Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 72 (2008) 513–520
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Purification and characterization of chitosanase from *Bacillus cereus* D-11

Xing-Ai Gao, Wan-Taek Ju, Woo-Jin Jung, Ro-Dong Park *

Glucosamine Saccharide Materials – National Research Laboratory (GSM – NRL), Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, Chonnam National University, Gwangju 500-757, South Korea

Received 7 August 2007; received in revised form 15 September 2007; accepted 21 September 2007
Available online 1 October 2007

Abstract

A chitosanase-producing bacterium was isolated from Taiwan soils and identified as *Bacillus cereus* D-11 based on the biochemical properties and 16S rRNA gene sequence. The optimal medium for enzyme production consisted of 0.7% colloidal chitosan, 1% yeast extract, and 1% NaCl at an initial pH of 7.0 with 0.5% inoculation concentration (1.4×10^8 CFU/ml). After cultivation at 30 °C for 3 days, the maximal activity of 4.85 U/ml was observed. An extracellular chitosanase produced from *B. cereus* D-11 was concentrated by lyophilization and purified by Sephadex G-150 gel filtration and CM-Sephadex ion exchange column chromatography. The molecular weight of the purified chitosanase was estimated to be 41 kDa by 12.5% SDS–PAGE. The optimal pH and temperature for the chitosanase were 6.0 and 60 °C, respectively. The enzyme was stable below 50 °C and from pH 5 to 10. N-terminal amino acid sequence exhibited highest homology to the chitosanases belonging to glycoside hydrolase family 8. The enzyme was inhibited by 10 mM 2-hydroxy-5-nitrobenzyl bromide but activated by phenylglyoxal and chloramine T. The K_m and V_{max} values were 7.5 mg/ml and 2.15×10^{-7} mol/mg/s for soluble chitosan (degree of deacetylation, DD 86%) as substrate. The D-11 chitosanase degraded chitosan with DD ranging from 70% to 100%, but did not degrade chitin. The most susceptible substrate was 86% deacetylated chitosan. Furthermore, the D-11 chitosanase inhibited the mycelial growth of *Rhizoctonia solani* on PDA medium.

Keywords: *Bacillus cereus* D-11; Chitosanase; Antifungal activity; Glycoside hydrolase family 8; Degree of deacetylation

1. Introduction

Chitosan is a linear polysaccharide composed of β-(1→4)-linked D-glucosamine residues. In nature, the polymer is partially acetylated and, in fact, the name chitosan describes a wide range of polymers with various proportions of D-glucosamine and *N*-acetyl-D-glucosamine residues (Pelletier & Sygusch, 1990).

Previously, several microorganisms including bacteria were reported to efficiently produce chitosanases (EC 3.2.1.132) to degrade chitosan to glucosamine oligomers (Jo et al., 2003; Kim, Kang, Chung, Kim, & Chung, 2004). Furthermore, fungi (Chen, Xia, & Yu, 2005; Zhang et al., 2000) and actinomycetes (Okajima, Ando, Shinoyama, & Fujii, 1994; Shimosaka, Nogawa, Wang, Kumehara, & Okazaki, 1995) also produce chitosan oligomers from chitosan. These chitosanases belong to various GH (glycosyl hydrolase) families including GH-5, GH-8, GH-46, GH-75, and GH-80, according to their amino acid sequences (Jung, Kuk, Kim, Park, & Park, 2005). The characteristics of the chitosanases produced from *Bacillus cereus* P16 (Jo et al., 2003), *Bacillus* sp. GM44 (Choi, Kim, Piao, Yun, & Shin, 2004), *Bacillus* sp. KSM-330 (Ozaki, Sumitomo, & Ito, 1991), *Bacillus circulans* WL-12 (Mitsutomi et al., 1998), and *Bacillus* sp. 7-M (Uchida & Ohtakara, 1988) have been well investigated and belong to the GH-8.

Chitosanases from individual organisms differ in their pattern of hydrolytic activity. Most microbial chitosanases

* Corresponding author. Tel.: +82 62 530 2133; fax: +82 62 530 0876.
E-mail address: rdpark@chonnam.ac.kr (R.-D. Park).

0144-8617/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.09.025

catalyze an endo-type cleavage reaction in which the relative velocity is highly dependent on the deacetylation of chitosan (Shimosaka et al., 1995). Recently, much attention has been paid to converting chitosan to safe and functional chitooligosaccharides, because chitooligosaccharides show strong physiological activities, such as antitumor effects (Maeda & Kimura, 2004) and antimicrobial activity (Wang et al., 2007). Application of novel and potential chitosanases from chitosanase-producing microorganisms has recently been one of the advanced and alternative approaches for producing chitosan oligomers.

The purpose of this study was to isolate chitosanase-producing bacteria, then to purify and characterize the chitosanase from *B. cereus* D-11, which has high chitosanolytic potential.

2. Materials and methods

2.1. Materials

Chitosans with various deacetylation degrees were purchased from Taehoon Bio Ltd. (Seoul, Korea). The chitosan used in this report was 86% deacetylated unless otherwise stated. Chitosan oligomers $(GlcN)_{1-7}$ were purchased from Wako Chemicals (Osaka, Japan). Sephadex G-150 and CM-Sephadex were purchased from Pharmacia and Sigma, respectively. Buffer solutions and other chemicals were reagent grade. *Rhizoctonia solani* AG-1 (B) Khun (KACC 40111) was obtained from Korea Agricultural Culture Collection (KACC).

2.2. Isolation and identification of chitosanolytic bacterium

One bacterium D-11 was screened from Taiwan soil samples. Serially diluted soil samples were inoculated on plates containing 1% NaCl, 1% tryptone, 0.5% colloidal chitosan (basic medium) and 2% agar and incubated at 30 °C for 3 days (Jo et al., 2003). A single colony showing prominent chitosanolytic activity was selected and subjected to taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology* (Sneath, Mair, Sharpe, & Holt, 1986). To further identify the bacterium, polymerase chain reaction (PCR) was performed to amplify part of the bacterial 16S rRNA gene. The forward and reverse primers were 5′-ACGGCTACCTTGTTACGACT-3′ and 5′-CCC ACTGCCTCCCGTAAGGAGT-3′, respectively. The PCR product was cloned using pGEM-T Easy vector (Promega, USA). The nucleotide sequence of the 16S rRNA gene of D-11 was determined by an ABI Prism 377 DNA Sequencer (PE Applied Biosystems, USA) and compared with published 16S rRNA sequences in a NCBI BLAST search.

2.3. Purification of chitosanase

D-11 was cultured in a medium containing 0.5% colloidal chitosan, 1% NaCl, and 1% tryptone (basic medium) at 30 °C for 3 days. The crude enzyme after concentration was lyophilized, dissolved in an appropriate volume of 50 mM sodium acetate buffer at pH 6.0, and then dialyzed. The solution was loaded onto a Sephadex G-150 gel filtration column (2.5 × 40 cm) which was equilibrated with the same buffer. The active fractions were further purified on a CM-Sephadex column (2.5 × 40 cm). The proteins were eluted at a flow rate of 0.9 ml/min, and 4-ml fractions were collected. The protein profile was monitored by the absorbance at 280 nm. Chitosanase activity was assayed using soluble chitosan (DD 86%) as a substrate (Imoto & Yagishita, 1971). Each reaction mixture contained 0.9 ml of 1% soluble chitosan, 80 μl of 100 mM sodium acetate buffer (pH 6), and 20 μl of enzyme solution. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 200 μl of 1 M NaOH. The amount of reducing sugar was determined by modification of Schale's method (Imoto & Yagishita, 1971) or the dinitrosalicylic acid method (Miller, 1959) with glucosamine as a standard. One unit of chitosanase was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute. Protein concentration was measured by Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

2.4. Characterization of the purified chitosanase

To determine optimum temperature, reaction mixtures were incubated at various temperatures for 30 min in the acetate buffer, pH 6.0, as described by Jo et al., 2003. To determine temperature stability, the residual activity was measured after pre-incubation of the enzyme at various temperatures for 1 h at pH 6.0 in the absence of substrate. For determination of the optimum pH, enzyme solutions were incubated with soluble chitosan in various pH buffers at 37 °C for 30 min. Mcllvaine buffer (pH 3.5), sodium acetate buffer (pH 4–6), Tris–HCl buffer (pH 7–8), and carbonate buffer (pH 9–11) were used for pH adjusting. For pH stability, the enzyme solutions were preincubated for 1 h at 4 °C in various pH buffers (50 mM). After adjusting to pH 6.0 with the acetate buffer, the enzyme solutions were incubated with substrate at 37 °C for 30 min. For substrate specificity, enzyme activity was determined toward several chitosan-derived and non-chitosan substrates (0.5% w v^{-1}). For metal ion effect, the chitosanase was pre-incubated with 10 mM metal ions, such as Mg^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+}, and Hg^{2+}. For kinetic constants, 20 μl of enzyme (4.2 U/ml) was incubated with soluble chitosan at concentrations between 0.5 and 2.5 mg/ml at 37 °C for 1 h. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined by Lineweaver–Burk transformation (Price & Storck, 1975).

2.5. Modification of amino acid residues

The amino acid residues or functional groups were modified by incubating the purified enzyme (2.0 mU) with several modifying reagents at 25 °C in the respective buffers

according to the procedures (In & Jung, 1998). After termination of reaction, residual enzyme activity was determined as describe above.

2.6. Mycelial growth inhibition

The inhibition of mycelial growth by the purified chitosanase was tested. *Rhizoctonia solani* AG-1 was grown on potato dextrose agar (PDA) medium, and a *R. solani* disk (1 cm diameter) was then placed in the center of the plates. The purified enzymes (1.6, 3.2, and 4.8 μg of protein) were then spotted on the plates. The plates were incubated at 27 °C for 3 days (Jung, Kuk, Kim, Kim, & Park, 2005).

2.7. Analytical methods

The chitosanase purity was examined by 12.5% SDS–PAGE using the Laemmli method (Laemmli, 1970). After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) nylon membrane (pore size, 0.45-μm; Hybond-N^+, Amersham Biosciences) using the electroblotting system. The N-terminal sequence of the purified protein was analyzed with the Applied Biosystems Procise Sequencer (Macintosh HD, USA). The viscosity change was measured according to Jo et al., 2003 using a Brookfield synchrolectric viscometer (Model LVT, Spindle No. 18 USA) at set intervals. The reaction mixtures contained 7.0 ml of 1% soluble chitosan (DD 86%) in 100 mM sodium acetate buffer (pH 6.0) and 200 μl of enzyme solution. The reactions were performed at 35 °C in the viscometer, and the viscosity of each mixture was measured at set intervals.

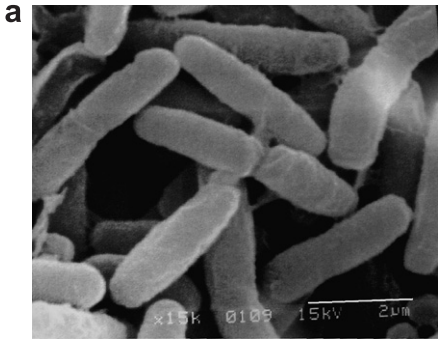
3. Results and discussion

3.1. Identification of microorganism

Bacterial isolate D-11 was subjected to a taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology* (Sneath et al., 1986), and identified as a member of the genus *Bacillus*. This taxonomic identification was based mainly on the following criteria. The organism was Gram-positive, aerobic, rod-shape (0.9–1 μm × 2.8–3.6 μm), motile, and catalase-positive, as shown in Table 1. The isolate was found not to grow in 5% NaCl. The nucleotide sequence of a conserved segment of 16S rRNA gene of D-11 was determined and submitted to GenBank (Accession No. EU050990). On the basis of the sequence, D-11 was identified as *B. cereus*. Within 1197, no base difference of 16S rRNA sequences between D-11 and *B. cereus* (ID AF176322) was detected. From the above results, D-11 was concluded to be most similar to *B. cereus* with more than 95% confidence (Fig. 1). Therefore, isolate D-11 was designated as *B. cereus* D-11. The previously described chitosanase-producing bacterial strains are members of the following genera: *Acinetobacter* (Shimosaka et al., 1995), *Amycolatopsis* (Okajima et al., 1994), *Bacillus*

Table 1
Morphological, biological and physiological characteristics of *Bacillus cereus* D-11

Characteristics	Result
Size	0.9–1 μm width 2.8–3.6 μm length
Gram staining	+
Sporangium	−
Spore	−
Catalase	+
Urease	+
Voges–Proskauer test	+
D-Glucose	+
D-Mannitol	+
Glycerol	−
Sucrose	−
Hydrolysis of casein	+
Hydrolysis of starch	+
Utilization of citrate	+
Formation of indole	−
Growth in NaCl (%)	
2	+
5	−
7	−
Growth at pH	
4.5	−
9	+
11	+
Growth at °C	
5	−
10	−
45	+
50	−



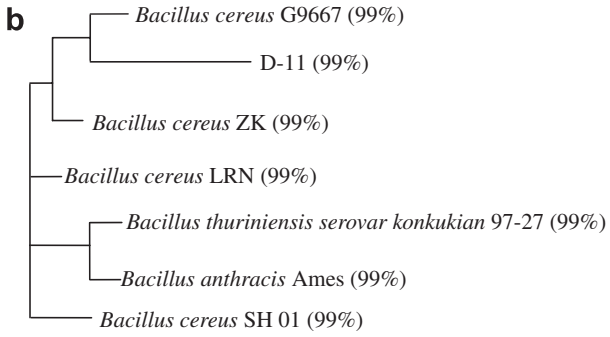


Fig. 1. (a) Scanning electron micrograph of *B. cereus* D-11. The bar indicates 2 μm. (b) Phylogenetic tree of 16S rRNA gene of the isolate D-11.

(Kim et al., 2002; Kwon, Park, Kim, & Nam, 2002; Lee et al., 1996; Mitsutomi et al., 1998; Pelletier & Sygusch, 1990; Seino, Tsukuda, & Shimasue, 1991; Tominaga & Tsujisaka, 1975; Uchida, Izume, & Ohtakara, 1989; Yabuki, Uchiyama, Suzuki, Andou, & Fujii, 1988), *Enterobacter* (Yamasaki et al., 1992), *Myxobacter* (Hedges & Wolfe, 1974), *Nocardia* (Sakai, Katsumi, Isobe, & Nanjo, 1991), *Pseudomonas* (Yoshihara, Hosokawa, Kubo, Nishiyama, & Koba, 1992), and *Streptomyces* (Boucher, Dupuy, Vidal, Neugehauer, & Brzezinski, 1992; Price & Storck, 1975).

3.2. Chitosanase production

The D-11 was investigated for extracellular chitosanase productivity in a media containing various carbon and nutrient sources. Colloidal chitosan (0.7%) and 1% yeast extract were the best carbon and nutrient sources found, respectively. Chitosanase activity was the highest at 30 °C among various temperatures (20–42 °C) tested in a shaking incubator (200 rpm) for the bacterium (data not shown). This strain grew well in a glucose medium that produced about 90% level of chitosanase compared with that in the chitosan-containing medium. It has been known that adding glucose will catabolically repress the chitosanase production, as in the *Aureobacterium* sp. (Lee, Lee, & Lee, 2000). The maximum level of chitosanase activity in the culture fluid after cultivation at 30 °C for 3 days was 4.85 U/ml (Fig. 2). It is exceptionally higher when compared with other *B. cereus* (2.0–3.8 U/ml) (Chang, Chen, & Jao, 2007; Jo et al., 2003), *Aureobacterium* sp. (15–30 mU/ml) (Lee et al., 2000), *Bacillus megaterium* P1 (1 U/ml) (Pelletier & Sygusch, 1990), and *Pseudomonas* sp. H-14 (650 mU/ml) (Yoshihara et al., 1992). In fact, this suggests its potential applicability for industrial production of chitooligosaccharides.

The chitosanase activity remained low (less than 0.5 U/ml) at the early stages of lag and exponential phases but it began to increase when the cultures reached the stationary phase. Chitosanase activity was hardly found in the homogenate of the cells, and what this implies is that all the chitosanase activity is secreted into the extracellular culture fluid just after synthesis and modification of the protein(s) in the bacterial cells. The culture supernatant contained marginal activities of chitinase and β-*N*-acetylhexosaminidase.

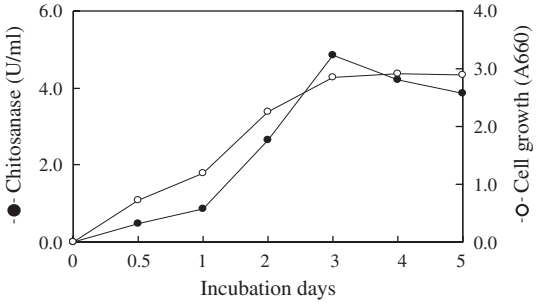


Fig. 2. Day-course of cell growth and chitosanase activity. D-11 was grown aerobically in broth medium at 30 °C for 5 days.

The culture supernatant of D-11 showed strong liquefying activity of the soluble chitosan. The liquefaction of chitosan largely resulted from the endo-cleavage activity of chitosanases. By activity staining after SDS–PAGE, 5 endo-type chitosanase isozymes were found in the culture supernatant (data not shown). Microbial multiple chitosanases are reported from *B. cereus* P16 (Jo et al., 2003), *B. circulans* WL-12 (Mitsutomi et al., 1998), *B. megaterium* P1 (Pelletier & Sygusch, 1990), *Aspergillus fumigatus* KH-94 (Kim, Shon, & Lee, 1998), *Mucor rouxii* (Alfonso, Martinez, & Reyes, 1992), and *Acinetobacter* sp. CHB101 (Shimosaka et al., 1995). Five chitosanases which were identified by active staining from the culture supernatant of P16 might be originated from multiple genes, but further study is needed to elucidate the relationship of the gene structure and isozyme multiplicity (Jo et al., 2003).

The D-11 grew in a medium containing not only colloidal chitosan and chitosan powder but also swollen chitin and chitin powder as carbon source, suggesting that the isolate also produced chitinolytic enzymes. In addition, since the culture supernatant of D-11 contained endo-type chitosanases, chitinase, and β-*N*-acetylhexosaminidase, it is suggested that this organism could degrade chitinous polymers with a broad range of DD values to support growth.

3.3. Purification of chitosanase

An extracellular chitosanase was purified from the culture supernatant (1820 ml) of *B. cereus* D-11 by Sephadex G-150 and CM-Sephadex chromatography (Fig. 3). The purification steps are summarized in Table 2. At the final step, the chitosanase was purified by 8.8-fold and the specific activity was 347.8 U/mg. The specific activities were 126 U/mg in *Bacillus* sp. Strain CK4 (Yoon et al., 2001)

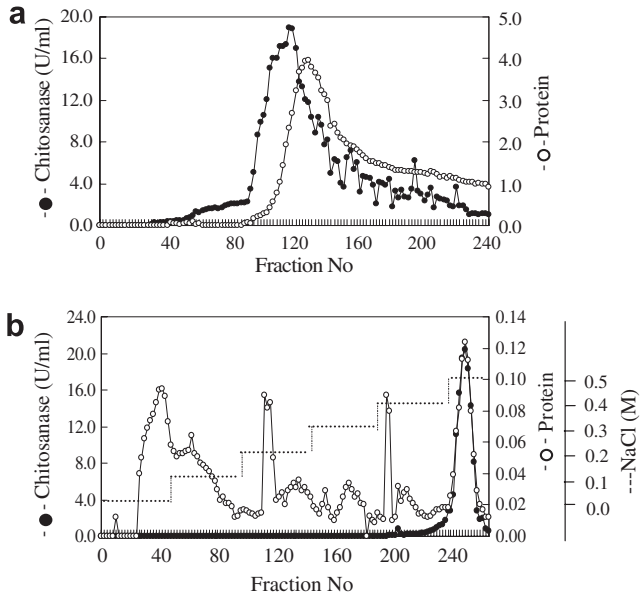


Fig. 3. (a) Sephadex G-150 column chromatography and (b) CM-Sephadex column chromatography.

Table 2
Chitosanase activity profile of *B. cereus* D-11 after purification

Purification steps	T-protein (mg)	T-activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	225.0	8834.0	39.3	1.0	100.0
Lyophilization and dialysis	87.0	7564.0	86.8	2.2	85.6
Sephadex G-150	13	1868.1	143.7	3.7	21.1
CM-Sephadex	1.0	347.8	347.8	8.8	3.9

and 93.6 U/mg in *Bacillus* sp. S65 (Su, Wang, Yao, & Yu, 2006). Protein and activity staining after SDS–PAGE revealed that the enzyme was homogeneous and the apparent molecular weight was 41 kDa (Fig. 4). The molecular weights of most endo-chitosanases have been reported to range from 20 to 50 kDa by SDS–PAGE, while exo-chitosanases ranged from 97 to 135 kDa (Chen et al., 2005). The N-terminal sequence of the D-11 chitosanase was A-A-A-K-E-M-K-P-F-P-Q-Q-V-N-Y-A-G-V-I-K and fully matched the chitosanases from *B. cereus* ATCC 14579 (Ivanova et al., 2003) and *Bacillus* sp. GM44 (Choi et al., 2004). Both they belong to family 8 glycosyl hydrosylase.

3.4. Effect of pH and temperature

The purified chitosanase D-11 had an optimal pH of 6.0 when assayed with soluble chitosan as a substrate (Fig. 5). A sharp drop in activity was observed at pH values higher than 7 or lower than 4. The enzyme was stable in the pH range of 6–10 for 1 h at 4 °C in the various pH buffers. Similar optimal pHs were obtained between pH 4.5 and 6.5 for *B. megaterium* strain p1 (Pelletier & Sygusch, 1990); between pH 5.2 and 7.8 for *Microbacterium* sp. OU01 (Sun, Liu, Han, Zhang, & Liu, 2006) and between pH 5.5 and 6.0 for *Bacillus* sp. HW-002 (Lee et al., 1996). When the enzyme was assayed at various temperatures, the optimal temperature was 60 °C. The chitosanase was stable below 50 °C. Furthermore about 70% of the initial activity remained after heating at 60 °C for 1 h (Fig. 5). The chitosanase from *Bacillus* sp. HW-002 (Lee et al., 1996) was stable up to 45 °C, and about 20% of its original activity remained after it was treated at 50 °C for 1 h.

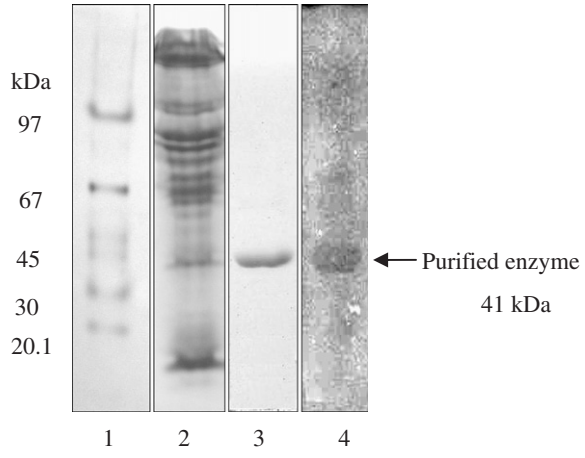


Fig.4. SDS–PAGE of proteins during purification. Lane 1, molecular weight marker; lane 2, crude enzyme; lane 3, purified enzyme obtained from CM-Sephadex column chromatography; lane 4, activity staining of the purified enzyme.

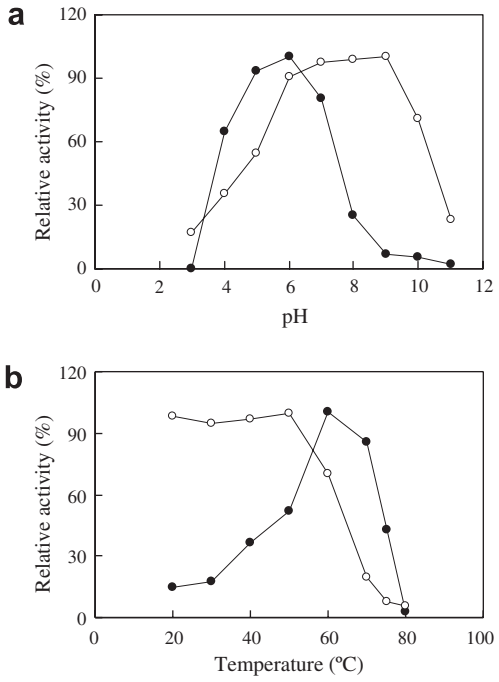


Fig. 5. (a) Optimal pH (-●-) and pH stability (-○-). (b) Optimal temperature (-●-) and temperature stability (-○-).

3.5. Effect of substrate specificity

For the substrate specificity of the purified enzyme, chitin, and chitosans with degree of deacetylation ranging from 74% to 98.5% were used as substrates, as summarized in Table 3. The enzyme efficiently hydrolyzed soluble and colloidal chitosans, but exhibited little activity on glycol chitosan, chitosan powder, and chitin. The chitosanases from

Table 3
Substrate specificity of the chitosanase purified from *B. cereus* D-11

Substrate	Relative activity (%)
Soluble chitosan (DD 98.5%)	84.1
Soluble chitosan (DD 86%)	100.0
Soluble chitosan (DD 74%)	65.3
Colloidal chitosan (DD 86%)	86.9
Carboxymethyl cellulose	75.6
Glycol chitosan	8.8
Chitosan powder (40 mesh)	1.9
Swollen chitin	0.2

Amycolatopsis sp. CsO-2 (Okajima et al., 1994), *Nocardioides* sp. K-01 (Okajima, Kinouchi, Mikami, & Ando, 1995), and *Bacillus* sp. CK4 (Yoon et al., 2001) were most active on approximately 100% deacetylated chitosan. The chitosanases from *Acinetobacter* sp. CHB101 (Shimosaka et al., 1995) and *Bacillus* sp. P16 (Jo et al., 2003) were most active on approximately 80% deacetylated chitosan. The most susceptible substrate for D-11 chitosanase was 86% deacetylated chitosan, suggesting that D-11 chitosanase has specificity to the linkages of GlcN-GlcN and GlcNAc-GlcN and/or GlcN- GlcNAc, and the *N*-acetylglucosamine residues are important in the recognition and reaction mechanism of the substrate by the enzyme (Jo et al., 2003). Surprisingly, D-11 chitosanase showed a hydrolysis activity for carboxymethylcellulose (CMC) comparable to soluble chitosan (DD 74%). The CMC activity is a criterion for family 8 hydrolases (Jung et al., 2005).

3.6. Effect of ions and inhibitors

The chitosanase was almost completely inactivated by Hg^{2+} and significantly inactivated by Pb^{2+} and Cu^{2+} at 10 mM concentration. However, the other ions little affected the enzyme activity (data not shown). The enzyme was inactivated when incubated with amino acid modifiers such as 2-hydroxy-5-nitrobenzylbromide (49%) and diethylpyrocarbonate (22%) at 10 mM (Table 4). 2-Hydroxy-5-nitrobenzylbromide is a tryptophan residue modifier, thus suggesting that a tryptophan residue may be involved in the catalytic activity of the enzyme. The chitosanases from *Bacillus* sp. P-16 (Jo et al., 2003) and *Aspergillus fumigatus* S-26 (Jung, Kuk, Kim, Jung, & Park, 2006) were also inhibited by 2-hydroxy-5-nitrobenzylbromide. However, phenylgloxal, chloramine T (a methionine residue modifier) and *p*-hydroxymercuribenzoic acid activated the enzyme activity. On the contrary, chloramine T once has been reported as an inhibitor for a chitosanase of *Bacillus* sp. P-16 (Jo et al., 2003).

3.7. Other characteristics

The purified enzyme (4.2 U/ml) was incubated with various concentrations of soluble chitosan (DD 86%) for 30 min at 37 °C and a Lineweaver–Burk plot was constructed. Kinetic parameters were then determined: K_m, 7.5 mg/ml; V_{max}, 2.15×10^{-7} mol/mg/s; K_{cat}, 1.73×10^4 s^{-1}; and K_{cat}/K_m, 2.31×10^3 ml/mg/s (Fig. 6).

The viscosity of soluble chitosan (DD 86%) rapidly decreased in accordance with increasing amounts of enzyme (Fig. 7). The chitosanase extensively reduced the viscosity of the chitosan solutions at an early stage of the reaction and the rate of viscosity reduction was slower

Table 4
Effect of inhibitors on the chitosanase from *B. cereus* D-11

Inhibitors (10 mM)	Relative activity (%)
None	100
Phenylglyoxal	138
Chloramine T	135
p-Hydroxymercuribenzoic acid	130
Iodoacetic acid	108
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide	101
N-Bromosuccinimide	97
N-Acetylimidazole	85
N-Ethylmaleimide	84
Diethylpyrocarbonate	78
2-Hydroxy-5-nitrobenzylbromide	51

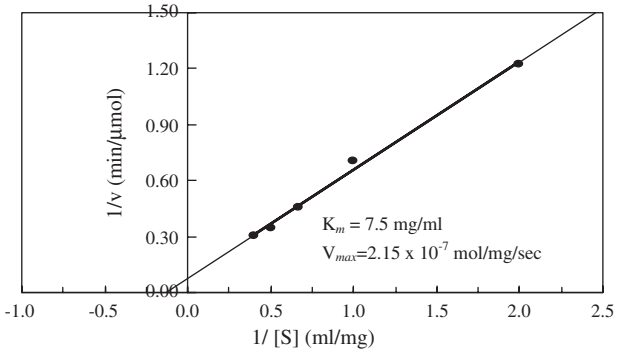


Fig. 6. Lineweaver–Burk plot of *B. cereus* D-11 chitosanase.

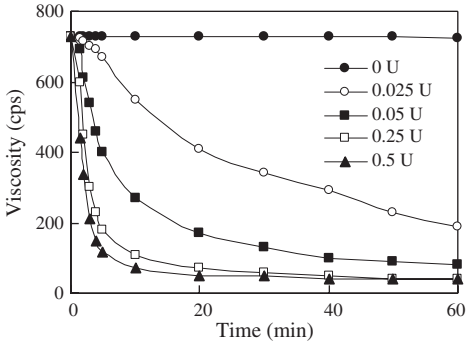


Fig. 7. Dependence of viscosity change on the concentration of *B. cereus* D-11 chitosanase. The reaction mixtures contained 7.0 ml of 1% soluble chitosan (DD 86%) in 100 mM sodium acetate buffer (pH 6.0) and 200 μl of enzyme solution. The viscosity was measured at set intervals as described in Section 2.

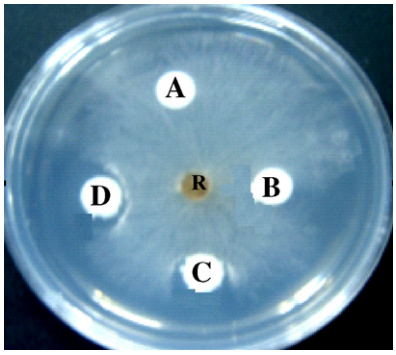


Fig. 8. Antifungal activity of *B. cereus* D-11 chitosanase. A, sterilized water (control); B, 1.6 μg; C, 3.2 μg; and D, 4.8 μg of the purified enzymes; R, *Rhizoctonia solani* AG-1 (KACC 40111).

down upon time elapse. These results indicate that the *B. cereus* D-11 chitosanase cleaves the polymeric chitosan chains in the endo-splitting manner. Analysis by TLC and HPLC of the enzymatic reaction products of soluble chitosan revealed that chitobiose, chitotriose, and chitorirose were major products (data not shown).

3.8. Mycelial growth inhibition

The inhibition of mycelial growth by the chitosanase was tested. *Rhizoetonia solani* AG-1 mycelia plugs were centered on PDA plates and challenged by the buffer containing 1.6, 3.2, and 4.8 μg of the enzyme. The purified chitosanase inhibited the mycelial growth of *R. solani* AG-1 at 3.2–4.8 μg after 3 days of inoculation, exhibiting an antifungal property (Fig. 8).

4. Conclusion

A 41-kDa chitosanase was purified from culture fluid of *B. cereus* D-11 and its properties were investigated. The enzyme showed high substrate specificity toward soluble chitosan with 70–100% deacetylation degree. However, this enzyme was unable to degrade chitin. The chitosanase D-11 showed antifungal activity against *R. solani* AG-1 on PDA medium. The purified chitosanase catalyzed an endo-type cleavage reaction, as demonstrated from the rapid reduction in the viscosity of chitosan solutions. Further studies are under progress to explain the hydrolysis mechanism for the purified endo-chitosanase to adapt it to large-scale commercial production.

Acknowledgements

This work was supported by Korea Science and Engineering Foundation (KOSEF) through the National Research Lab. Program funded by the Ministry of Science and Technology (No. M10300000322-06J0000-32210).

References

Alfonso, C., Martinez, M. J., & Reyes, F. (1992). Purification and properties of two endochitosanases from *Mucor rouxii* implicated in its cell wall degradation. *FEMS Microbiology Letters, 95*, 187–194.

Boucher, I., Dupuy, A., Vidal, P., Neugehauer, W. A., & Brzezinski, R. (1992). Purification and characterization of a chitosanase from *Streptomyces* N174. *Applied Microbiology and Biotechnology, 38*, 188–193.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Chang, W. T., Chen, Y. C., & Jao, C. L. (2007). Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. *Bioresource Technology, 98*, 1224–1230.

Chen, X., Xia, W. S., & Yu, X. B. (2005). Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326. *Food Research International, 38*, 315–322.

Choi, Y. J., Kim, E. J., Piao, Z., Yun, Y. C., & Shin, Y. C. (2004). Purification and characterization of chitosanase from *Bacillus* sp. Strain KCTC 0377BP and its application for the production of chitosan oligosaccharides. *Applied and Environmental Microbiology, 70*, 4522–4531.

Hedges, A., & Wolfe, R. S. (1974). Extracellular enzyme from *Myxobacter* AL-1 that exhibits both β-1,4-glucanase and chitosanase activities. *Journal of Bacteriology, 120*, 844–853.

Imoto, T., & Yagishita, K. (1971). A simple activity measurement of lysozyme. *Agricultural and Biological Chemistry, 35*, 1154–1156.

In, M. J., & Jung, J. (1998). Characterization of *β*-galactosidase from a *Bacillus* sp. with high catalytic efficiency for transgalactosylation. *Journal of Microbiology and Biotechnology, 8*, 318–324.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., et al. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature, 423*, 87–91.

Jo, Y. Y., Jo, K. J., Jin, Y. L., Jung, W. J., Kuk, J. H., Kim, K. Y., et al. (2003a). Characterization of endochitosanases-producing *Bacillus cereus* P16. *Journal of Microbiology and Biotechnology, 13*, 960–968.

Jo, Y. Y., Jo, K. J., Jin, Y. L., Kim, K. Y., Shim, J. H., Kim, Y. W., et al. (2003b). Characterization and kinetics of 45 kDa chitosanase from *Bacillus* sp. P16. *Bioscience, Biotechnology, and Biochemistry, 67*, 1875–1882.

Jung, W. J., Kuk, J. H., Kim, K. Y., Park, Z. Y., & Park, R. D. (2005). Enzymatic characterization and classifications of chitosanases. *Journal of the Korean Society for Applied Biological Chemistry, 48*, 16–22.

Jung, W. J., Kuk, J. H., Kim, K. Y., Kim, T. H., & Park, R. D. (2005). Purification and characterization of chitinase from *Paenibacillus illinoisensis* KJA-424. *Journal of Microbiology and Biotechnology, 15*, 274–280.

Jung, W. J., Kuk, J. H., Kim, K. Y., Jung, K. C., & Park, R. D. (2006). Purification and characterization of exo-b-D-glucosaminidase from *Aspergillus fumigatus* S-26. *Protein Expression and Purification, 45*, 125–131.

Kim, P. I., Kang, T. H., Chung, K. J., Kim, I. S., & Chung, K. C. (2004). Purification of a constitutive chitosanase produced by *Bacillus* sp. MET 1299 with cloning and expression of the gene. *FEMS Microbiology Letters, 240*, 31–39.

Kim, S. Y., Shon, D. H., & Lee, K. H. (1998). Purification and characterization of two types of chitosanase from *Aspergillus fumigatus* KH-94. *Journal of Microbiology and Biotechnology, 8*, 568–574.

Kim, J. S., Lee, I. S., Lee, S., Lee, Y. P., Jung, C., Kim, H. C., et al. (2002). Cloning and expression of the aminopeptidase gene from the *Bacillus licheniformis* in *Bacillus subtilis*. *Journal of Microbiology and Biotechnology, 12*, 773–779.

Kwon, M. J., Park, S. L., Kim, S. K., & Nam, S. W. (2002). Overproduction of *Bacillus macerans* cyclodextrin glucanotransferase in *E.coli* by coexpression of GroEL/ES chaperone. *Journal of Microbiology and Biotechnology, 12*, 1002–1005.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 277*, 680–685.

Lee, H. W., Choi, J. W., Han, D. P., Park, M. J., Lee, N. W., & Yi, D. H. (1996). Purification and characteristics of chitosanase from *Bacillus* sp. HW-002. *Journal of Microbiology and Biotechnology, 6*, 19–25.

Lee, D., Lee, E. L., & Lee, K. M. (2000). Isolation and characterization of chitosanase-producing microorganism, *Aureobacterium* sp. YL, from crab shells. *Journal of Microbiology and Biotechnology, 10*, 208–214.

Maeda, Y., & Kimura, Y. (2004). Antitumor effects of various low-molecular-weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180-bearing mice. *American Journal of Clinical Nutrition, 134*, 945–950.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry, 31*, 426–428.

Mitsutomi, M., Isono, K., Uchiyama, A., Nikaidou, N., Ikegami, T., & Watanabe, T. (1998). Chitosanase activity of the enzyme previously reported as b-1,3-1,4-glucanase from *Bacillus circulans* WL-12. *Bioscience, Biotechnology, and Biochemistry, 62*, 2107–2114.

Okajima, S., Ando, A., Shinoyama, H., & Fujii, T. (1994). Purification and characterization of an extracellular chitosanase produced by *Amycolatopsis* sp. CsO-2. *Journal of Fermentation and Bioengineering, 77*, 617–620.

Okajima, S., Kinouchi, T., Mikami, Y., & Ando, A. (1995). Purification and some properties of a chitosanase of *Nocardioides* sp.. *The Journal of General and Applied Microbiology, 41*, 351–357.

Ozaki, K., Sumitomo, N., & Ito, S. (1991). Molecular cloning and nucleotide sequence of the gene encoding an endo-1,4-beta-glucanase from *Bacillus* sp. KSM-330. *Journal of General Microbiology, 137*, 2299–2305.

Pelletier, A., & Sygusch, J. (1990). Purification and characterization of three chitosanase activities from *Bacillus megaterium* p1. *Applied and Environmental Microbiology, 56*, 844–848.

Price, J. S., & Storck, R. (1975). Production, purification and characterization of an extracellular chitosanase from *Streptomyces*. *Journal of Bacteriology, 124*, 1574–1585.

Sakai, K., Katsumi, R., Isobe, A., & Nanjo, F. (1991). Purification and hydrolytic action of a chitosanase from *Nocardia orientalis*. *Biochimica et Biophysica Acta, 1079*, 65–72.

Seino, H., Tsukuda, K., & Shimasue, Y. (1991). Properties and action pattern of a chitosanase from *Bacillus* sp. P-7S. *Agricultural and Biological Chemistry, 55*, 2421–2423.

Shimosaka, M., Nogawa, M., Wang, X. Y., Kumehara, M., & Okazaki, M. (1995). Production of two chitosanases from a chitosan-assimilating bacterium, *Acinetobacter* sp. Strain CHB101. *Applied and Environmental Microbiology, 61*, 438–442.

Sneath, P. H. A., Mair, N. S., Sharpe, M. E., & Holt, J. G. (1986). Bergey's manual of systematic bacteriology (Vol. 2). Baltimore, MD, USA: Williams and Wilkins.

Su, C. X., Wang, D. M., Yao, L. M., & Yu, Z. L. (2006). Purification characterization and gene cloning of a chitosanase from *Bacillus* species strain S65. *Journal of Agricultural and Food Chemistry, 54*, 4208–4214.

Sun, Y. Y., Liu, W. S., Han, B. Q., Zhang, J. Q., & Liu, B. (2006). Purification and characterization of two types of chitosanase from a *Microbacterium* sp.. *Biotechnology Letters, 28*, 1393–1399.

Tominaga, Y., & Tsujisaka, Y. (1975). Purification and some enzymatic properties of the chitosanase from *Bacillus* R-4 which lyses *Rhizopus* cell walls. *Biochimica et Biophysica Acta, 410*, 145–155.

Uchida, Y., & Ohtakara, A. (1988). Chitosanase from *Bacillus species*. In W. A. Wood & S. T. Kellogg (Eds.), *Methods in enzymology,* (Vol. 161, pp. 501–505). London: Academic Press, Inc.

Uchida, Y., Izume, M., & Ohtakara, A. (1989). Purification and enzymatic properties of chitosanase from Bacillus sp. No. 7-M. *Bulletin of the Faculty of Agriculture. Vol. 66* (pp. 105–116). Saga University.

Wang, Y., Zhou, P. G., Yu, J. X., Pan, X. R., Wang, P. P., Lan, W. Q., et al. (2007). Antimicrobial effect of chitooligosaccharides produced by chitosanase from *Pseudomonas* CUY8. *Asia Pacific Journal of Clinical Nutrition, 16*, 174–177.

Yabuki, M., Uchiyama, A., Suzuki, K., Andou, A., & Fujii, T. (1988). Purification and properties of chitosanase from *Bacillus circulans* MH-K1. *The Journal of General and Applied Microbiology, 34*, 255–270.

Yamasaki, Y., Ohta, Y., Morita, K., Nakagawa, T., Kawamukai, M., & Matsuda, H. (1992). Isolation, identification and effect of oxygen supply on cultivation of chitin and chitosan degrading bacterium. *Bioscience, Biotechnology, and Biochemistry, 56*, 1325–1326.

Yoon, H. G., Kim, H. Y., Kim, H. K., Hong, B. S., Shin, D. H., & Cho, H. Y. (2001). Thermostable chitosanase from *Bacillus* sp. Strain CK4: Its purification, characterization, and reaction patterns. *Bioscience, Biotechnology, and Biochemistry, 65*, 802–809.

Yoshihara, K., Hosokawa, J., Kubo, T., Nishiyama, M., & Koba, Y. (1992). Purification and properties of a chitosanase from *Pseudomonas* sp. H-14. *Bioscience, Biotechnology, and Biochemistry, 56*, 972–973.

Zhang, X. Y., Dai, A. L., Zhang, X. K., Kuroiwa, K. J., Kodaira, R., Shimosaka, M., et al. (2000). Purification and characterization of chitosanase and exo-b-D-glucosaminidase from a koji mold, *Aspergillus oryzae* IAM2660. *Bioscience, Biotechnology, and Biochemistry, 64*, 1896–1902.