J. Microbiol. Biotechnol. (2009), **19**(4), 358–361
doi: 10.4014/jmb.0805.306
First published online 3 October 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Reaction Pattern of *Bacillus cereus* D-11 Chitosanase on Chitooligosaccharide Alcohols

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Received: May 4, 2008 / Revised: August 11, 2008 / Accepted: August 12, 2008

The purified endochitosanase (Mw 41 kDa) from bacterium *Bacillus cereus* D-11 hydrolyzed chitooligomers $(GlcN)_{5\text{-}7}$ into chitobiose, chitotriose, and chitotetraose as the final products. The minimal size of the oligosaccharides for enzymatic hydrolysis was a pentamer. To further investigate the cleavage pattern of this enzyme, chitooligosaccharide alcohols were prepared as substrates and the end products of hydrolysis were analyzed by TLC and HPLC. The chitosanase split $(GlcN)_4GlcNOH$ into $(GlcN)_3$+ $(GlcN)_1GlcNOH$, and $(GlcN)_5GlcNOH$ into $(GlcN)_4$+ $(GlcN)_1GlcNOH$ and $(GlcN)_3$+$(GlcN)_2GlcNOH$. The heptamer $(GlcN)_6GlcNOH$ was split into $(GlcN)_5$ [thereafter hydrolyzed again into $(GlcN)_3$+$(GlcN)_2$]+$(GlcN)_1GlcNOH$, $(GlcN)_4$+$(GlcN)_2GlcNOH$, and $(GlcN)_3$+$(GlcN)_3GlcNOH$, whereas $(GlcN)_{1\text{-}3}GlcNOH$ was not hydrolyzed. The monomers GlcN and GlcNOH were never detected from the enzyme reaction. These results suggest that D-11 chitosanase recognizes three glucosamine residues in the minus position and simultaneously two residues in the plus position from the cleavage point.

Keywords: *Bacillus cereus* D-11, endochitosanase, chito-oligosaccharides, chito-oligosaccharide alcohols, cleavage pattern

Chitosanase (E.C. 3.2.1.132) is an enzyme that belongs to the glycoside hydrolase family, and is characterized by its ability to catalyze the hydrolytic cleavage of chitosan [14]. Chitosanases have been purified from many microorganisms, and the mode of action has been studied by several investigators [2, 5, 10]. Most chitosanases are characterized as endo-type enzymes that randomly cleave chitosan and its oligosaccharides. Chitosanases from individual organisms differ in their hydrolytic pattern [12].

Chito-oligosaccharide alcohols have been used for the elucidation of cleavage patterns of glycosidases, because the reaction products from the nonreducing and reducing ends of the alcohols could be distinguishable; one contains aldehyde residue and the other still alcohol residue. Chitinases A1 and D from *B. circulans* WL-12 hydrolyze chitin saccharide alcohol $(GlcNAc)_3GlcNAcOH$ to β-chitobiose and chitobitol [1]. Tanaka *et al.* [15] demonstrated that an exo-glucosaminidase from *Thermococcus kodakaraensis* KOD1 hydrolyzed the first exo-β-D-glucosaminide bond from the nonreducing end of the chitooligosaccharide alcohol $(GlcN)_{1\text{-}4}GlcNOH$. However, little is still known about the degradation pattern and the cleavage point for most chitosanases. In this study, we focused on elucidating the cleavage pattern of an extracellular 41-kDa chitosanase purified from the culture supernatant of *B. cereus* D-11 [4] using chitooligosaccharide alcohols as novel substrates.

Chitooligosaccharide alcohols were derived from chitooligomers by borohydride reduction according to the method of Kazuaki and Kazuhiro. [7]. Chitooligomers $(GlcN)_{2\text{-}7}$ were obtained from Wako (Japan). For the reaction, 2–4 mg of chitooligomers $(GlcN_{1\text{-}7})$ or chitooligosaccharide alcohols $[(GlcN)_{1\text{-}6}GlcNOH]$ was each dissolved in 0.1 ml of 0.05 M sodium acetate buffer (pH 6) and then 0.02 ml of purified enzyme (4 U/ml) was added. Then 0.38 ml of the sodium acetate buffer was added to adjust the total volume to 0.5 ml. The mixture was incubated at 37°C for 24 h. After boiling in 100°C water and brief centrifugation, the supernatant was spotted on TLC plates (Silica gel $60F_{254}$; Merck, Germany) and developed with *n*-propanol:ethylacetate:ammonia water:water (6:3:3:1 v/v) as described previously [6]. The sugars on the plates were visualized by spraying with 0.2% ninhydrin dissolved in 99% ethanol and holding the plates on a hotplate for 1 min [16]. The products were also analyzed by HPLC (Shimadzu Model 10 AD, Japan) with a carbohydrate column (3.9×300 mm; Waters, Ireland) with a mobile phase of acetonitrile:water (70:30 v/v) at a flow rate of 1.0 ml/min and detected with a refractive index (RI) detector.

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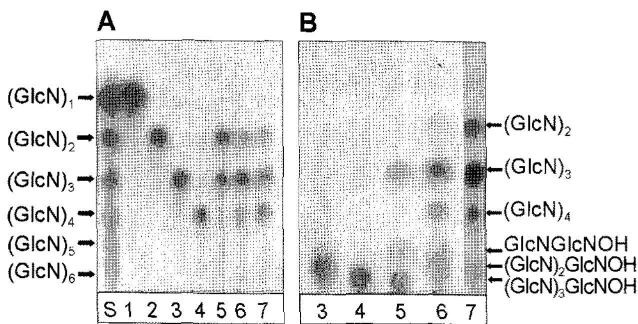

Fig. 1. TLC analysis of enzymatic hydrolyzates of chito-oligosaccharides (**A**) and their alcohols (**B**).

Panel **A**: lane S, standard $(GlcN)_{1\text{-}6}$ mixture; lanes 1–7, enzymic hydrolysis products from the substrate $(GlcN)_{1\text{-}7}$. Panel **B**: lanes 3–7, enzymic hydrolysis products from the substrate $(GlcN)_{2\text{-}6}$GlcNOH.

Enzyme Reaction Products from Chitooligosaccharides

Hydrolysis products of the chitooligomers are shown in Fig. 1A. Chitooligomers [$(GlcN)_{2\text{-}4}$] were not degraded by the enzyme even after a prolonged reaction time (lanes 2, 3, and 4 in Fig. 1A; B in Fig. 2 for the tetramer). The chitosanase split $(GlcN)_5$ into $(GlcN)_2$ and $(GlcN)_3$ (lane 5), and $(GlcN)_6$ and $(GlcN)_7$ oligomers were split into $(GlcN)_{2\text{-}4}$ (lanes 6 and 7). However, GlcN was not detected. These results indicate that *B. cereus* D-11 chitosanase cleaves the oligomeric chains in the endo-splitting manner. In $(GlcN)_6$ hydrolysis, as shown in Fig. 2, the amount of $(GlcN)_3$ was almost two-times higher than the sum of $(GlcN)_2$ and $(GlcN)_4$, suggesting that the frequency of cleavage to $(GlcN)_3$+$(GlcN)_3$ was higher than that to $(GlcN)_4$+$(GlcN)_2$.

The D-11 chitosanase cleaved soluble chitosan to result in $(GlcN)_{2\text{-}6}$ by endo-type action, but the major products were $(GlcN)_{2\text{-}4}$ (data not shown). The hydrolysis pattern of this enzyme is similar with the most known chitosanases. *Bacillus* sp. HW-002 chitosanase [8] produced $(GlcN)_2$ from chitosan; *Nocardioides* sp. K-01 [11] and *Acinetobacter* sp. CHB101 [13] finally produced $(GlcN)_{2\text{-}3}$; *Streptomyces* sp. N174 [3] and *Bacillus* sp. DAU101 [9] produced $(GlcN)_{2\text{-}4}$; *Bacillus* sp. KFB-C108 [17] produced $(GlcN)_{3\text{-}5}$; and *Bacillus* sp. P16 [6] produced $(GlcN)_{2\text{-}5}$ as major products.

Enzyme Reaction Products from Chitooligosaccharide Alcohols

In order to further analyze the hydrolysis pattern of the enzyme, the prepared chitooligosaccharide alcohols were used as substrates and the hydrolysis products were analyzed by TLC (Fig. 1B). The chitosanase hydrolyzed chitooligosaccharide alcohols into chitooligomers and residual saccharide alcohols. $(GlcN)_4$GlcNOH (pentamer) resulted in 2 products $(GlcN)_3$ and GlcNGlcNOH (lane 5, Fig. 1B), and $(GlcN)_5$GlcNOH (hexamer) resulted in 4 products, $(GlcN)_{3\text{-}4}$ and $(GlcN)_{1\text{-}2}$GlcNOH (lane 6, Fig. 1B), suggesting two points cleavage into $(GlcN)_3$+$(GlcN)_2$GlcNOH and $(GlcN)_4$+$(GlcN)_1$GlcNOH.

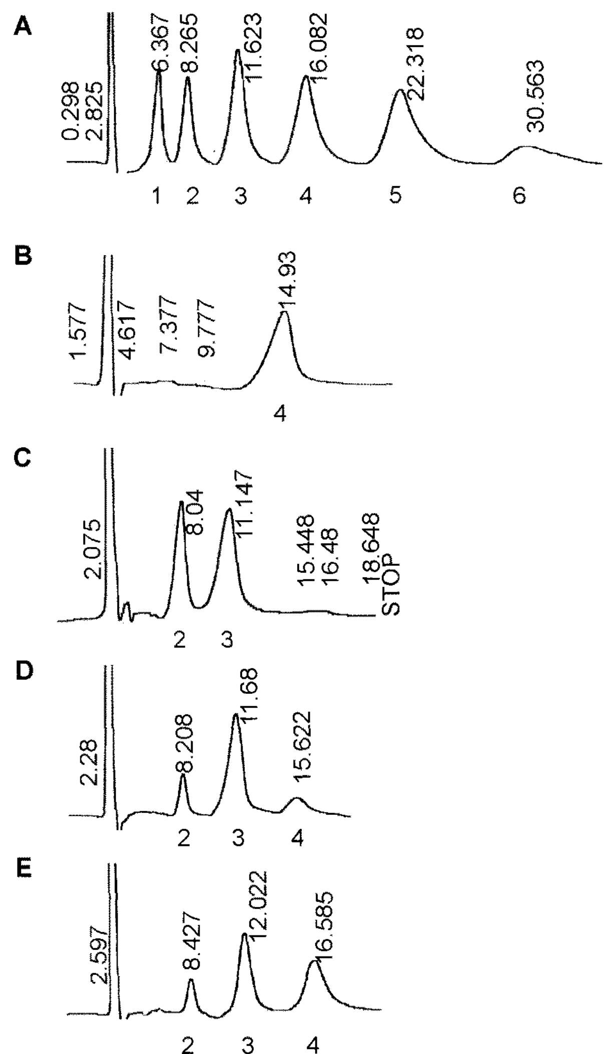

Fig. 2. HPLC chromatograms of enzymic hydrolyzates of chitooligosaccharides.

A. Standard $(GlcN)_{1\text{-}6}$ mixture. **B–E.** Enzymic hydrolysis products from the tetramer, pentamer, hexamer, and heptamer, respectively.

HPLC analysis obtained the same results as expected from TLC analysis. For examples, the 2 products $(GlcN)_3$ and GlcNGlcNOH were clearly shown as the hydrolyzates of $(GlcN)_4$GlcNOH (Fig. 3A), which supported one point cleavage in the oligosaccharide alcohol chain. The 4 products $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_1$GlcNOH, and $(GlcN)_2$GlcNOH were shown as the hydrolyzates of $(GlcN)_5$GlcNOH (Fig. 3B), which supported two points cleavage in the hexamer chain. $(GlcN)_3$ and $(GlcN)_2$GlcNOH were eluted at the same retention time in the solvent system, and the amount of both was 2-times higher than the sum of $(GlcN)_4$+$(GlcN)_1$GlcNOH, in accordance with the hydrolytic result of $(GlcN)_6$ as described above.

$(GlcN)_6$GlcNOH (heptamer) resulted in 7 products (not 6 products), $(GlcN)_{2\text{-}4}$ and $(GlcN)_{1\text{-}3}$GlcNOH (lane 7, Fig. 1B), suggesting the cleavages into $(GlcN)_3$+$(GlcN)_3$GlcNOH,

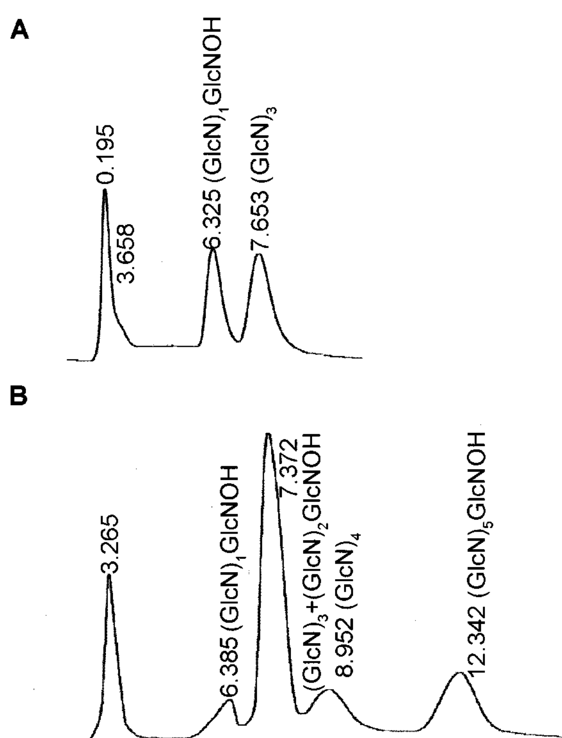

Fig. 3. HPLC chromatograms of enzymic hydrolyzates of chitooligosaccharide alcohols.
A. Hydrolysis products from $(GlcN)_4GlcNOH$ (pentamer). **B**. Hydrolysis products from $(GlcN)_5GlcNOH$ (hexamer).

Table 1. The suggested cleavage points of *B. cereus* D-11 chitosanase deduced from the reaction products.

Chito-oligosaccharide alcohols	Chito-oligosaccharides
○–●	○–∅
○–○–●	○–○–∅
○–○–○–●	○–○–○–∅
○–○–○↓○–●	○–○–○↓○–∅
○–○–○↓○–○–●	○–○–○↓○–○–∅
○–○–○–○↓○–●	○–○–○–○↓○–∅
○–○–○↓○–○–○–●	○–○–○↓○–○–○–∅
○–○–○–○↓○–○–●	○–○–○–○↓○–○–∅
○–○–○(↑)○–○↓○–●	○–○–○(↑)○–○–○↓○–∅

○, GlcN residue; ∅, GlcN reducing end; ●, GlcNOH residue; ↓, The cleavage point; (↑), The second cleavage point on the resulting product after the first cleavage.

$(GlcN)_4$+$(GlcN)_2GlcNOH$, and $(GlcN)_5$+$(GlcN)_1GlcNOH$. However, $(GlcN)_5$ did not appear in lane 7, assuming that the product $(GlcN)_5$ hydrolyzed again into $(GlcN)_2$ and $(GlcN)_3$. That is why $(GlcN)_2$ had appeared additionally. Chitooligosaccharide alcohols ($GlcN_{2\text{-}3}GlcNOH$, trimer and tetramer) were not degraded by the enzyme (lanes 3 and 4, Fig. 1B), as expected from the results with chitooligomers [$(GlcN)_{3\text{-}4}$]. All these results are summarized in Table 1.

Elucidation of Enzyme Hydrolysis Pattern

From these results, it was confirmed that the hydrolysis patterns of the chitosanase for chitooligosaccharide alcohols and chitooligomers were exactly the same, as shown in Table 1. $(GlcN)_5$ was split into $(GlcN)_3$ and $(GlcN)_2$, and $(GlcN)_4GlcNOH$ into $(GlcN)_3$ and $(GlcN)_1GlcNOH$, suggesting the catalytic domain of D-11 chitosanase recognizes and needs at least 5 glucosamine units for hydrolytic catalysis of the glycosidic linkages. Thus, the dimer, trimer, and tetramer of both chitooligomers and chitooligosaccharide alcohols were not degraded by D-11 chitosanase.

It was assumed that D-11 chitosanase recognized at least three glucosamine units from the nonreducing end and simultaneously at least two glucosamine units from the reducing end of the chitooligosaccharides. Thus, $(GlcN)_4GlcNOH$ would be cleaved to $(GlcN)_3$ and $(GlcN)_1GlcNOH$, but never into $(GlcN)_2$ and $(GlcN)_2GlcNOH$. Along the same logic, one can deduce that D-11 chitosanase recognizes the third β-glycosidic linkage from the nonreducing end of $(GlcN)_5$ to degrade it into $(GlcN)_{3nr(nonreducing\ end)}$+$(GlcN)_{2r(reducing\ end)}$, but never into $(GlcN)_{2nr}$+$(GlcN)_{3r}$; that is, the dimer always resulted from the reducing end residues but not from the nonreducing end residues.

In the case of $(GlcN)_5GlcNOH$, this oligomer was cleaved at two points, resulting in $(GlcN)_3$+$(GlcN)_2GlcNOH$ and $(GlcN)_4$+$(GlcN)_1GlcNOH$. In the same way, $(GlcN)_6$ was cleaved into $(GlcN)_{3nr}$+$(GlcN)_{3r}$ and $(GlcN)_{4nr}$+$(GlcN)_{2r}$. Furthermore, $(GlcN)_6GlcNOH$ was hydrolyzed by the chitosanase at three points, as shown from the products, $(GlcN)_3$+$(GlcN)_3GlcNOH$, $(GlcN)_4$+$(GlcN)_2GlcNOH$, and $(GlcN)_5$+$(GlcN)_1GlcNOH$, in accordance with chitooligosaccharide $(GlcN)_7$ cleavages (Table 1). The product $(GlcN)_5$ from the heptamers was further degraded into $(GlcN)_{3nr}$+$(GlcN)_{2r}$, as summarized in Table 1. From the suggested cleavage pattern of D-11 chitosanase, the monomers GlcN and GlcNOH are never expected as the hydrolytic product.

Taken together, it was found that the purified D-11 chitosanase needed at least five glucosamine residues for cleavage and recognized at least three glucosamine units from the nonreducing (minus) end and simultaneously at least two glucosamine units from the reducing (plus) end of the chitooligosaccharides. That is why the minimal size

of the oligosaccharides for enzymatic hydrolysis should be a pentamer and $(GlcN)_4GlcNOH$ be cleaved to $(GlcN)_3$ and $(GlcN)_1GlcNOH$. It was confirmed that chitooligosaccharide alcohols were useful for elucidating the hydrolytic pattern of chitosanase. Presently, study for elucidating the subsite structure of D-11 chitosanase is undertaken.

Acknowledgment

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the National Research Lab. Program funded by the Ministry of Science and Technology. [No. R0A-2003-000-10322-0 (2007).]

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