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Characterization of Endochitosanases-Producing *Bacillus cereus* P16

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Abstract A bacterial isolate showing a strong endochitosanase activity was isolated from soil and then characterized. The isolate was identified and designated as *Bacillus cereus* P16, based on morphological and biochemical properties, assimilation tests, cellular fatty acids pattern, along with 16S rRNA gene sequence. The optimized medium for producing extracellular chitosanase in a batch culture contained 1% tryptone, 0.5% chitosan, and 1% NaCl (pH 7.0). Powder chitosan and tryptone served the best as carbon and nitrogen sources, respectively, for the chitosanase production. Chitosanase activity was the highest when culture was completed at 37°C among various temperatures (20–42°C) tested in a shaking incubator (200 rpm). The levels of chitosanase activity in the culture fluid were 2.0 U/ml and 3.8 U/ml, respectively, when incubated in a flask for 60 h and in a jar fermenter for 24 h. The culture supernatant showed a strong liquefying activity on the soluble chitosan. The viscosity of 1% chitosan solution, that was incubated with the culture supernatant, was rapidly decreased, suggesting the secretion of endochitosanolytic enzymes by P16. The culture fluid revealed six endo-type chitosanase isozymes, two major (38 and 45 kD), and four minor (54, 65, 82, and 96 kD) forms by staining profile. The crude enzymes were very stable, and full activity was maintained for 4 weeks at 4°C or −20°C in the culture supernatant, suggesting a highly desirable stability rate for making an industrial application of the crude enzymes. The supernatant also cleaved the insoluble chitosan powder, but the hydrolysis rate was much lower. The enzymic degradation products of chitosan contained $(GlcN)_n$ (n=2–8). The concentration of chitosan in the reaction mixture of the crude enzyme affected the chitooligosaccharides composition of the hydrolysis products. When the higher concentration of chitosan was used, the higher degree of polymerized chitooligosaccharides were produced. By comparison with other commercial chitosanase preparations, P16 was indeed found to be a valuable enzyme source for industrial production of chitooligosaccharides from chitosan.

Key words: Chitosanase, *Bacillus cereus* P16, chitooligosaccharides, active staining, enzyme stability

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An interest in chitosan, a linear polymer of β-(1→4)-linked D-glucosamine units with various degrees of *N*-acetylation (D.A.), has been continuously increasing, due to a large number of potentials in medical, industrial, and agricultural applications [33]. Low-molecular weight chitosan oligosaccharides have also received much attention, because of their interesting properties including antifungal [4, 10], antibacterial [5, 36], and antitumoral [32, 34] activities, in addition to their ability to elicit pathogenesis-related proteins and phytoalexins in higher plants [8, 10, 14]. Chitooligosaccharides can also be used as a starting material by *N*-acetylation [1] for preparation of *N*-acetylchitooligosaccharides, which have physiological activities such as growth-inhibitory effects against sarcoma 180 [34], MM-46 [34], and Meth-A [35] solid tumors, and protective effect against infection with some microorganisms in mice [17].

Chitosanolytic enzymes which cleave macromolecular chitosan into chitooligosaccharides have been found in a variety of microorganims, including both bacteria [6, 9, 13, 21–29, 37, 39–42] and fungi [2, 7, 32]. The microbial chitosanases have received special attention, because they are known to be important in maintaining the ecological balance and the recycling of chitinous biopolymers, and also for enzymatic preparation of the biofunctional chitooligosaccharides.

Some chitosanases have hydrolytic activity on substrates other than chitin and chitosan, such as cellulose and lichenan [22]. More detailed knowledge on the primary and three-

dimensional structures, their common structural elements, mode of action, and classification along with natural occurrence are needed for further understanding of the role of enzymes in the degradation of β-1,4-glycan in nature and to apply in preparation of chitosan oligomers by the enzymatic procedure.

Thus, it is important to acquire novel chitosanase-producing microorganisms to gain more detailed knowledge on chitosanases and for enzymatic preparation of chitooligosaccharides. In this study, a bacterium which showed a strong chitosanolytic activity was isolated from soil. The physiological properties of this bacterial strain and the potential application of the bacterial chitosanases in the production of chitooligosaccharides are fully described.

MATERIALS AND METHODS

Screening and Identification of Bacterial Strains

The samples to screen for chitosan-assimilating bacterial strains were obtained from soils around Gimje, Jeollabuk-Do, Korea. Microbial cells were washed out of the soil samples with 1% NaCl solution, and 5 ml of the resulting cell suspensions were incubated into 100-ml Erlenmeyer flasks containing 50 ml of the medium supplemented with 0.5% chitosan with a D.A. (degree of acetylation) of 15.4% as the sole carbon source. After the samples were incubated at 37°C for 5 days on a shaking incubator (200 rpm), 2 ml portion of each preparation was transferred into 50 ml of fresh chitosan medium, and the cultures were incubated under the same conditions for an additional 5 days. During that time, chitosanolytic activity in the culture supernatant was measured. Subsequently, the samples were spread onto the LB-chitosan agar medium that was solidified with 1.5% agar and colloidal chitosan. Clear zones (halos) were observed around some microbial colonies after incubation for several days at 30°C. Pure cultures were obtained by successively isolating single colonies and growing them on a chitosan agar medium.

The morphological and sporulated characteristics were investigated by using both the light microscope and scanning electron microscope (Hatachi Model S-2400). The bacterial isolate was taxonomically classified as described in *Bergey's Manual of Systematic Bacteriology* [31]. Profiles of cellular fatty acid composition were analyzed by using a Hewlett-Packard model 5890A gas-liquid chromatograph and a Microbial Identification System Library for aerobes (ver. 3.90). Carbon source assimilation was examined by the Biolog GP test kit (Biolog Inc., Hayward, Co.) according to the manufacturer's specifications.

To identify the bacterium at the gene level, polymerase chain reaction (PCR) was performed to amplify a part of the 16S rRNA gene of the bacterium. Three primer pairs were synthesized and used for the PCR reactions. The PCR product was cloned by using a pGEM-T Easy vector (Promega, U.S.A.). The nucleotide sequence of 16S rRNA gene of the *Bacillus* sp. P16 was determined by an ABI Prism 377 DNA Sequencer (PE Applied Biosystem, U.S.A.) at Gwangju Branch of KBSI (Korea Basic Science Institute). After that, it was compared with published 16S rRNA sequences by using a Blast Search at NCBI. The sequence of 16S rRNA gene was deposited at GenBank (Accession number AY048782).

Culture Conditions for Chitosanase Production

The medium contained 0.5% chitosan, 1% tryptone, and 1% NaCl (pH 7.0) [24]. Cells were grown at 37°C while shaking, and culture fluid was obtained by centrifugation. The culture supernatant was used for chitosanase assay after overnight dialysis against 100 mM sodium acetate buffer (pH 5.5). Cell growth was measured by absorbance at 660 nm.

Chitosanase Assay

Unless otherwise indicated, chitosan with a D.A. of 15.4% was used as the substrate in a standard chitosanase assay [25]. Each reaction mixture contained 1 ml of 1.0% soluble chitosan, 1 ml of 100 mM sodium acetate buffer (pH 5.5), and 100 μl of the enzyme solution. After incubation at 37°C for 30 min, the reaction was terminated by using 200 μl of 1 M NaOH. The amounts of reducing sugars which were liberated during the hydrolysis of chitosan were determined by the modified Schale's method [11]. One unit of activity was defined as the amount of enzyme to liberate 1 μmol of reducing sugar per min. Glucosamine was used as the standard. A viscometric chitosanase assay was performed by using a Brookfield synchrolectric viscometer (Model LVT, Spindle number 18). The reaction mixtures contained 7.8 ml of 1% soluble chitosan (D.A. 15.4%) in 100 mM sodium acetate buffer (pH 5.5) and 200 μl of enzyme solution. The reactions were performed at 37°C in the viscometer, and the viscosity of each mixture was determined at appropriate intervals [24].

Analytical Methods

Protein contents were determined with Bradford assay kit (Bio-Rad), using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [19]. An activity staining of the enzyme was performed by following the method of Trudel and Asselin [38]. The products of enzymatic hydrolysis were analyzed by thin-layer chromatography (TLC) [25]. In a case of crude proteins obtained from the culture fluid, a reaction mixture contained 1 ml of 1% soluble chitosan and 20 μl of the culture supernatant. After incubation at 37°C for 10 or 30 min, the reaction was terminated by immersing the

reaction tube in boiling water for 3 min. The reaction products were spotted on a TLC plate (Merck TLC silica Gel G-25). After developing in *n*-propanol:ethylacetate: ammonia solution:water (6:3:3:1, v/v), amino sugars were detected by the ninhydrin reaction. The products of enzymatic hydrolysis were also analyzed by using a high-performance liquid chromatography (HPLC) with RI detector, as previously reported [24].

Chemicals

Chitosan was purchased from Taehoon Bio (Korea). Chitobiose, chitotriose, chitotetraose, chitopentaose, chitohexaose, and chitoheptaose were purchased from Wako Chemicals (Japan). Soluble chitosan was prepared by the method of Izume and Ohtakara [12]. Chitosanases were purchased from Wako Chemicals (Japan), Meiji (Japan), Doo Sung Food Co. (Korea), and Korea New Biomaterials (Korea) for comparative study. All other chemicals were of reagent grade.

RESULTS

Screening of the Chitosan-Assimilating Bacterium

Most of the suspensions which were washed out of the soil samples with 1% NaCl solution produced extracellular chitosanase activity, when inoculated into the medium supplemented with 0.5% chitosan with a degree of acetylation (D.A.) of 15.4% as the sole carbon source. During the subsequent screening experiments, various kinds of microbial colonies that formed halos on the LB-chitosan agar were observed. Several bacterial cultures, in which the colonies formed large and clear halos, were purified and then tested for extracellular chitosanolytic activity after grown in the LB-chitosan liquid medium. Most of these isolates exhibited significant levels of chitosanase activity in the liquid medium.

Among them, bacterial isolate P16 showed the highest endochitosanase activity. This was identified with 1) precipitation test by alkalization of enzymatic reaction mixtures, 2) reduction in viscosity of chitosan solutions, and 3) TLC and HPLC analyses of enzymatic reaction products. The isolate P16 was chosen for further study, because of its apparent suitability for a large-scale production of endochitosanase to produce high DP (degree of polymerization) chitooligosaccharides.

Physicochemical Characteristics of Strain P16

Bacterial isolate P16 was subjected to a taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology* [31], 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 (0.4-0.6 μm width×1.6-2.2 μm length), spore shape ellipsoidal, motile, and catalase-positive, as shown in Table 1 and Fig. 1.

Table 1. Morphological, biological, and physiological characteristics of *Bacillus* sp. P16.

Characteristics	result
Size	0.4-0.6 μm width×1.6-2.2 μm length
Gram staining	positive
Mobility	+
Sporangium	not swollen
Spore shape	ellipsoidal
Spore position	central
Catalase	+
Urease	+
Voges-Proskauer test	+
Acid from	
D-Glucose	+
L-Arabinose	-
D-Xylose	-
D-Mannitol	-
Hydrolysis of casein	+
of starch	+
Utilization of citrate	-
Nitrate reduction	+
Formation of indole	-
Growth in NaCl 5%	+
7%	+
10%	-
Growth at pH 4.5	+
9.0	+
11.0	+
Growth at 5°C	-
10°C	+
42°C	+
50°C	-

The isolate P16 was found to grow in 7% NaCl, and was resistant to 50 ppm ampicillin and novobiocin, but susceptible to 10 ppm tetracycline and chlorampenicol (data not shown). Assimilation tests using the Biolog system identified the isolate P16 as *Bacillus cereus* (with similarity index of 0.64).

Fatty Acid Composition of the Cell and rRNA Gene Sequence

A profile of cellular fatty acid composition was also analyzed (Table 2) and compared with that of the *B. cereus* group. A similarity index for the fatty acid relatedness between the P16 and *B. cereus* was found to be 0.95. The nucleotide sequence of the 16S rRNA gene of P16 was determined and submitted to GenBank (Accession number AY048782). On the basis of the nucleotide sequence of 16S rRNA gene, P16 was identified as *Bacillus cereus*. Within 1,197 bp that was sequenced and compared, no base difference between P16 (ID AY048782) and *B. cereus*

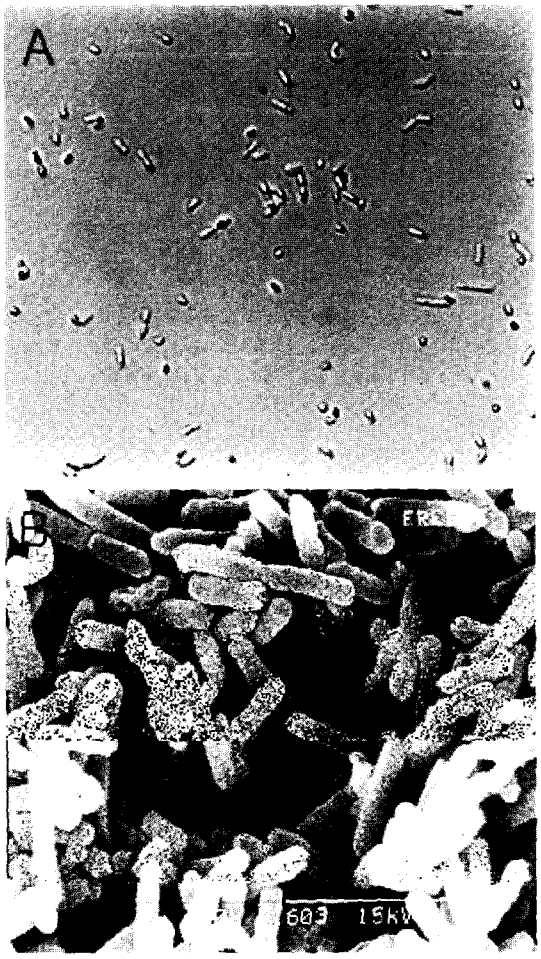

Fig. 1. Differential interference contrast micrograph (A) and scanning micrograph (B) of P16.

16S rRNA sequences (ID AF176322) was detected. From the above results, the P16 was concluded to be most similar to *B. cereus* with more than 95% confidence.

Table 2. Composition of major cellular fatty acids in *Bacillus* sp. P16.

Name	%
C12:0alde	3.95
C13:0iso	12.67
C13:0anteiso	2.15
C14:0	3.43
C14:0iso	5.5
C15:0iso	25.4
C15:0iso2OH/16:1w7c	10.05
C15:0anteiso	5.92
C16:0	3.99
C17:0iso	6.66
C17:1w5c	5.02
C17:1w10c	2.65

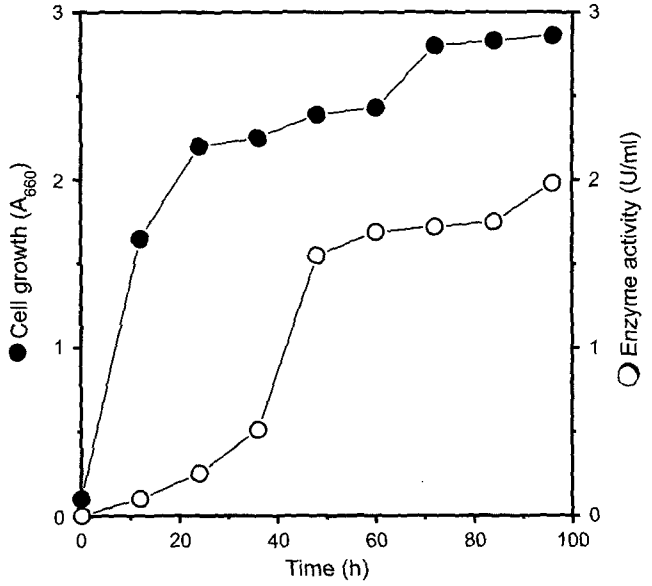

Fig. 2. Cell growth and extracellular chitosanolytic activity of *Bacillus cereus* P16.
Cells were grown in a chitosan medium at 37°C with shaking. At appropriate intervals, cell density was measured at wavelength 660 nm, and the chitosanase activity in the culture supernatant was determined by measuring the total reducing sugars as in Materials and Methods.

Therefore, isolate P16 was designated as *Bacillus cereus* P16.

Chitosanase Production by *Bacillus cereus* P16

The P16 was investigated for extracellular chitosanase productivity by growing in a media containing various carbon and nitrogen sources. The optimized medium for production of chitosanase in a batch culture contained 1% tryptone, 0.5% chitosan, and 1% NaCl (pH 7.0). Powder chitosan (0.5%, less than 40 mesh) and 1% tryptone were the best carbon and nitrogen sources, respectively, for chitosanase production. Chitosanase activity was the highest when culture was completed at 37°C among various temperatures (20–42°C) tested in a shaking incubator (200 rpm) for the soil bacterium. Interestingly, the enzyme production was slightly suppressed in colloidal chitosan or in yeast extract-containing medium. Glucosamine, the end-product, also suppressed enzyme production. This strain grew well in a glucose medium, which produced 85% of that in the chitosan medium. The maximum level of chitosanase activity in the culture fluid was 2.0 U/ml after incubation in a flask for 60 h (Fig. 2). The chitosanase activity in the culture fluid was almost doubled (3.8 U/ml) after being incubated in a jar fermenter for 24 h (data not shown). The chitosanase activity remained low (less than 0.1 U/ml) at the early lag and exponential growth phases of culture, but it began to increase, when the culture reached the stationary phase. Chitosanase activity was hardly found in the homogenate of the cells, implying that all the

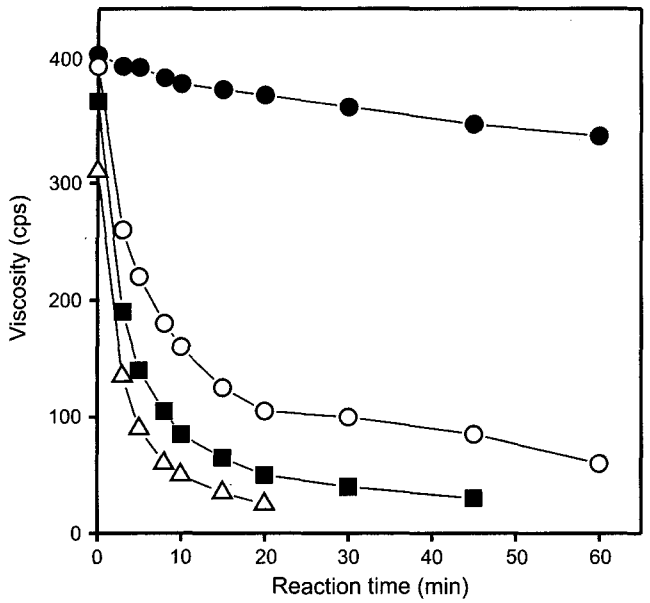

Fig. 3. Increase of endochitosanolytic activity in the culture supernatant during cultivation of *Bacillus cereus* P16.
Viscosity of 1% chitosan solution was drastically decreased upon cultivation. ●, one-day culture; ○, 2-day culture; ■, 3-day culture; △, 4-day culture.

chitosanase activity was secreted into the extracellular culture fluid just after synthesis and modification of the protein(s) in the bacterial cells.

Properties of the Crude Chitosanases

The culture supernatant showed a strong liquefying activity on soluble chitosan. When 0.1 ml of supernatant was incubated with 1% soluble chitosan (1.0 ml) at 37°C for 24 h and then neutralized with NaOH, not much precipitation was observed, suggesting endochitosanolytic cleavage of chitosan by the chitosanases in the supernatant. Figure 3 shows an increase of the endochitosanolytic activity in the culture supernatant during the cultivation process of *Bacillus cereus* P16. The viscosity of 1% chitosan solution, when incubated with the culture supernatant, was rapidly decreased as the days of cultivation increased, and this suggested that the P16 produced and secreted endochitosanolytic enzyme(s) to the media. The culture exhibited significant degradation activity toward partially acetylated chitosan with 15.4% D.A., and fully deacetylated chitosan (D.A. 0%), however, it showed lower activity toward chitin or glycol chitin.

The supernatant also cleaved insoluble chitosan powder, but the hydrolysis rate was much lower. Soluble chitosan is easily degraded due to its higher accessibility to the enzyme. The culture fluids showed roughly 100-times higher activity on soluble chitosan than with insoluble powered chitosan. In fact, this was based on the rate of reducing sugar production, and no differences in the chitooligosaccharides composition of the hydrolysis products were observed by TLC and HPLC (data not shown). An analysis of the degradation products of chitosan with crude enzymes in the culture fluid revealed the production of $(GlcN)_n$ (n=2-8) (see below).

Isozyme Pattern of the Crude Chitosanases

To observe the isozyme pattern of crude chitosanases, the culture supernatant was concentrated, electrophoresized in a chitosan-containing SDS-PAGE, and visualized with Calcofluor White M2R, according to Trudel and Asselin [38]. Figure 4 shows that the culture supernatant contained six endotype chitosanase isozymes, two major (38 and 45 kD) and four additional (54, 65, 82, and 96 kD) forms.

Stability and Applicability of the Crude Enzymes

For achieving an industrial applicability of the crude enzyme in preparation of chitooligosaccharides from chitosan, the stability of the crude enzyme was tested after the storage for certain periods at 4°C and -20°C. The crude enzymes were highly stable to maintain full activity for 4 weeks at 4°C or -20°C as in the culture supernatant (data not shown), suggesting that the chitosanases possess high stability for various industrial uses.

The effect of chitosan concentration (0.5-7%) on the chitooligosaccharides composition of the hydrolysis products

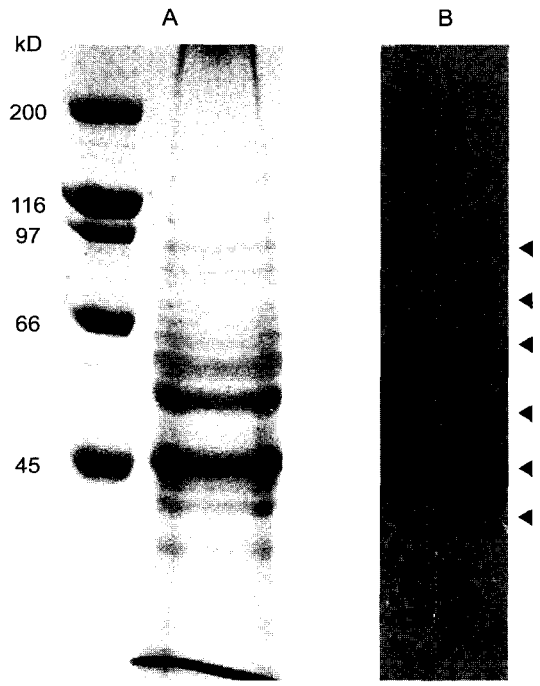

Fig. 4. Activity staining of endochitosanases in the culture supernatant.
SDS-PAGE was performed in 10% polyacrylamide gel containing 0.01% soluble chitosan and stained with Coomassie Blue (A). For activity staining (B), the gel was renaturated, incubated, and stained with Calcofluor white M2R. Arrowheads indicate the bands with chitosanolytic activity. The left lane in panel A indicates molecular markers as indicated.

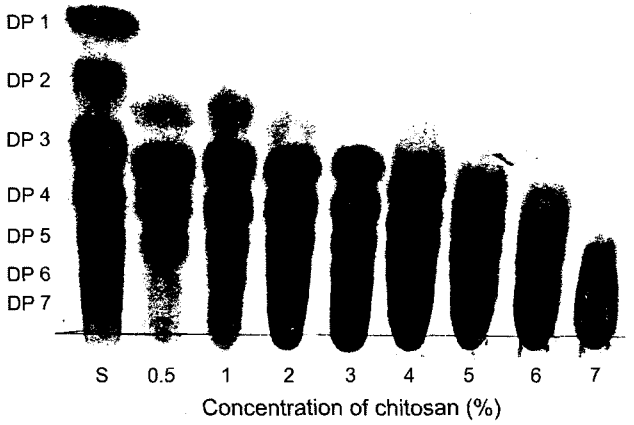

Fig. 5. The effect of chitosan concentration on the chitooligosaccharides composition of the hydrolysis products by the crude enzyme.
One-hundred ml of various chitosan solutions (0.5-7%) were prepared (pH 5.5) and incubated with a culture supernatant (5 ml) at 37°C for 24 h. Aliquots were thin-layer chromatographed as in Materials and Methods. From left, authentic glucosamine oligomers $(GlcN)_{1-7}$ as indicated; 0.5, 1, 2, 3, 4, 5, 6, and 7% chitosan.

by the crude enzyme was also tested: The highest concentration prepared and tested was 7% chitosan. As shown in Fig. 5, the chitooligosaccharide composition varied with the chitosan concentration. When the higher concentration of chitosan was applied, the higher DP of chitooligosaccharide was produced. This implies that it is necessary to incubate

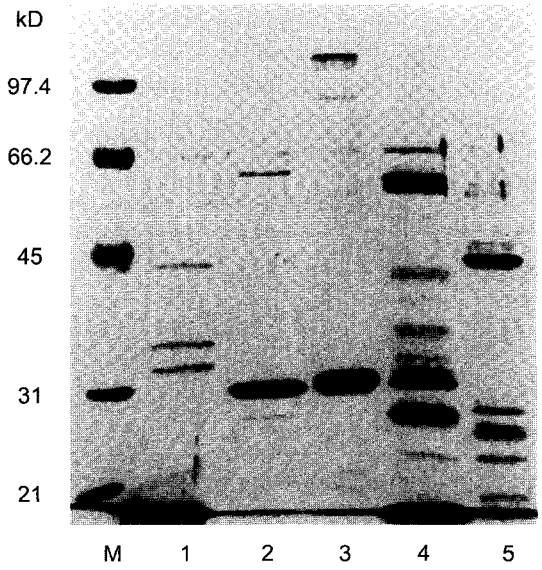

Fig. 6. SDS-PAGE of P16 and commercial chitosanase preparations.
Enzyme preparation of P16 was obtained by acetone precipitation of the culture supernatant. Other chitosanase preparations were purchased as in Materials and Methods. M, molecular markers; 1, Doo Sung (20 μg); 2, Meiji (200 μg); 3, Korea New Biomaterials (200 μg); 4, Wako (200 μg); 5, P16 (120 μg).

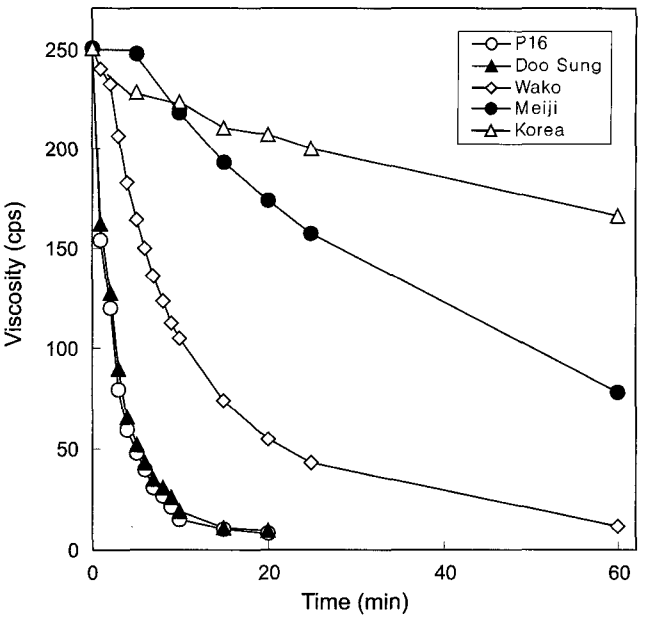

Fig. 7. The endocleavage activities of P16 and commercial chitosanase preparations.
Decrease in viscosity of 1% chitosan solution was recorded after incubation with the preparations.

the crude enzyme with a higher concentration of chitosan to produce high DP of chitooligosaccharides. Surprisingly, major chitooligosaccharides were trimer to heptamer forms, and dimer forms was very limited.

Comparison with Other Preparations

In order to compare P16 chitosanases with other commercial preparations, crude P16 enzyme was prepared by acetone precipitation and freeze-drying of the culture supernatant. As shown in Fig. 6, SDS-PAGE showed the major protein bands which were quite different from those of other commercial preparations: The 45 kDa band was the most prominent and responsible for the chitosanase activity of P16 (Fig. 2 and Fig. 6). Next, the endocleavage activities were compared with other chitosanase preparations by using viscometry (Fig. 7). As expected from Fig. 3, the P16 preparation showed a strong endochitosanolytic activity, which was comparable with the activity of a commercial preparation (Doo Sung). The preparations from Meiji and Korea New Biomaterials showed much less endo-activities than those from P16 and Doo Sung. However, the reducing sugar-producing power was in decreasing order of P16> Meiji>Korea New Biomaterials=Doo Sung>Wako.

DISCUSSION

A novel bacterial strain P16, which was found to be a member of the genus *Bacillus*, was isolated from soil, and

it was able to use chitosan as a sole carbon source. This strain was identified as *B. cereus,* based on the light and electron microscopic observations, Biolog assimilation tests, cellular fatty acids composition, and the analysis of 16S rRNA gene; therefore, it was designated as *Bacillus cereus* P16. The previously described chitosanase-producing bacterial strains are members of the following genera: *Acinetobacter* [30], *Amycolatopsis* [23], *Bacillus* [15, 18, 20, 22, 26, 29, 37, 39, 40], *Enterobacter* [41], *Myxobacter* [9], *Nocardia* [28], *Pseudomonas* [42], and *Streptomyces* [6, 27].

The P16 showed a maximal extracellular chitosanase activity in the medium containing 0.5% chitosan, 1% tryptone, and 1% NaCl (pH 7.0) at 37°C. The P16 grew adequately in the chitosan-added medium as the sole carbon source (Fig. 2), suggesting that exogenously added chitosan was rapidly degraded to low-molecular-weight chitooligosaccharides which supported the growth of bacterial cells. The level of extracellular chitosanases remained low during both lag and exponential growth phases, and rapidly increased when cultures reached the stationary phase. This indicates that the production and/or secretion of chitosanase are dependent on the growth phase of the batch culture.

This strain grew well in a glucose medium and produced 85% level of chitosanase when compared with that in the chitosan medium. It has been known that adding glucose will catabolically repress the chitosanase production, as in the *Aureobacterium* sp. [21]. The levels of chitosanase activity in the culture fluid were 2.0 U/ml after a 60 h incubation in a flask and 3.8 U/ml after 24 h incubation in a jar fermenter. It is exceptionally higher than those with others, such as *Aureobacterium* sp. (15-30 mU/ml) [21], *Bacillus megaterium* P1 (1 U/ml) [26], and *Pseudomonas* sp. H-14 (650 mU/ml) [42]. In fact, this suggests its potential applicability for industrial production of chitooligosaccharides.

The P16 grew in a medium containing colloidal chitosan, powdered chitosan, powdered chitin, or swollen chitin used as a sole carbon source, suggesting that the isolate also produces chitinolytic enzymes. In addition, since the culture supernatant of P16 contained at least 6 endotype chitosanases as shown in Fig. 4, it is suggested that this organism could degrade chitinous polymers with a broad range of D.A. values (from 0 to 100%) to support growth.

The culture supernatant of P16 showed strong liquefying activity of the soluble chitosan. The liquefaction of chitosan largely resulted from the endocleavage activity of chitosanases. By activity staining, 6 endotype chitosanase isozymes, two major (38 and 45 kD) and four additional (54, 65, 82 and 96 kD), were found in the culture supernantant by SDS-PAGE (Fig. 4B). Microbial multiple chitosanases have been reported from *B. circulans* WL-12 [22], *B. megaterium* P1 [26], *Aspergillus fumigatus* KH-94 [16], *Mucor rouxii* [2], and *Acinetobacter* sp. CHB101 [30]. The culture supernatant of *B. circulans* WL-12 contained more than 10 chitinases when grown in the presence of chitin. It was found that these chitinases are derived from only 3 genes encoding 3 chitinases A1, C1, and D1, and the proteolytic cleavage results in many chitinases with different sizes [3]. Unfortunately, in the case of chitosanases, not much information is available on its gene structure, compared to chitinases. Six chitosanases which were identified by activity staining from the culture supernatant of P16 might have been originated from multiple genes, but further study is needed to elucidate the relationship of the gene structure and isozyme multiplicity.

The crude enzymes were so stable that full activity was maintained for 1 month at 4°C or -20°C as in the culture supernatant. Therefore, the stability of chitosanases is a highly desirable characteristics for industrial use in preparation of chitooligosaccharides.

An analysis of the degradation products by crude enzymes in the culture fluid revealed the production of $(GlcN)_n$ (n=3-8). Only a trace amount of monomer glucosamine and dimer were detected in HPLC analysis. Interestingly, no activity of β-*N*-acetylhexosaminidase was detected in the crude supernatant. As shown in Fig. 5, TLC analysis revealed that the major degradation products were trimer to heptamer, and dimer was very limited. This property along with the enzyme stability is highly desirable for industrial use in preparation of high DP chitooligasaccharides.

The effect of chitosan concentration (0.5-7%) on the chitooligosaccharides composition of the hydrolysis products by the crude enzyme is of interest. When higher concentration of chitosan was applied, higher DP of chitooligosaccharide was observed (Fig. 5). Thus, it is desirable to incubate the crude enzyme with higher concentration of chitosan to produce high DP level of chitooligosaccharides.

Enzyme preparation of P16 was also prepared by acetone precipitation and freeze-drying of the culture supernatant, and compared with other commercial preparations for the protein band pattern and enzyme activity. The protein pattern of the P16 enzyme was unique, and the chitosan-endosplitting activity was comparable with or more favourable than those of commercial preparations in preparation of chitooligosaccharide (Fig. 7).

In conclusion, the isolate P16 which produces an exceptionally high endochitosanolytic activity has been isolated from soil, identified, and named as *Bacillus cereus* P16. The properties of the crude enzyme indicate that P16 is indeed a valuable enzyme source for industrial production of chitooligosaccharides from chitosan by enzymatic cleavage.

Acknowledgments

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