Journal of Bioscience and Bioengineering
VOL. 127 No. 4, 425–429, 2019

www.elsevier.com/locate/jbiosc

Characterization of a novel exo-chitosanase, an exo-chitobiohydrolase, from *Gongronella butleri*

Kiyohiko Seki, Yasue Nishiyama, and Masaru Mitsutomi*

Department of Applied Biochemistry and Food Science, Saga University, 1 Honjo-machi, Saga 840-8502, Japan

Received 13 July 2018; accepted 12 September 2018
Available online 10 October 2018

An exo-chitosanase was purified from the culture filtrate of *Gongronella butleri* NBRC105989 to homogeneity by ammonium sulfate precipitation, followed by column chromatography using CM-Sephadex C-50 and Sephadex G-100. The enzyme comprised a monomeric protein with a molecular weight of approximately 47,000 according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme exhibited optimum activity at pH 4.0, and was stable between pH 5.0 and 11.0. It was most active at 45°C, but was stable at temperatures below 30°C. The enzyme hydrolyzed soluble chitosan and glucosamine (GlcN) oligomers larger than tetramers, but did not hydrolyze *N*-acetylglucosamine (GlcNAc) oligomers. To clarify the mode of action of the enzyme, we used thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) to investigate the products resulting from the enzyme-catalyzed hydrolysis of chitosan and N^1-acetylchitohexaose [$(GlcN)_5$-GlcNAc] with a GlcNAc residue at the reducing end. The results indicated that the enzyme is a novel exo-type chitosanase, exo-chitobiohydrolase, that releases $(GlcN)_2$ from the non-reducing ends of chitosan molecules. Analyses of the hydrolysis products of partially *N*-acetylated chitooligosaccharides revealed that the enzyme cleaves both GlcN-GlcNAc and GlcNAc-GlcN bonds in addition to GlcN-GlcN bonds in the substrate.

[**Key words:** Exo-chitobiohydrolase; Chitosanase; Mode of action; Cleavage specificity; *Gongronella butleri*]

Chitosan is a polysaccharide composed of D-glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc). It is industrially produced by the alkaline deacetylation of chitin. Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the endohydrolysis of β-1,4 linkages between GlcN residues in a partially *N*-acetylated chitosan (Enzyme Nomenclature, http://www.chem.qmul.ac.uk/iubmb/enzyme/). Chitooligosaccharides produced by the hydrolysis of partially *N*-acetylated chitosan have many biological activities, including antibacterial activity, antifungal activity, immune-enhancing effects, and elicitor activity (1). Hence, a number of chitosanases have been isolated from various bacteria (2–8) and fungi (9–12) to prepare functional chitooligosaccharides. On the other hand, exo-β-D-glucosaminidase (EC 3.2.1.165), an exo-type chitosanase that releases GlcN from the non-reducing end of the chitosan, has been purified and characterized from microorganisms (13–17). The enzyme is very useful for the structural analysis of chitooligosaccharides and the production of glucosamine. Using thin-layer chromatography (TLC) analysis, Watanabe (18) reported that chitobiose [a glucosamine dimer, $(GlcN)_2$] was detected as hydrolysis product of chitosan with a culture filtrate of *Gongronella butleri* C6-2. The results suggest that the strain produces an exo-chitosanase that hydrolyzes chitosan in an exo-splitting manner. However, exo-chitobiohydrolase has not yet been purified or characterized.

Recently, we found that *G. butleri* NBRC105989 produced an extracellular chitosanase when grown on chitooligosaccharides. The enzyme hydrolyzed chitosan to produce only $(GlcN)_2$. In the present study, we purified and characterized the chitosanase from *G. butleri* NBRC105989, and found that it hydrolyzed chitosan and chitosan oligosaccharides to remove successive $(GlcN)_2$ from the non-reducing end.

In the present paper, we describe the purification, characterization, and mode of action of a novel exo-chitosanase, exo-chitobiohydrolase, produced by *G. butleri* NBRC105989. To the best of our knowledge, this is the first report on the purification and mode of action of exo-chitobiohydrolase.

MATERIALS AND METHODS

Materials The chitooligosaccharides mixture was provided by Yaegaki Bioindustry, Inc. (Himeji, Japan). A series of $(GlcN)_n$ (n = 2–6) and $(GlcNAc)_6$ was supplied by Yaizu Suisankagaku Industry (Shizuoka, Japan). Powdered chitosans (75% and 98% deacetylated) were provided by the Katakura & Co-op Agri Corporation (Tokyo, Japan). Chitosans 7B (70% deacetylated), 8B (80% deacetylated), 9B (90% deacetylated), and 10B (100% deacetylated), and powdered chitin (Chitin EX) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Carboxymethyl cellulose (CMC) was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). N^1-Acetylchitooligosaccharides [$(GlcN)_n$-GlcNAc, n = 1–5], N^1,N^4-diacetylchitotetraose [GlcNAc-$(GlcN)_2$-GlcNAc], and N^1,N^6-diacetylchitohexaose [GlcNAc-$(GlcN)_4$-GlcNAc] were prepared by the method described by Mitsutomi et al. (19). N^4-Acetylchitohexaose

* Corresponding author. Tel.: +81 952 28 8786; fax: +81 952 28 8709.
E-mail address: mitsutom@cc.saga-u.ac.jp (M. Mitsutomi).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.09.009

[$(GlcN)_2$-GlcNAc-$(GlcN)_3$] and N^1,N^4-diacetylchitohexaose [$(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc] were prepared by digesting 75% deacetylated chitosan with chitosanase from *Bacillus amyloliquefaciens* UTK (20,21). N^3-Acetylchitopentaose [GlcN-GlcNAc-$(GlcN)_3$] and N^1,N^3,N^4-triacetylchitopentaose [GlcN-$(GlcNAc)_2$-GlcN-GlcNAc] were prepared by digesting 75% deacetylated chitosan with chitosanase from *Bacillus circulans* MH-K1 (22) and chitinase from *Trichoderma* sp. OMI5074, respectively. CM-Sephadex C-50 and Sephadex G-100 were purchased from GE Healthcare (Tokyo, Japan).

Microorganism and enzyme production *G. butleri* NBRC105989 was used in the present study. The strain was grown and stocked on a potato glucose agar slant. Approximately 10 mm^2 of the mycelia was inoculated into a 300-mL conical flask containing 50 mL of a medium (pH 6.5) comprising a 0.5% chitooligosaccharides mixture, 0.1% yeast extract, 0.2% polypeptone, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. The mixture was incubated at 25°C and 200 rpm for 3 days. The seed culture (30 mL) was transferred to a 3-L conical flask containing 1 L of the same medium, and incubated at 25°C and 200 rpm for 7 days. Subsequently, the culture broth was centrifuged for 20 min at 6000 × *g*, and the supernatant was used in enzyme purification.

Enzyme assay Exo-chitobiohydrolase/chitosanase activity was assayed using soluble chitosan (98% deacetylated) as the substrate. The reaction mixture comprised 0.9 mL of 0.11% chitosan solution in 0.1 M sodium acetate buffer (pH 4.0), and 0.1 mL of enzyme solution. It was incubated at 40°C for 20 min. The reaction was stopped by the addition of 2 mL of 0.5 M sodium carbonate containing 0.05% potassium ferricyanide. The final volume of the reaction mixture was adjusted to 3.5 mL by the addition of 0.5 mL of water. The amount of liberated reducing sugar was determined using a modification of the Schales' method (23). One unit of exo-chitobiohydrolase activity was defined as the amount of enzyme that produces reducing sugar corresponding to 1 μmol of $(GlcN)_2$ per min.

When the GlcN oligomer and the partially *N*-acetylated chitooligosaccharides were used as the substrates, the reaction mixture comprised 20 μL of 4 mM substrate solution, 10 μL of 0.1 M acetate buffer (pH 4.0), and 10 μL of enzyme solution; the mixture was incubated at 37°C for 20 min, and the amount of liberated $(GlcN)_2$ was determined using the method described by Rondle and Morgan (24).

Protein measurement During column chromatography, the protein concentration in each fraction was monitored by measuring the absorbance at 280 nm. The protein concentration was determined by the Bradford method (25) using a Coomassie Plus Better Bradford Assay kit (Thermo Science, Kanagawa, Japan) with bovine serum albumin as standard.

Electrophoresis Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method described by Laemmli (26) with 12.5% polyacrylamide gel containing 0.1% SDS. The protein bands were detected by staining with Coomassie Brilliant Blue R-250. The molecular weight of the enzyme was estimated by SDS-PAGE.

Enzyme purification All chitosanase purification steps were performed at 4°C. The culture filtrate was dialyzed against 50 mM phosphate buffer (pH 6.8). The dialyzed solution was concentrated to approximately one-third of its original volume by dialysis against ammonium sulfate, and brought to saturation by adding powdered ammonium sulfate. The obtained precipitate was dissolved in distilled water, and the concentrated enzyme solution was dialyzed against 50 mM acetate buffer (pH 5.0). The dialyzed solution was introduced to a CM-Sephadex C-50 column (2.6 × 25 cm) equilibrated with 50 mM acetate buffer (pH 5.0) at a flow rate of 25 mL/h. After washing the column with the same buffer, the adsorbed protein was eluted with a linear gradient of NaCl (0–0.3 M) in the same buffer. After passing through a Sephadex G-100 column (2.6 × 90 cm) previously equilibrated with 50 mM phosphate buffer (pH 6.8) containing 0.1 M NaCl, the chitosanase-active fractions were pooled and used as the purified enzyme preparation.

Thin-layer chromatography The products of the enzymatic hydrolysis of chitosan were analyzed by TLC. A reaction mixture comprising 90 μL of 0.11% chitosan solution in 0.1 M sodium acetate buffer (pH 4.0) and 10 μL of chitosanase (0.012 units) was incubated for various times at 40°C. After the mixtures had been boiled for 5 min to terminate the reaction, each sample (2 μL) was subjected to TLC. The sugars were developed on a silica gel plate (TLC aluminum sheets silica gel 60; Merck, Darmstadt, Germany) with an *n*-propanol–water–28% ammonia (70:15:15) mixture as the developing solvent. The spots were made visible using diphenylamine–aniline reagent.

High-performance liquid chromatography The hydrolysis products of partially *N*-acetylated chitooligosaccharide were analyzed by high-performance liquid chromatography (HPLC). A reaction mixture comprising 20 μL of 10 mM substrate solution, 10 μL of 5 mM phosphate buffer (pH 6.0), and 10 μL of exo-chitobiohydrolase (2.63 units/mL) was incubated at 40°C. The reaction was terminated by boiling for 5 min. The hydrolysate of each sample (10 μL) was injected into an HPLC column. The HPLC system comprised a PU-2089 plus pump, an 830-RI detector, and ChromNAV software (Japan Spectroscopic, Tokyo, Japan). The sugars were separated on a Carbohydrate High Performance column (4.6 × 250 mm; Waters, Milford, MA, USA) using an acetonitrile–water (65:35) mixture as the mobile phase at a flow rate of 1.0 mL/min. The chitooligosaccharides were detected by monitoring the refractive index.

RESULTS AND DISCUSSION

Purification of chitosanase from *G. butleri* *G. butleri* NBRC105989 produced an extracellular chitosanase when grown in a medium containing chitooligosaccharides. We purified the enzyme from the culture filtrate of *G. butleri* by ammonium sulfate precipitation, followed by column chromatography using CM-Sephadex C-50 and Sephadex G-100. The purification procedure and the results are summarized in Table 1. We used SDS-PAGE to confirm that the purified enzyme was homogeneous (Fig. 1). The molecular mass of the enzyme was estimated to be approximately 46,000 by gel filtration on Sephadex G-100, and approximately 47,000 by SDS-PAGE, suggesting that the enzyme is a monomeric protein. These values differed from those of endo-chitosanases Csn 1 (Mr 90,000) and Csn 2 (Mr 28,000) from *Gongronella* sp. JG (27,28).

Effect of pH on activity and stability The effects of pH on the chitosanase are shown in Fig. 2. The chitosan substrate was completely dissolved during the experiment. The optimum pH for chitosanase activity was 4.0 (Fig. 2A). The stability of the enzyme at various pH values was determined after preincubation of the

TABLE 1. Purification of chitosanase from *G. butleri*.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Culture filtrate	74.9	258	3.44	1	100
$(NH_4)_2SO_4$ ppt.	38.9	209	5.37	1.56	81.0
CM-Sephadex C-50	8.01	115	14.4	4.19	44.6
Sephadex G-100	1.12	72.4	64.6	18.8	28.1

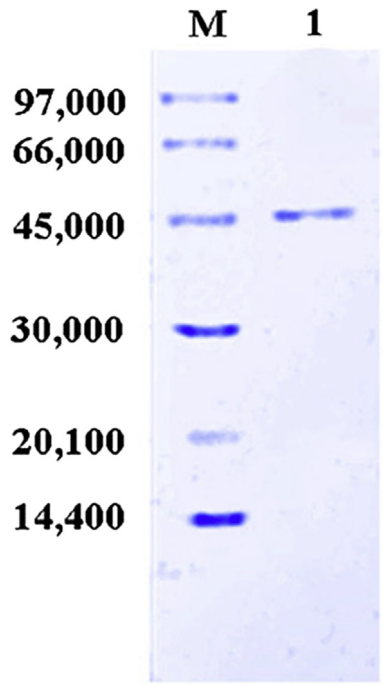

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the purified chitosanase. Lane M, molecular weight markers; lane 1, purified chitosanase. The standard proteins used were β-lactalbumin (Mr 14,400), trypsin inhibitor (Mr 20,100), carbonic anhydrase (Mr 30,000), ovalbumin (Mr 45,000), albumin (Mr 66,000), and phosphorylase b (Mr 97,000) (Protein Molecular Weight Markers, GE Healthcare, Tokyo, Japan).

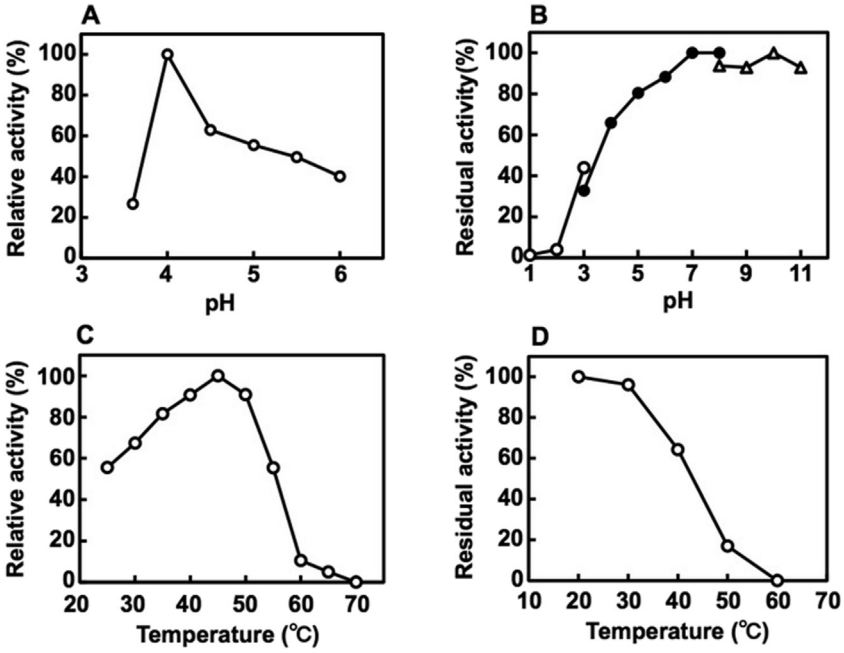

FIG. 2. Effects of pH and temperature on the activity and stability of the chitosanase. (A) The enzyme activities were measured under standard assay conditions with McIlvain's buffers at pH 3.6 to 6.0. (B) The enzymes were incubated for 2 h at 30°C in various buffers at pH 1.0 to 11.0, and the residual activities were assessed under standard conditions. The buffers used were 0.1 M sodium citrate buffer (pH 1.0 to 3.0), McIlvain's buffer (pH 3.0 to 8.0), and Atkins and Pantin's buffer (pH 8.0 to 11.0). Symbols: open circles, 0.1 M sodium citrate buffer; closed circles, McIlvain's buffer; open triangles, Atkins and Pantin's buffer. (C) The enzyme activities were assessed under standard conditions at various temperatures from 30°C to 70°C. (D) The enzymes were incubated for 15 min at various temperatures in 50 mM phosphate buffer (pH 6.8), and the residual activities were assessed under standard conditions.

enzyme at pH 2.0–11.0 and 30°C for 2 h. The enzyme was stable between pH 5.0 and 11.0 (Fig. 2B).

Effect of temperature on activity and stability The optimum temperature for the activity of the enzyme was 45°C (Fig. 2C). The thermal stability of the enzyme was examined after incubation in 50 mM phosphate buffer (pH 6.8) containing 0.1 M NaCl at various temperatures for 15 min. The enzyme was stable up to 30°C (Fig. 2D).

Substrate specificity The activities of the chitosanase on chitosan-related compounds are shown in Table 2. The enzyme hydrolyzed partially *N*-acetylated chitosan, but did not hydrolyze chitin or CMC. The chitosanase activity of the enzyme increased as the degree of chitosan deacetylation increased. We determined the rates at which the enzyme hydrolyzed GlcN oligosaccharides. The rates of hydrolysis of $(GlcN)_4$, $(GlcN)_5$, and $(GlcN)_6$ were 39.4, 50.2, and 62.3 μmol/min/mg, respectively. The enzyme did not hydrolyze $(GlcN)_3$ or $(GlcNAc)_6$. Therefore, the enzyme hydrolyzed chitooligosaccharides larger than tetra-saccharides. The activity of the enzyme increased as the degree of polymerization of GlcN increased.

TABLE 2. Substrate specificity of the chitosanase.

Substrate	Specific activity (units/mg)	Relative activity (%)
100% deacetylated chitosan	43.3	100
90% deacetylated chitosan	3.9	9.0
80% deacetylated chitosan	2.8	6.5
70% deacetylated chitosan	1.4	3.2
Powdered chitosan	0	0
Powdered chitin	0	0
CMC	0	0

The reactions were conducted under standard conditions with shaking. The amounts of reducing sugars liberated were measured as described in Materials and methods.

Mode of action of chitosanase The hydrolysates of chitosan produced by the purified enzyme were analyzed by TLC (Fig. 3). The enzyme produced only $(GlcN)_2$ throughout the entire course of chitosan hydrolysis. The TLC analysis suggested that the chitosan-degrading enzyme catalyzed exo-type cleavage to release a $(GlcN)_2$ molecule from the end of the chitosan. To determine whether the purified enzyme released $(GlcN)_2$ from the non-reducing terminal or the reducing terminal, we used HPLC to investigate the hydrolysis products of N^1-acetylchitohexaose [$(GlcN)_5$-GlcNAc] with a GlcNAc residue at the reducing end. As shown in Fig. 4A, the enzyme hydrolyzed $(GlcN)_5$-GlcNAc into $(GlcN)_2$ and $(GlcN)_3$-GlcNAc, which was further hydrolyzed into $(GlcN)_2$ and

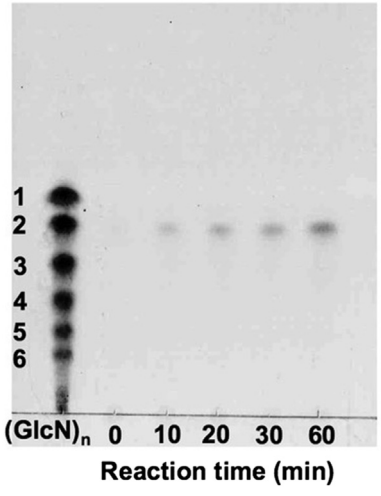

FIG. 3. Thin-layer chromatography (TLC) of the products resulting from the hydrolysis of chitosan by chitosanase. The experimental details are described in Materials and methods.

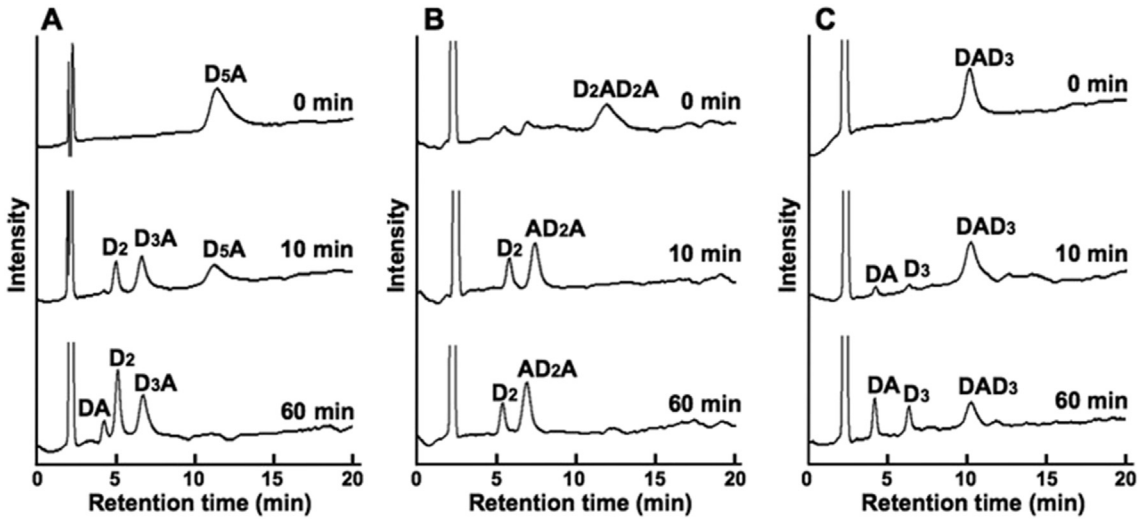

FIG. 4. High-performance liquid chromatography (HPLC) of the products resulting from the hydrolysis of $(GlcN)_5$-GlcNAc (A), $(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc (B), and GlcN-GlcNAc-$(GlcN)_3$ (C) by chitosanase. The experimental details are described in the MATERIALS AND METHODS section. D, GlcN; D_2, $(GlcN)_2$; DA, GlcN-GlcNAc; D_3, $(GlcN)_3$; D_3A, $(GlcN)_3$-GlcNAc; AD_2A, GlcNAc-$(GlcN)_2$-GlcNAc; DAD_3, GlcN-GlcNAc-$(GlcN)_3$; D_5A, $(GlcN)_5$-GlcNAc; D_2AD_2A, $(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc.

GlcN-GlcNAc. We did not detect $(GlcN)_4$ as a hydrolysis product. These results indicate that the chitosanase hydrolyzes β-1,4-glucosaminidic linkages in chitosan and chitooligosaccharides to remove successive $(GlcN)_2$ units from the non-reducing ends of the chains. Therefore, the new exo-type chitosanase was characterized as an exo-chitobiohydrolase. Sun et al. (29) reported that the chitosanase from *Paenibacillus dendritiformis* hydrolyzed chitosan to yield $(GlcN)_2$ as the major product. However, this chitosanase exhibited an endo-type cleavage pattern, which is different to that of exo-chitobiohydrolase. The exo-chitobiohydrolase would be an efficient tool for the production of chitobiose.

Hydrolysis of partially *N*-acetylated chitooligosaccharides by exo-chitobiohydrolase We also investigated the action of the exo-chitobiohydrolase on partially *N*-acetylated chitooligosaccharides. The hydrolysis products of chitooligosaccharides were analyzed by HPLC (Fig. 4B, 4C). The enzyme hydrolyzed $(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc into $(GlcN)_2$ and GlcNAc-$(GlcN)_2$-GlcNAc (Fig. 4B). The hydrolysis products of GlcN-GlcNAc-$(GlcN)_3$ were GlcN-GlcNAc and $(GlcN)_3$ (Fig. 4C). GlcN-$(GlcNAc)_2$-GlcN-GlcNAc was not hydrolyzed by the enzyme (data not shown). These results indicate that the exo-chitobiohydrolase splits the second β-1,4-glycoside linkages from the non-reducing end of the chitooligosaccharides, and can split GlcN-GlcN, GlcN-GlcNAc, and GlcNAc-GlcN linkages in the substrates. Endo-chitosanase can be classified into four subclasses I, II, III, and IV according to their hydrolytic specificity toward N^1,N^4-diacetylchitohexaose; subclass I chitosanases split both GlcN-GlcN and GlcNAc-GlcN bonds; subclass II chitosanases split only the GlcN-GlcN bond; subclass III chitosanases split both GlcN-GlcN and GlcN-GlcNAc bonds; and subclass IV chitosanases split GlcNAc-GlcN, GlcN-GlcNAc, and GlcN-GlcN bonds (21). Exo-β-D-glucosaminidase hydrolyzes both GlcN-GlcN and GlcN-GlcNAc bonds (14,17). The hydrolytic specificity of the exo-chitobiohydrolase was similar to the specificities of subclass IV chitosanases from *Amicolatopsis* sp. CsO-2 and *Pseudomonas* sp. A-01 (21). Exo-β-D-glucosaminidase catalyzes transglycosylation reactions as well as hydrolytic reactions (17,30). The exo-chitobiohydrolase had no transglycosylation activity (data not shown).

As shown in Table 3, the rates of hydrolysis of $(GlcN)_2$-GlcNAc-$(GlcN)_3$, $(GlcN)_5$-GlcNAc, and $(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc were higher than the rate of hydrolysis of $(GlcN)_6$. However, the rate of hydrolysis of GlcN-GlcNAc-$(GlcN)_3$ was lower than that of $(GlcN)_6$. The results suggest that the *N*-acetyl groups of the sugar residues located at the (−1), (+1), and (+4) sites influence the catalytic reaction. Further kinetic analyses are required to clarify the sugar recognition specificity of the enzyme. The enzyme did not hydrolyze partially *N*-acetylated chitooligosaccharides with GlcNAc residues at the non-reducing ends, such as GlcNAc-$(GlcN)_2$-GlcNAc and GlcNAc-$(GlcN)_4$-GlcNAc (Fig. 4B, Table 3). The results indicated that the subsite (−2) must have absolute specificity for GlcN. However, more information is required for a full understanding of substrate recognition and the catalytic mechanism of the exo-chitobiohydrolase based on its three-dimensional structure.

Most fungal cell walls are primarily composed of chitin, glucan, and glycoproteins. Chitin synthase, chitinase, and β-*N*-acetylhexosaminidase are actively involved in cell wall synthesis and remodeling (31,32). However, *G. butleri*, belonging to Mucoromycotina, contains chitosan in its cell wall (33). The exo-chitobiohydrolase produced by *G. butleri* may play a role in cell wall remodeling.

We are now investigating the cloning and nucleotide sequence of this exo-chitobiohydrolase, in an attempt to clarify the structure and biological function of this unique chitosanase family.

TABLE 3. Hydrolysis of partially *N*-acetylated chitooligosaccharides with the exo-chitobiohydrolase.

Substrate	Relative rate (%)
$(GlcN)_6$	50.6
$(GlcN)_2$-GlcNAc-$(GlcN)_3$	80.4
$(GlcN)_5$-GlcNAc	98.5
$(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc	100
GlcN-GlcNAc-$(GlcN)_3$	10.8
GlcNAc-$(GlcN)_2$-GlcNAc	0
GlcNAc-$(GlcN)_4$-GlcNAc	0
$(GlcNAc)_6$	0

The reaction mixture, consisting of 20 μL of 10 mM substrate solution, 10 μL of 5 mM phosphate buffer (pH 6.0), and 10 μL of enzyme solution, was incubated at 40°C for 20 min. The amounts of hydrolysis products were determined by HPLC.

ACKNOWLEDGMENT

We wish to thank Ms. Saori Chihara for technical assistance. The authors declare no conflict of interest.

References

1. **Kim, S. K., Rajapakse, N., and Shahidi, F.:** Production of bioactive chitosan oligosaccharides and their potential use as nutraceuticals, pp. 183–194, in: Barrow, C. and Shahidi, F. (Eds.), Marine nutraceuticals and functional foods. CRC Press, Boca Raton (2008).
2. **Yabuki, M., Uchiyama, A., Suzuki, K., Ando, A., and Fujii, T.:** Purification and properties of chitosanase from *Bacillus circulans* MH-K1, J. Gen. Appl. Microbiol., **34**, 255–270 (1988).
3. **Boucher, I., Dupuy, A., Vidal, P., Neugebauer, W. A., and Brzezinski, R.:** Purification and characterization of a chitosanase from *Streptomyces* N174, Appl. Microbiol. Biotechnol., **38**, 188–193 (1992).
4. **Izume, M., Nagae, S., Kawagishi, H., Mitsutomi, M., and Ohtakara, A.:** Action pattern of *Bacillus* sp. No. 7-M chitosanase on partially *N*-acetylated chitosan, Biosci. Biotechnol. Biochem., **56**, 448–453 (1992).
5. **Okajima, S., Ando, A., Shinoyama, H., and Fujii, T.:** Purification and characterization of an extracellular chitosanase produced by *Amycolatopsis* sp. CsO-2, J. Ferment. Bioeng., **77**, 617–620 (1994).
6. **Tanabe, T., Morinaga, K., Fukamizo, T., and Mitsutomi, M.:** Novel chitosanase from *Streptomyces griseus* HUT 6037 with transglycosylation activity, Biosci. Biotechnol. Biochem., **67**, 354–364 (2003).
7. **Ando, A., Saito, A., Arai, S., Usuda, S., Furuno, M., Kaneko, N., Shida, O., and Nagata, S.:** Molecular characterization of a novel family-46 chitosanase from *Pseudomonas* sp. A-01, Biosci. Biotechnol. Biochem., **72**, 2074–2081 (2008).
8. **Kobayashi, T., Koide, O., Deguchi, S., and Horikoshi, K.:** Characterization of chitosanase of a deep biosphere *Bacillus* strain, Biosci. Biotechnol. Biochem., **75**, 669–673 (2011).
9. **Kim, S. Y., Shon, D. H., and Lee, K. H.:** Purification and characterization of two types of chitosanase from *Aspergillus fumigatus* KH-94, J. Microbiol. Biotechnol., **8**, 568–574 (1998).
10. **Cheng, C. Y. and Li, Y. K.:** An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides, Biotechnol. Appl. Biochem., **32**, 197–203 (2000).
11. **Eom, T. K. and Lee, K. M.:** Characteristic of chitosanase from *Aspergillus fumigatus* KB-1, Arch. Pharm. Res., **26**, 1036–1041 (2003).
12. **Chen, X., Xia, W., and Yu, X.:** Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326, Food Res. Int., **38**, 315–322 (2005).
13. **Nanjo, F., Katsumi, R., and Sakai, K.:** Purification and characterization of an exo-β-D-glucosaminidase, a novel type of enzyme, from *Nocardia orientalis*, J. Biol. Chem., **265**, 10088–10094 (1990).
14. **Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H., and Morikawa, Y.:** Purification and characterization of exo-β-D-glucosaminidase from a cellulolytic fungus, *Trichoderma reesei* PC-3-7, Appl. Environ. Microbiol., **64**, 890–895 (1998).
15. **Zhang, X.-Y., Dai, A.-L., Zhang, X.-K., Kuroiwa, K., Kodaira, M., Shimosaka, M., and Okazaki, M.:** Purification and characterization of chitosanase and exo-β-D-glucosaminidase from a Koji mold, *Aspergillus oryzae* IAM2660, Biosci. Biotechnol. Biochem., **64**, 1896–1902 (2000).
16. **Côté, N., Fleury, A., Dumont-Blanchette, E., Fukamizo, T., Mitsutomi, M., and Brzezinski, R.:** Two exo-β-D-glucosaminidases/exochitosanases from actinomycetes define a new subfamily within family 2 of glycoside hydrolases, Biochem. J., **394**, 675–686 (2006).
17. **Fukamizo, T., Fleury, A., Côté, N., Mitsutomi, M., and Brzezinski, R.:** Exo-β-D-glucosaminidase from *Amycolatopsis orientalis*: catalytic residues, sugar recognition specificity, kinetics, and synergism, Glycobiology, **16**, 1064–1072 (2006).
18. **Watanabe, K.:** Isolation and identification of a microorganism which produces a novel chitosan-hydrolyzing enzyme, exo-chitobiohydrolase, Chitin Chitosan Res, **8**, 252–258 (2002) (in Japanese).
19. **Mitsutomi, M., Ueda, M., Nakazawa, M., Miyatake, K., and Seki, K.:** Enzymatic preparation of mono-*N*-acetylated chitooligosaccharides, pp. 516–521, in: Domard, A., Guibal, E. K., and Vårum, K. M. (Eds.), Advances in chitin science IX (2006).
20. **Seki, K., Tateishi, K., and Uchida, Y.:** Action pattern of chitosanase from *Bacillus amyloliquefaciens* UTK on partially *N*-acetylated chitosan, Bull. Fac. Agric. Saga Univ., **85**, 109–116 (2000).
21. **Hirano, K., Watanabe, M., Seki, K., Ando, A., Saito, A., and Mitsutomi, M.:** Classification of chitosanases by hydrolytic specificity toward N^1,N^4-diacetylchitohexaose, Biosci. Biotechnol. Biochem., **76**, 1932–1937 (2012).
22. **Saito, J., Kita, A., Higuchi, Y., Nagata, Y., Ando, A., and Miki, K.:** Crystal structure of chitosanase from *Bacillus circulans* MH-K1 at 1.6-Å resolution and its substrate recognition mechanism, J. Biol. Chem., **274**, 30818–30825 (1999).
23. **Imoto, T. and Yagishita, K.:** A simple activity measurement of lysozyme, Agric. Biol. Chem., **35**, 1154–1156 (1971).
24. **Rondle, C. J. M. and Morgan, W. T. J.:** The determination of glucosamine and galactosamine, Biochem. J., **61**, 586–589 (1955).
25. **Bradford, M. M.:** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., **72**, 248–254 (1976).
26. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, **227**, 680–685 (1970).
27. **Zhou, W., Yuan, H., Wang, J., and Yao, J.:** Production, purification and characterization of chitosanase produced by *Gongronella* sp. JG, Lett. Appl. Microbiol., **46**, 49–54 (2008).
28. **Wang, J., Zhou, W., Yuan, H., and Wang, Y.:** Characterization of a novel fungal chitosanase Csn 2 from *Gongronella* sp. JG, Carbohydr. Res., **343**, 2583–2588 (2008).
29. **Sun, H., Mao, X., Guo, N., Zhao, L., Cao, R., and Liu, Q.:** Discovery and characterization of a novel chitosanase from *Paenibacillus dendritiformis* by phylogeny-based enzymatic product specificity prediction, J. Agric. Food Chem., **66**, 4645–4651 (2018).
30. **Matsumura, S., Yao, E., and Toshima, K.:** One-step preparation of alkyl β-D-glucosaminide by the transglycosylation of chitosan and alcohol using purified exo-β-D-glucosaminidase, Biotechnol. Lett., **21**, 451–456 (1999).
31. **Rast, D. M., Baumgartner, D., Mayerc, C., and Hollenstein, G. O.:** Cell wall-associated enzymes in fungi, Phytochemistry, **64**, 339–366 (2003).
32. **Bowman, S. M. and Free, S. J.:** The structure and synthesis of the fungal cell wall, Bioessays, **28**, 799–808 (2006).
33. **Maw, T., Tan, T. K., Khor, E., and Wong, S. M.:** Selection of *Gongronella butleri* strains for enhanced chitosan yield with UV mutagenesis, J. Biotechnol., **95**, 189–193 (2002).