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Isolation, Purification, and Enzymatic Characterization of Extracellular Chitosanase from Marine Bacterium *Bacillus subtilis* CH2

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A *Bacillus subtilis* strain was isolated from the intestine of *Sebastiscus marmoratus* (scorpion fish) that was identified as *Bacillus subtilis* CH2 by morphological, biochemical, and genetic analyses. The chitosanase of *Bacillus subtilis* CH2 was best induced by fructose and not induced with chitosan, unlike other chitosanases. The strain was incubated in LB broth, and the chitosanase secreted into the medium was concentrated with ammonium sulfate precipitation and purified by gel permeation chromatography. The molecular mass of the purified chitosanase was detected as 29 kDa. The optimum pH and temperature of the purified chitosanase were 5.5 and 60°C, respectively. The purified chitosanase was continuously thermostable at 40°C. The specific acitivity of the purified chitosanase was 161 units/mg. The N-terminal amino acid sequence was analyzed for future study.

Keywords: Chitosan, chitosanase, chito-oligosaccharide, *Bacillus subtilis*, *Sebastiscus marmoratus*, purification

Chitosans are high-molecular-weight polysaccharides consisting of 1,4-ß-linked D-glucosamine residues, the glycosidic bonds of which could be hydrolyzed by chitosanase. Chitosan was first identified as a minor component of cell walls of *Phycomyces blakesleeanus* [8] and can be produced from chitin through chemical *N*-deacetylation. Chitosan and its partially degraded oligosaccharides are becoming important because of their potential usefulness and novel applications in the fields of functional foods, medical aids, pharmaceuticals, and agricultural agents [9].

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In addition, chitosanases may find important industrial applications in the utilization of the enormous chitosan substrates, as it is reported that chitooligosaccharides have antibacterial activity [5, 18, 21], antifungal activity [7], antitumor activity [20, 23], osteoporosis effect [6], and immuno-enchancing effects [22].

Three main classes of chitosanases are identified according to their substrate specifities. The first class is the chitosanases that degrade chitosan upon recognizing a GlcNAc-GlcN bond, those that recognize both the GlcNAc-GlcN and the GlcNGlcN bond, and those that are specific to the GlcNGlcN bond only. The final group of chitosanases has been purified from *Bacillus* sp. PI-7S [19] and *Bacillus* sp. No. 7-M [4].

Chitosanase activity has been detected in a variety of prokaryotes [13], fungi [2], plants [12], and viruses [25]. Among these, bacterial chitosanases await a breakthrough as it could be especially useful in obtaining large amounts of chitosan oligomers and the enzyme can be easily prepared. Most bacteria and fungi producing chitosanases are known to secrete chitosanases extracellularly [1, 13, 17, 26], which are mostly induced by chitosan as the carbon source. Intracellular chitosanases are found in plants and zygomycete fungi.

In this study, our aim was to isolate a microorganism from the intestine of *Sebastiscus marmoratus* that produces highly active chitosanase, and purify and characterize the enzyme in order to obtain the maximum use in industry.

Materials and Methods

Screening of Chitosanase-Producing Bacterium

In order to isolate chitosanase-producing bacterial strains, samples from the intestine of *Sebastiscus mamoratus* (Jeju, Korea) were used

and the samples were diluted with autoclaved seawater. All the samples were spread on minimal medium containing $(NH_4)_2HPO_4$ 0.5%, K_2HPO_4 0.21%, KH_2PO_4 0.09%, $MgSO_47H_2O$ 0.05%, $FeSO_4$ 0.001%, $ZnSO_4$ 0.0001%, $MnCl_2$ 0.0001%, and agar 1.5%, and chitosan (Sigma co., USA) was used as the only carbon source. The plates were incubated at 30°C for 2~3 days. Positive colonies were inoculated in Luria-Bertani (LB) broth for 16 h at 30°C and at 200 rpm. The cultured bacteria were centrifuged at 15,000 rpm for 1 min and the supernatants were tested for chitosanase activity. Bacterial strains with high chitosanase activity were selected.

Identification of Chitosanase-Producing Bacteria by Morphological, Chemical, and Genetic Methods

The morphological characteristics of the selected bacteria were observed under a scanning electron microscope (SEM) (Hitachi S-2460N, Japan). The bacteria was incubated on LB agar plate for SEM and grown at 30°C for 4 and 12 h. The appeared (12 h) and not appeared (4 h) bacterial colonies were cut (0.5 × 0.5 cm) from the LB agar plate and treated with 2.5% glutaraldehyde solution for 2 h. The colonies were washed two times with 1× PBS for 5 min and dehydrated in 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol for 1 h in each solution. The colonies were moved into a mixing solution (isoamyl acetate:ethanol = 1:3, 1:1, 3:1) for 1 h and kept in 100% isoamyl acetate for more than 1 h. A photograph of the colony was taken by the SEM after treatment and drying with CO_2 and coating with gold.

The biochemical characteristics of the bacteria were tested by the method described in Mac-Faddin [10].

To identify the 16S rRNA gene from the bacteria, chromosomal DNA was extracted with a QIAamp DNA Mini Kit (Qiagen Inc., Germany). The 16S rRNA gene was amplified using polymerase chain reaction (PCR) with the primer 5'-CCAGACTCCTACGGGA GGCAGCA-3' as the sense primer and 5'-TTGACGTCRTCCCCAC CTTCCTC-3' as the antisense primer. The PCR mixture included 0.2 μg of template, 20 pmole of each primer, 10 mM of dNTP mixture, 5 μl of 10× *pfu* DNA polymerase buffer (Mg^{2+} included), and 3 units of *pfu* DNA polymerase (Bioneer Co., Korea) in a 50 μl reaction volume. The initial denaturation step was 5 min at 94°C, and 30 cycles of amplification were carried out with the following cycles: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Finally, an extension step was carried out for 10 min at 72°C. The PCR product was identified on 1% agarose gel and purified with a Gel Purification Kit (Bioneer Co., Korea). The purified PCR product was cloned into the pBlueScript SK(-) vector and transformed into *Escherichia coli* DH5α. Plasmid extraction was carried out with a plasmid extraction kit (Bioneer Co., Korea). Sequencing of the 16S rRNA gene was conducted by Macrogen (Korea). The sequencing result was compared with published 16S rRNA sequences using NCBI Blast search.

Enzyme Assay and Protein Measurement

Chitosanase activity was assayed using soluble chitosan as the substrate. Soluble chitosan was prepared by adding 1 ml of acetic acid into 80 ml of distilled water, followed by adding 1 g of chitosan. The solution was mixed and the pH was adjusted to 5.5 with 10 N NaOH. Finally, the volume was made up to 100 ml with distilled water. Enzyme solution and 1% soluble chitosan were mixed to initiate the reaction. The reaction was carried out at 50°C for 10 min, according to the method described by Rondle and Morgan [16]. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute. Glucosamine was used as a standard.

The protein concentration was measured using the BCA Protein Assay Kit (Pierce, USA) with bovine serum albumin as a standard.

Induction of Chitosanase

Starch, soluble chitosan, chitosan powder, chitin powder, mannitol, lactose, galactose, glycerol, fructose, maltose, glucose, and sucrose were used in order to identify the optimal carbon source. To this end, 0.5% of each component in minimal medium containing 0.3% yeast extract as the nitrogen source was used in this experiment. The chitosanase-producing bacteria was cultured in each broth at 30°C for 10 h. Activity was checked from the supernatant of the samples.

Purification of Chitosanase

Chitosanase-producing bacteria were cultured in 1 l of LB broth at 30°C for 20 h, and the supernatant was collected by centrifugation (4,000 rpm for 10 min at 4°C). Crude proteins were precipitated by adding ammonium sulfate to the final concentration of 90% (w/v) and proteins were harvested by centrifugation (15,000 rpm for 30 min at 4°C). The pellet was dissolved in 1× PBS and dialyzed in order to desalt crude protein using dialysis membrane (MWCO: 12–14,000). After desalting, the solution was added to a Sephadex-G 100 column for gel permeation chromatography, and 1 ml fractions were collected. Each fraction was assayed for activity, and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to detect the purified chitosanase.

Characterization of Purified Chitosanase

In order to identification of temperature effect with purified chitosanase, soluble chitosan (1%) was mixed with purified chitosanase and kept at a range of different temperatures from 30 to 80°C with 5°C intervals for 10 min. The samples were checked for activies. To test the thermostability, enzyme samples were subjected to 40°C, 50°C, 60°C, 70°C, and 80°C for 10~60 min with 10 min intervals. Chitosanase activity was also measured at different pHs (3.5~7.5). Different buffers used to test the pH were 0.1 M acetate buffer (pH 3.5~5.5) and 0.2 M phosphate buffer (pH 6.5~7.5). Specific activity was checked on optimum temperature and pH.

Analysis of N-Terminal Amino Acid Sequence

Purified chitosanase was electrophoresed on 12% acrylamide gel followed by electroblotting onto a PVDF membrane (Millipore) using 10 mM CAPS (3-cyclohexylamino-1-propane sulfonic acid) transfer buffer in 10% methanol (pH 11). Then, the membrane was briefly stained with Coomassie brilliant blue R250 and destained to excise the chitosanase band from the membrane. Finally, the N-terminal amino acid sequence of the chitosanase was carried out by the Genetic Engineering Research Center (Pusan National University, Korea) with an Automatic Protein Sequencer (ABI Procise 491).

RESULTS

Screening and Identification of Chitosanase-Producing Bacteria Strain

A bacterial strain that produces chitosanase was found from the intestine of *S. marmoratus*. The strain showed high chitosanase activity with chitosan. The strain was

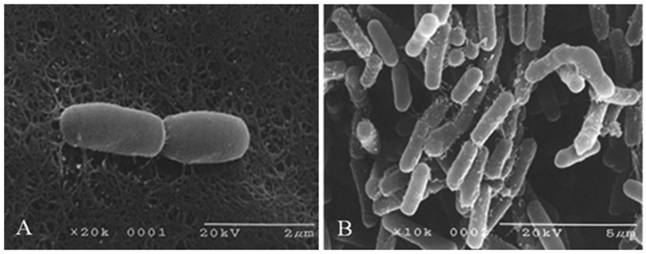

Fig. 1. Scanning electron micrograph (SEM) of *B. subtilis* CH2 after 4 h (**A**) and 12 h (**B**) of incubation at 30°C.

identified by morphological, biochemical, physiological, and genetic methods.

The strain was observed as a Gram-positive rod bacterium with 1.5~2.5 μm size (Fig. 1). Taxonomic characteristics are shown in Table 1. The 16S rRNA sequence of the strain showed 100% identity to *Bacillus subtilis* strain FMG-3 (Accession No. GU124639). With these results, the strain was named *Bacillus subtilis* CH2.

Production of Chitosanase from *Bacillus subtilis* CH2

B. subtilis CH2 was incubated in minimal broth with differential carbon sources at 30°C for 10 h. The highest chitosanase activity of *B. subtilis* CH2 was observed with fructose, and it showed high activity with other substrates such as lactose, galactose, maltose, and glucose as well. However, the chitosanase was not induced by chitosan, unlike other chitosanases. The results are shown in Fig. 2.

Purification of Chitosanase

B. subtilis CH2 was inoculated in LB broth and the supernatant collected for chitosanase purification. Crude protein solution was obtained by salting out the enzyme solution with ammonium sulfate. Chitosanase was purified by gel permeation chromatography using a Sephadex-G 100 column after salt removal. The molecular mass of the purified chitosanase when analyzed with SDS–PAGE showed around 29 kDa (Fig. 3).

Table 1. Taxonomic characteristics of *B. subtilis* CH2 compared with *B. subtilis* CH1.

	B. subtilis CH1	*B. subtilis* CH2
Colony shape	Round	Irregular
Colony margin	Entire	Undulate
Colony color	White, shiny	White, dull
Colony texture	Moist	Dry
Gram stain	+	+
Shape of cell	Rod	Rod
Spore	+	+
Cell size	1.5~3.0 μm	1.5~2.5 μm
Catalase	+	+
Oxidase	-	-
Methyl red test	-	-
Voges–proskauer test	-	-
Indole	-	-
Oxidation/fermentation	O	O
Triple suger iron agar	K/A	K/A

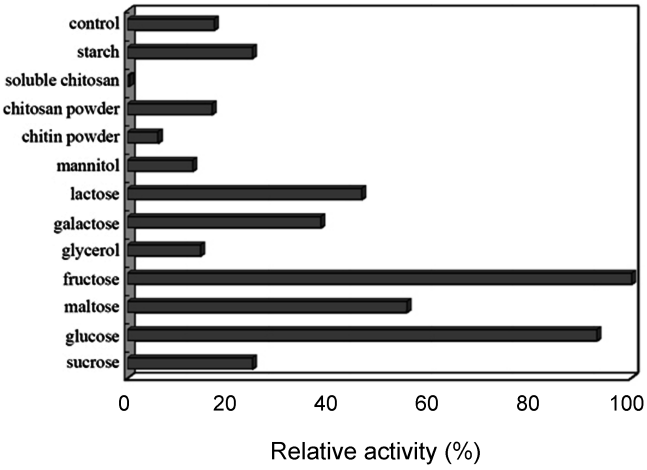

Fig. 2. Induction of chitosanase, according to carbon sources, from *B. subtilis* CH2.
Incubation was at 30°C for 10 h. Each carbon source was used at 0.5% concentration. The basal medium alone was used in the control.

Functional Characterization of *B. subtilis* CH2

The optimal temperature and pH of the purified chitosanase was 60°C (Fig. 4) and 5.5 (Fig. 5), respectively. The thermal stability of chitosanase at different incubation temperatures showed that chitosanase was stable upto

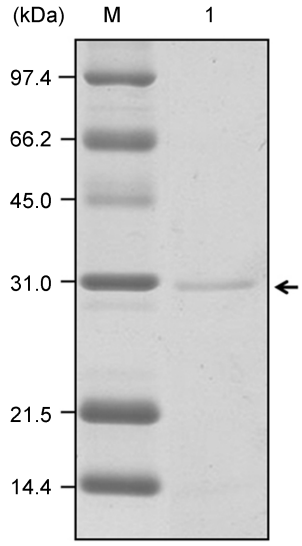

Fig. 3. SDS–PAGE of the purified chitosanase from *B. subtilis* CH2.
Purified chitosanase by gel permeation chromatography was separated on 12% SDS–PAGE and stained with Coomassie brilliant blue. M: molecular mass marker; Lane 1: purified chitosanase.

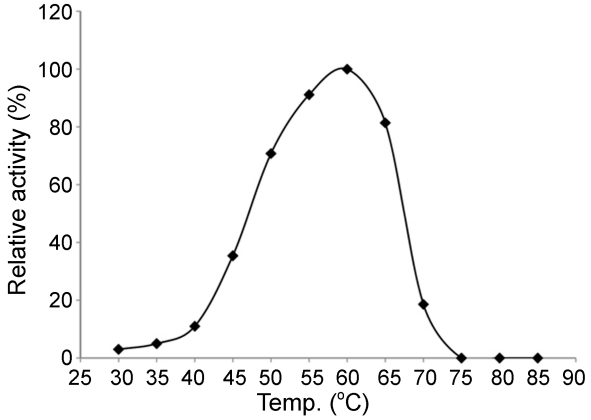

Fig. 4. Effect of temperature on the activity of purified chitosanase from *B. subtilis* CH2.
The effect of temperature on enzyme activity was determined under standard assay conditions as described in Methods, at temperatures ranging between 30 and 85°C.

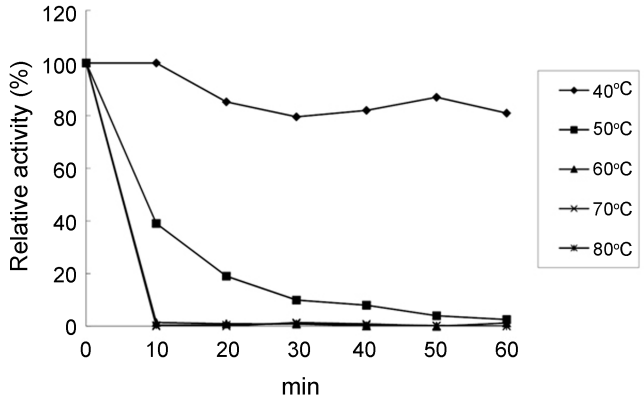

Fig. 6. Effect of thermostability on purified chitosanase from *B. subtilis* CH2 at different temperatures at different time points.
Thermostability was determined by measurement of residual activity under standard assay conditions at temperatures between 40 and 55°C for 10~60 min.

40°C, and the stability was gradually decreased when the temperature was increased to 50°C but rapidly decreased at 60°C (Fig. 6). The specific acitivity of the purified chitosanase was 161 units/mg on optimum conditions.

N-Terminal Amino Acid Sequence of Chitosanase

SDS–PAGE was carried out with purified chitosanae and transferred to PVDF membrane. PVDF blotting was used for analysis of the N-terminal amino acid sequence. Analysis of the N-terminal amino acid sequence resulted in 15 amino acids from the purified chitosanase (AGLNKDQKRRAEQLT). It was similar to other chitosanases of *Bacillus* species.

DISCUSSION

A bacterial strain that secretes chitosanase extracellularly from the intestine of *S. marmoratus* was isolated. The chitosanase secreted by the strain had high activity towards chitosan. The selected strain was a Gram-positive rod bacterium and the size was 1.5~2.5 μm in length. The colony form was irregular and dry in shape. In our previous study, we isolated *B. subtilis* CH1, which was round in shape and the mucus was secreted in large quantity [11]. The isolated strain was similar to *B. subtilis* according to morphological, biochemical, and 16S rRNA sequence analyses. Therefore, we conclude that this strain belongs to *B. subtilis* and we named it as *B. subtilis* CH2.

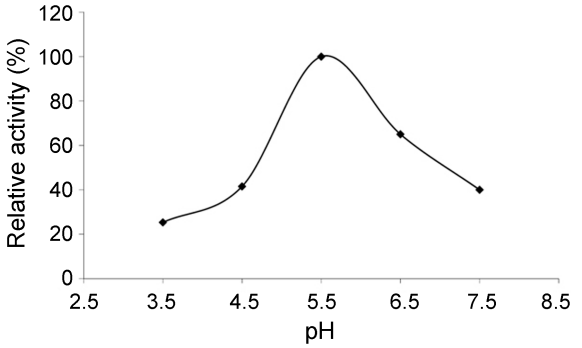

Fig. 5. Effect of pH on the activity of purified chitosanase from *B. subtilis* CH2.
Optimum pH for chitosanase activity was examined from pH 3.5~7.5 at pH 1 intervals at 40°C using acetate (pH 3.5~5.5) and 0.2 M phosphate buffer (pH 6.5~7.5).

According to carbon sources in minimal medium, chitosanase activity was checked on bacteria grown. The highest activity was shown when fructose used, and lactose, galactose, maltose, and glucose also showed high activity. Most of the other studies have reported that bacteria need chitosan as an inducer for production of chitosanase [13, 14, 19, 24, 27]. However, the chitosanase production of *B. subtilis* CH2 was not induced by chitosan, by rather it was induced highly by other carbon sources.

The chitosanase was purified by gel permeation chromatography and the molecular mass of purified chitosanase detected on SDS–PAGE was about 29 kDa. The size of the chitosanase from *B. subtilis* CH2 is similar to the size of chitosanases from *Myxobacter* AL-1 [3], *Bacillus* R4 [24], and *Penicillium islandicum* [2]. The optimal temperature of the purified chitosanase was 60°C; however, it was not stable at this temperature. It was more stable at 40°C continuously. The optimal pH of the purified chitosanase was 5.5. The specific activity was 161 units/mg at the optimum condition. The enzyme characterization of this chitosanase was similar to *B. subtilis* 168 [15]. However, the specific activity of chitosanase from *B. subtilis* CH2 was higher than *B. subtilis* 168 chitosanase (66.3 units/mg). The high active chitosanase produced by the *B. subtilis* CH2 could be useful in the industries provided the enzyme conditions.

The N-terminal amino acid sequence was analyzed for further study. We are planning to conduct future studies in analyzing the gene responsible for chitosanase and to overexpress it in a suitable recombinant expression system.

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