. The 6×His-tagged rBTBCHITO was purified using immobilized metal affinity chromatography (IMAC). Followed by staining, the results of SDS-PAGE showed that only one specific protein band was observed, and it migrated at approximately 47 kDa (Fig. 1). After purification, about 300 mg rBTBCHITO were obtained from the culture supernatant, and specific activity of rBTBCHITO was approximately 240 U/mg. A comparison of the enzyme activity between rBTBCHITO with other reported chitosanases was showed in Table 1.

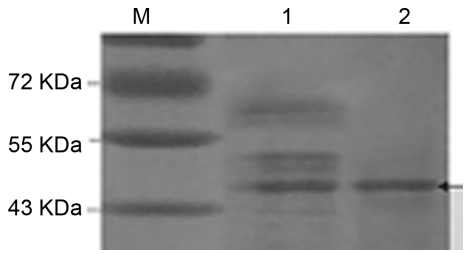

Fig. 1. SDS-PAGE of the rBTBCHITO expression. M: molecular weight marker; Lane 1: crude chitosanase; Lane 2: purified rBTBCHITO. Proteins were separated by 12% SDS-polyacrylamide gel.

The homology of rBTBCHITO with the relevant chitosanases was compared on amino acid level in NCBI database using the BLAST program. The rBTBCHITO showed high levels of similarity to chitosanases from *B. cereus* (WP_098398473.1), *B. cereus* (WP_042515026.1), *B. cereus* (WP_047956305.1), *B. thuringiensis* JAM-GG01 (BAJ05248.1) [22] and *B. thuringiensis* var. sotto (ABO61893.1) [23], with 98%, 98%, 98%, 97% and 98% identities, respectively.

Hydrolysis Products Analysis

The final hydrolysis products of 70%, 80%, and 90% DDA chitosan by rBTBCHITO were analyzed by TLC. After overnight of incubating at 45℃, the major products were (GlcN)3-7 (Fig. 2). Furthermore, the hydrolysis products were subjected to MS analysis, the results showed that (GlcN)3 (m/z 524), (GlcN)4 (m/z 685), (GlcN)5 (m/z 846), (GlcN)6 (m/z 1007), and (GlcN)7 (m/z

Table 1. Comparison of the induction and characteristic of chitosanases produced by bacteria of the Bt and Bc subspecies.

Strain	Chitosanase activity	Major hydrolysis products	Index
B. thuringiensis BMB171	240 U/mg	trimers to heptamers	This work
B. thuringiensis JAM-GG01	412 U/mg	chitotriose and chitobiose	22
B. thuringiensis var. sotto	-	trimer and tetramer	23
B. thuringiensis 105	4.67 nkat/mg	-	28
B. cereus QQ308	2.7 U/ml	-	29
B. cereus TKU027	0.022 U/ml	hetero-chitooligosaccharides of DP 4-9	30
B. cereus GU-02	-	dimers to pentamers	18
B. cereus TKU022	7.0 U/mg	(GlcNAc)2, (GlcNAc)4, (GlcNAc)5, and (GlcNAc)6	31
B. cereus S1	196 U/mg	chitobiose, chitotriose, and chitotetraose	32
B. cereus KU030	0.177 U/mg	-	33

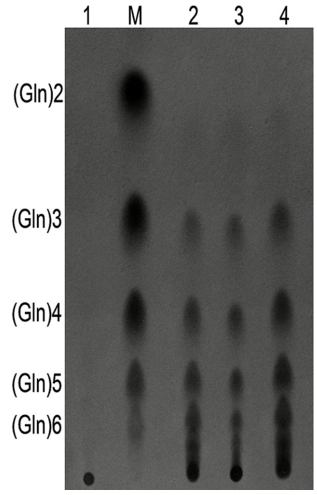

Fig. 2. TLC analysis of the hydrolysis products of different DDA chitosan incubation with rBTBCHITO. Lane M: the standard oligosaccharides of (GlcN)2-6. Lane 1: the control of chitosan incubated without any enzyme. Lane 2: the products of enzymatic hydrolysis of 70% DDA chitosan. Lane 3: the products of enzymatic hydrolysis of 80% DDA chitosan. Lane 4: the products of enzymatic hydrolysis of 90% DDA chitosan.

1168) were major peaks with various DDA chitosan hydrolysates (Fig. 3). These results were not the same in all chitosanolytic enzyme-producing bacteria, such as *B. thuringiensis* JAM-GG01 [22], *B. thuringiensis* var. sotto [23], and *B. cereus* S1 [32], but they were similar to bacteria such as *B. cereus* TKU027 [30] and *B. cereus* TKU022 [31] (Table 1).

The enzymatic hydrolysis of 70%, 80%, and 90% DDA chitosan was identical (Fig. 3), and the acetyl content of the substrate had no effect on the formation of the products; however, of those that had a relative enzymatic reaction rate, 90% DDA chitosan was found to the best substrate for COS production (data not shown). During the hydrolysis, the proportion of pentamers, hexamers, and heptamers were relatively high (Fig. 2). Therefore, this enzyme can be a useful tool for production of COSs (GlcN)n (n > 4) in industrial field and studies of biological functions of (GlcN)n on various applications.

The acetyl content of these specific oligosaccharides was also determined and deduced. Although the major

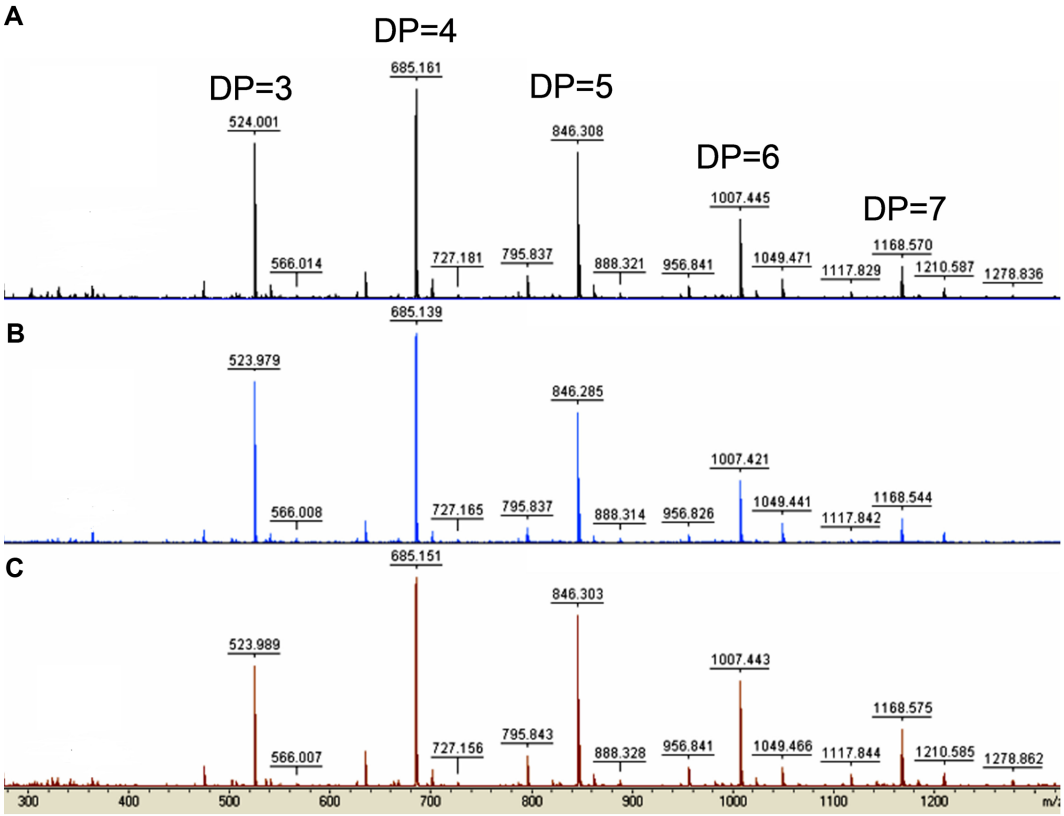

Fig. 3. MS analysis of COSs produced by hydrolysis of different DDA chitosan. (A) COSs from 70% deacetylated chitosan hydrolysis, (B) COSs from 80% deacetylated chitosan hydrolysis, and (C) COSs from 90% deacetylated chitosan hydrolysis.

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Table 2. Ion composition of the MALDI-TOF MS spectra (m/z) of the oligosaccharides produced by chitosan hydrolysis.

m/z	COS types	Ion composition	Acetyl content
524	chitotriose (DP=3)	$(GlcN)_2$-GlcNAc	1
566 (524+42_Ac)	chitotriose	GlcN-$(GlcNAc)_2$	2
685	chitotetraose (DP=4)	$(GlcN)_3$-GlcNAc	1
729 (685+42_Ac)	chitotetraose	$(GlcN)_2$-$(GlcNAc)_2$	2
846	chitopentaose (DP=5)	$(GlcN)_4$-GlcNAc	1
888 (846+42_Ac)	chitopentaose	$(GlcN)_3$-$(GlcNAc)_2$	2
1007	chitohexaose (DP=6)	$(GlcN)_5$-GlcNAc	1
1049 (1007+42_Ac)	chitohexaose	$(GlcN)_4$-$(GlcNAc)_2$	2
1168	chitoheptaose (DP=7)	$(GlcN)_6$-GlcNAc	1
1210 (1168+42_Ac)	chitoheptaose	$(GlcN)_5$-$(GlcNAc)_2$	2

products of chitotriose (m/z 524), chitotetraose (m/z 685), chitopentaose (m/z 846), chitohexaose (m/z 1049), and chitoheptaose (m/z 1168) were detected, other clear mass signals (m/z 566, 727, 888, 1049, and 1210) were also appeared (Fig. 3). There was a difference of 42 atomic mass units between these corresponding COSs in m/z values, which corresponded well to the molecular mass of acetyl.

Chitosanase from *B. thuringiensis* is a family 8 glycosyl hydrolase, which primarily cleaves the linkage of GlcN-GlcN bond. Conventionally, the amount of acetyl of the COSs obtained by enzyme hydrolysis of chitosan should be from 0 to n-1 for (GlcNAc)n. In fact, the results showed that the numbers of acetyls of COSs (n) were from 1 to 2, and the non-acetyl COSs (GlcN)n (m/z 483, 644, 805, 966, and 1126) and COSs with three or more acetyls were not detected (Fig. 3 and Table 2).

Taken together, these results indicate that the chitosanase enzyme from *B. thuringnisis* BMB171 shows remarkable specificity toward the chitosan substrate (70%, 80% and 90% DDA); therefore, it can be a useful tool for specific structural and high molecular weight COSs production in the industrial scale and may be used for further research on its potential biological application functions in nature.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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