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Purification, Characterization, and Gene Cloning of Chitosanase from *Bacillus cereus* H-1

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A 1.3-kb of chitosanase gene (*choA*) encoding 45-kDa polypeptide was cloned, expressed, and characterized from a newly isolated *Bacillus cereus* H-1. The chitosanase protein (ChoA) of *B. cereus* H-1 was purified to homogeneity by ammonium sulfate precipitation and CM-sephadex column chromatography. Optimum pH was around 7, and stable pH range in the incubation at 50°C was 4-11. Optimum temperature was around 50°C, and enzyme activity was relatively stable below 45°C. ChoA showed the activities toward carboxymethyl cellulose (CMC) in addition to soluble or glycol chitosan. Based on MALDI-TOF MS analysis of purified ChoA, the entire amino acid sequence of ChoA was interpreted by database searching of previously known *Bacillus* chitosanases. A 1.6 kb of PCR product of corresponding chitosanase gene was obtained and its DNA sequence was determined. The deduced amino acid of *choA* revealed that ChoA have a 98% homology with those of *Bacillus* sp. No.7-M strain and *Bacillus* sp. KCTC0377BP. The recombinant ChoA protein was expressed in *E. coli* DH5α. Deduced amino acid comparison of *choA* with other chitosanases suggested that it belongs to family 8 microbial endo-chitosanase with chitosanase-cellulase activity.

Key words: *Bacillus cereus*, chitosanase, cellulase, gene cloning

Chitosanases (EC3.2.1.132), which is a member of glycosyl hydrolase family 46, are hydrolytic enzymes acting on chitosan, a polymer composed of β-(1-4)-linked glucosamine residues [27]. Chitosanolytic enzymes have found in a variety of microorganisms, including bacteria and fungi [3, 12, 17]. Oligomeric chitosans obtained by enzymatic depolymerization have many potential biotechnological applications including medical and food materials, antifungal agents, and elicitor of pathogenesis-related proteins in higher plants [5, 17]. The conversion of chitosan oligomers could be achieved by either chemical method or enzymatic method. Enzymatic hydrolysis by chitosanase is very desirable for producing chitooligosaccharides because the concentrated hydrochloric acid is used in the chemical depolymeriazation of chitosan [2].

Several microbial chitosanases have been purified [4, 6-8, 10, 15, 16, 18] and characterized with gene cloning over the past decade [11, 14, 18, 22, 23]. Most of the bacterial chitosanases are induced by the substrate chitosan and act in the degradation. Their molecular mass is low, in the range of 10-50 kDa. The optimum pH and temperature of chitosanases activity are in the range of 4.0-8.0 and 30-60°C [17]. Chitosanases can be divided into three classes according to their specificity for the hydrolysis of the β-glycosidic linkages in partially N-acetylated chitosan molecules. Class I degrades chitosan upon recognizing a GlcNAc-GlcN bond, class II recognizes both GlcnAc-GlcN and GlcN-GlcN bonds, and class III is specific to the GlcN-GlcN bond [25]. The family 46 chitosanses degrade only chitosan, in contrast, the family 8 chitosanases hydrolyze both chitosan and CM-cellulose. Among the chitosanases so far sequenced, family 8 chitosanases from *Bacillus* sp. No, 7-M and *Bacillus circulans* WL-12 degraded CM-cellulose [10, 11, 19].

Recently, in the course of screening for microorganisms that produce a novel chitosanase, we isolated a chitosanlolytic bacterium belong to *Bacillus cererus* from a contaminated colloidal chitosan plate and it was named as H-1 strain. H-1 chitosanase was produced extracellularly only when colloidal chitosan or soluble chitosan are added in a medium, which represent the inducible characteristic of ChoA. This chitosanase (ChoA) from *B. cereus* was purified and further

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characterized by MALDI-TOF MS analysis. The entire amino acid sequence of ChoA identified from MALDI-TOF MS analysis was compared with other previously reported *Bacillus* chitosanases and used for designing degenerated oligonucleotides for PCR cloning.

In this paper, we describe the purification of *B. ceresus* H-1 chitosanase, its enzymatic characterization, and genetic analysis of chitosanase gene.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strain used in this work was accidentally isolated from a colloidal chitosan plate. This isolation was presumably caused by contamination from the air during media preparation and it was named as H-1. This strain was tentatively assigned to *B. cereus* H-1 by API50 CHB/E (Biomerieux, France) Kit analysis and 16S rRNA sequencing [26] (data not shown). It was grown aerobically in a medium containing 1% Tryptone, 1% NaCl, and 0.5% colloidal chitosan at 37°C. Chitosan (95% deacetylated) was obtained from Korea Chitosan (Youngdeok, Korea) and used for colloidal chitosan preparation. *E. coli* DH5 was used as the host strain for the plasmid maintenance and gene cloning experiment. Ampicillin was added to the media at a final concentration of 50 μg/ml. Plasmid pGEM T-easy (Promega, U.S.A.) and pUC18 (Gibco-BRL, U.S.A.) were used for the cloning vector. The 1.6 kb PCR product of *choA* gene was cloned into pGEM T-easy vector and subsequently subcloned into *EcoRI* site of pUC18. The resulting plasmid, *pChoA,* which harbored *choA* gene was used for recombinant ChoA expression. The morphological characteristics were studied using a scanning electron microscope (JSM5410, Jeol, Japan).

Purification of chitosanase

The chitosanase containing in the supernatant of 200 ml culture was salted out by adding ammonium sulfate to make 90% saturation. The precipitated protein separated by centrifugation was dissolved with 15 ml of 20 mM sodium phosphate buffer (pH 6.8) and dialyzed with same buffer. The crude enzyme solution was applied to CM-sephadex C-50 (Pharmacia, Sweden) column chromatography employing Biologic LP System (Bio-Rad, U.S.A.). Column was extensively washed with pH gradient (pH 4-8) to remove the contaminated proteins and eluted with a linear gradient of 0 to 1.0 M NaCl solution. Fractions (each 4 ml) with chitosanase activity were checked by activity staining and were used for further enzymatic characterizations.

Chitosanase assay

Chitosanase activity was assayed by using colloidal chitosan as a substrate [14]. The reaction mixture consisted of 0.8 ml of 1% colloidal chitosan, and 0.2 ml of the enzyme solution, and the mixtures were incubated at 37°C for 30 min. Reactions were stopped by its boiling for 15 min. The reaction mixtures were centrifuged, and the supernatants were retained. The amount of reducing sugars produced was determined at Abs_{575nm} by the modified Schales method [14]. One unit of chitosanase activity was taken as the amount of enzyme that produced 1 μmole of reducing sugars (glucosamine equivalents) as glucose per minute under the reaction conditions.

In-gel digestion and MALDI-TOF MS analyses of ChoA

Complete amino acid sequencing of ChoA using MALDI-TOF MS was conducted at Korea Amersham Biosciences Proteome Facility. Stained ChoA band was excised from 12% SDS-PAGE and in gel digested by Trypsin (Promega, U.S.A.). The vacuum dried peptide samples were purified by Zip-tip (Waters, U.S.A.) and further analyzed by Ettan MALDI-TOF Pro. Mass Spectrometry (Amersham Biosciences). Bromophenol blue and trypsin molecules were used for internal mass markers of mass analyses.

DNA manipulation and amplification (PCR) reactions

The extraction of *Bacillus* genomic DNA was done as described previously [22]. The plasmid DNA was isolated by the alkaline lysis procedure. PCR amplication of the chitosanase gene was performed by GeneAmp PCR System 2700 (Applied Biosytems, U.S.A.). The two primers, designated Cho-F and Cho-R (5'-GGAATTCGCATATGAATGGAAAAAGAA-3' and 5'-CCGCTCGAGTTAATTATCGTATCCTT-3'), were used to amplify coding DNA region of the *choA* gene from *B. cereus* H-1. Another two primers, designated Cho-PF and Cho-TR (5'-GCAAATAATCAATCCGTAT-3' and 5'-GAATGGATAATGTTTAAC-3') were used for amplication of promoter and termination region of the *choA*. For the analysis of 16S rRNA gene, two universal primers (fD1 and rP2) were used to amplify partial 16S rRNA gene [26].

Nucleotide sequence analysis

The nucleotide sequence analysis of *choA* was conducted at the Macrogen Co. DNA sequencing facility (Seoul, Korea) using the double-stranded dideoxy sequencing method on an ABI 377 DNA sequencer.

Gel electrophoresis and activity staining

The SDS-PAGE was performed in a 12% SDS polyacrylamide gel containing 0.1% glycol-chitosan or 0.5% CMC [7]. The samples were heat-treated for 5 min at 80°C before being loaded and run at 40 mA for 40 min using a Bio-Rad Mini-Protein III Gel Kit. After the electrophoresis, the gels were washed for 1 hr at 25°C with shaking in a 10 mM sodium phosphate buffer (pH 7.0) with 1% Triton X-100 and further incubated in a 10 mM sodium phosphate buffer for 1 hr at 25°C. The protein gels were stained with Comassie brilliant blue G, while the activity gels were stained with a 0.1% Congo red solution for 10 min and destained with a 1 M NaCl solution.

Effect of pH on enzyme activity and stability

The activity was measured by incubating the reaction mixture at different pHs for 30 min, as described in the enzyme assay. To measure the pH stability, the enzyme was incubated at room temperature for 30 min in different buffers. The residual activity was then measured, as described in the enzyme assay. The buffer solutions used were 0.1 M Citric acid plus 0.2 M Na_2HPO_4 buffer (pH 2.0 to 8.0), 0.1 M Tris plus 0.1 M HCl buffer (pH 9.0 to 10.0), and 0.05 M Na_2HPO_4 plus 0.1 M NaOH buffer (pH 10.0 to 12.0).

Effect of temperature on enzyme activity and stability

The effect of temperature was measured by incubating the reaction mixture at different temperatures for 30 min, as described for the enzyme assay. For the thermostability measurement, the enzyme was incubated at different temperatures for 30 min in a 20 mM sodium phosphate buffer (pH 7.0) and the residual activity measured, as described in the enzyme assay.

Phylogenic analyses of *Bacillus cereus* H-1

Bacillus sp. H-1 was further analyzed using an API Bacterial Identification Kit and 16S rDNA analysis, and identified as *Bacillus cereus* according to computer analyses of the API Kit (BioMerieux) results (69.1% identity) and the NCBI Blast search result of a partial 1.5 kb of the 16s rDNA sequence data (99%) (data not shown).

DNA Sequence accession number

The DNA sequence reported here was deposited in the GenBank under accession number of AY207001 (*choA*)

Results and Discussion

Taxonomic analysis of strain H-1

Bacillus cereus H-1 had been tentatively identified as a member of the Genus *Bacillus* by brief taxonomic tests using API 50 CHB/E bacterial identification kit. This identification was based on acids production from various carbohydrates and related compounds. The DNA sequencing of PCR products of 16S rRNA gene further confirmed that H-1 strain belongs to *Bacillus cereus* because it showed 99% homology with 16S rRNA gene sequences of other previously known *Bacillus cereus*. The results of morphological, cultural, and biochemical characteristics of this strain (Fig. 1) supported that it belongs to *Bacillus* sp. (Fig. 2).

Purification of chitosanase

Crude enzymes solution prepared by salting out with ammonium sulfate and dialyzed was applied to gel chromatography on CM-Sephadex C-50 column (1.5×5cm). Using BioLogic LP (Bio-Rad) chromatography system, one single peak was eluted with a linear gradient of 0 to 1.0 M NaCl. Chitosanase activities of the eluted active fractions were confirmed by activity staining and it was almost homogeneous on SDS-PAGE (Fig. 3A). The molecular

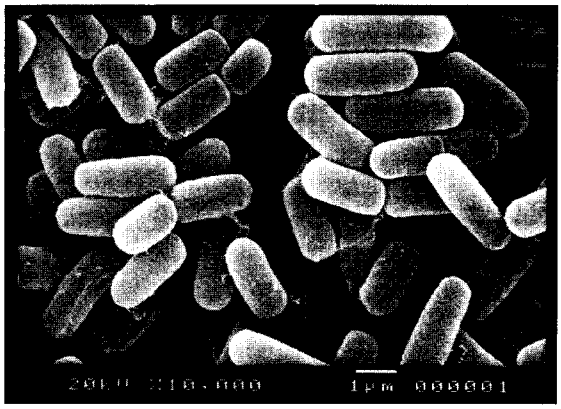


Fig. 1. Scanning electron microscopy (SEM) micrograph (×10,000) of *Bacillus cereus* H-1. The size bar indicates 1 μm.

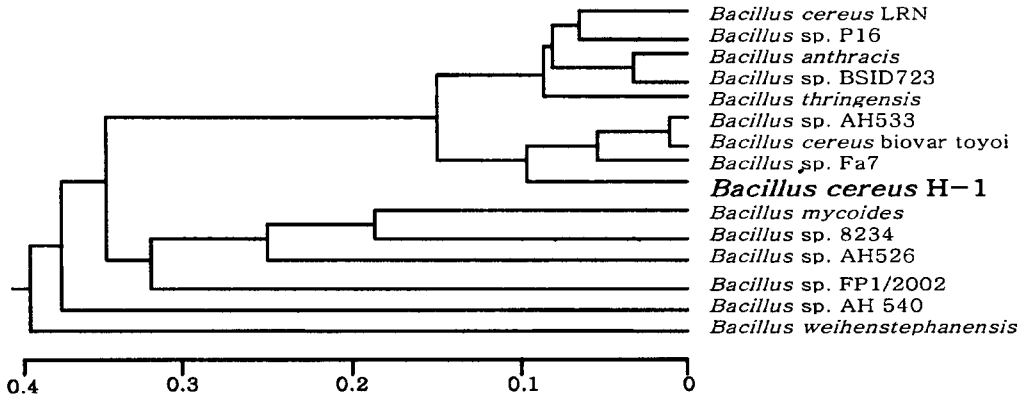


Fig. 2. Phylogenic position of strain H-1 based on 16S rRNA gene sequence. 16S rRNA gene sequence of strain H-1 was determined and compared with complete 16S rRNA gene sequences of other related genera. Sequences were aligned with Clustal W (DNAstar) program, and tree was constructed by neighbor-joining method based on Kimur's evolution distance. Horizontal lines indicate the evolutionary distance.

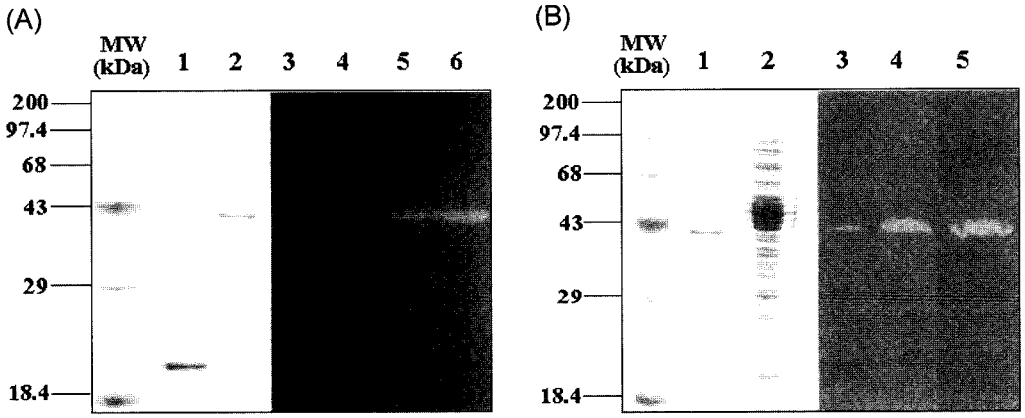


Fig. 3. Detection of chitosanase-cellulase activity of purified ChoA and recombinant ChoA. (A) Chitosanase-cellulase activity of purified ChoA. Lane MW, molecular mass marker; lane1, ammonium-sulfate precipitated culture broth of *B. cereus* H-1; lane2, Purified of *Bacillus* ChoA protein band; lane 3 and 4, chitosanase activity staining on glycol chitosan (0.01%); lane 5 and 6, cellulase activity staining on CMC (0.5%) (B) Chitosanase-cellulase activity of recombinant ChoA. Lane 1, purified *Bacillus* ChoA; lane 2, crude extracts of recombinant *E. coli* harboring p*Cho*A; lane 3 and 4, chitosanase activity staining of purified *Bacillus* ChoA and recombinant ChoA; lane 5, cellulase activity of recombinant ChoA.

weight of purified ChoA was estimated to about 42,000 Dalton.

Enzymatic characterization of ChoA

Chitosanase activity was measured at various pHs and temperatures. The optimum temperature of the purified enzyme under standard assay conditions was 50°C. Chitosanase activity was found to be stable below 45°C (Fig. 4A). The purified chitosanase (ChoA) had an optimal pH of 7.0 for chitosan hydrolysis. When the enzyme was kept at 25°C for 30 min, the enzyme was stable at pH 5-10 (Fig. 4B). These enzymatic characteristics are almost similar as the results of previously reported chitosanases from other *Bacillus cereus* [7, 8] except lowered optimum temperature compared with others (60°C).

MALDI-TOF MS analysis of ChoA protein

Complete amino acid sequence of purified ChoA protein was identified by mass-spectrometry employing Ettan MALDI-TOF Pro. The footprinting of several in-gel digested peptides provided the tentative complete amino acid sequence of ChoA (Fig. 5). Amino acid sequences determined by MALDI-TOF were further compared with amino acid sequences of other reported *Bacillus* chitosanases (GenBank accession numbers: AB051575, AF334682). Subsequently, the degenerated primers, which correspond to the N-terminal and C-terminal amino acids, were synthesized and used for PCR cloning of chitosanase gene.

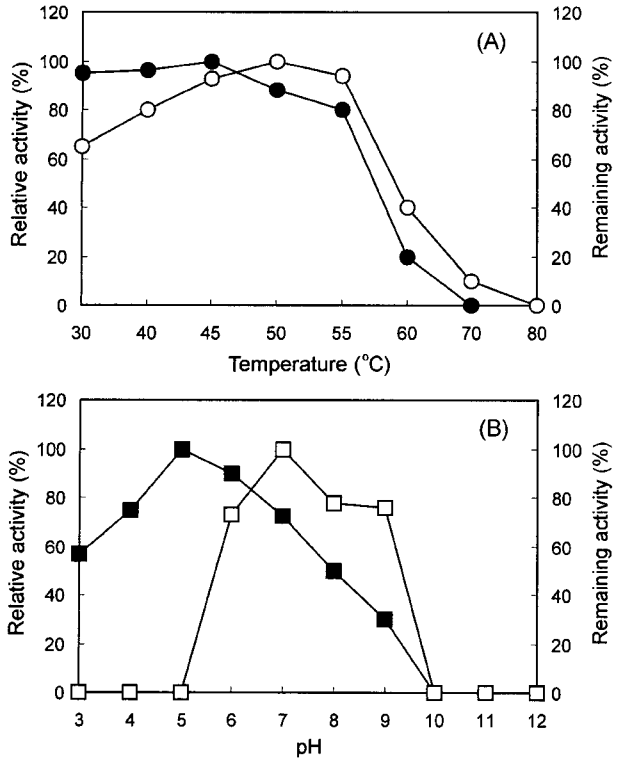
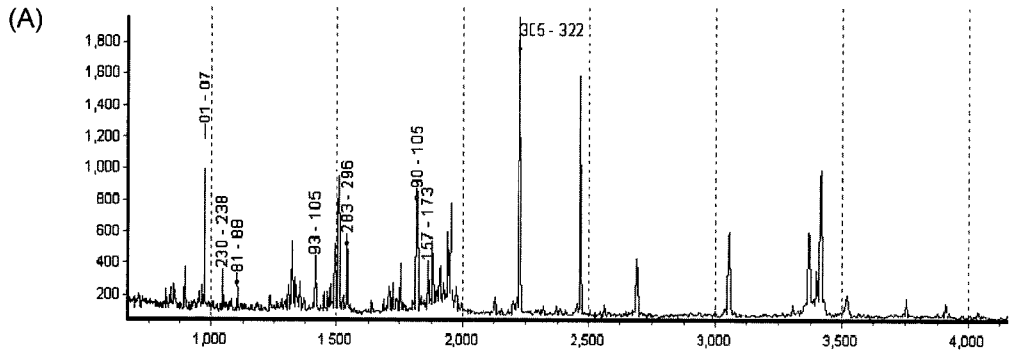


Fig. 4. Enzymatic properties of purified *Bacillus* ChoA protein. (A) Temperature effect (○) and thermostability (●), (B) pH effect (□) and pH stability (■).

Cloning of the chitosanase gene (*choA*)

Based on the two primers covering the full amino acid sequence of ChoA, a 1.36 kb of DNA fragment was obtained by PCR amplification and then cloned into pGEM T-easy vector. The estimated molecular weight of cloned *choA* was about 50 kDa which is somewhat larger than that of purified H-1 chitosanase (42 kDa). This fact suggests that ChoA protein may undergo the posttranslational cleavage. When the deduced amino acid sequence of *choA* was aligned with the previous amino acid sequences determined by MALDI-TOF of ChoA, there were mismatched amino acid sequences. These discrepancies between the interpreted amino acid sequence and deduced amino acid sequence of *choA* might come from internal errors that most footprinting analysis of MADLI-TOF data use only previously deduced amino acid sequences from other known genes. The computer graphic N-terminal analyses of *choA* using a signal sequence prediction program (Swiss-prot, Geneva, Switzerland) revealed the existence of a putative signal sequence cleaved between Ala-27 and Ala-28. This putative signal peptide (27 amino acids) was similar to those of other *Bacillus* species. The deduced amino acid sequence of choA had high homology (98%) with the previously reported chitosanase of *Bacillus* sp. No 7-M and *Bacillus* sp. KCTC0377BP [2]. For the purpose of characterizing promoter and terminator region of *choA* gene, another primer set based on previously known untranslated 5 and 3 region of other *Bacillus* chitosanase gene was designed. A 1.6 kb PCR product was obtained and its DNA sequence was determined. DNA sequencing analysis



(B) Matched peptides

	Meas. mass	Ave/Isotopic	Comp. mass	Abs[Error]	Res. start	Res. end	M. cut	Peptide sequence
▶	974.475	Mono	974.413	0.0624	81	87	0	SYYDNWK
	1046.495	Mono	1046.504	0.0093	230	238	0	LNLGDWDSK
	1102.427	Mono	1102.508	0.0806	81	88	1	SYYDNWKK
	1411.685	Mono	1411.699	0.0144	93	105	0	NDLSSLPGGYYVK
	1538.807	Mono	1538.798	0.0087	283	296	0	YSPNTGLISDFVVK
	1815.932	Mono	1815.941	0.0095	90	105	1	YLKNDLSSLPGGYYVK
	1859.894	Mono	1859.917	0.0229	157	173	1	SSQNPNLMGWVVADSKK
	2219.679	Mono	2219.928	0.0490	305	322	0	DFLDESEYTNAYYYNASR

Fig. 5. MALDITOF mass fingerprinting analysis of ChoA. (A) Mass spectrometry data of ChoA protein. The number appeared at the peaks denote the number of the identical amino acid in other *Bacillus* chitosanases. (B) Partial amino acid sequences of ChoA identified from mass spectrometry.

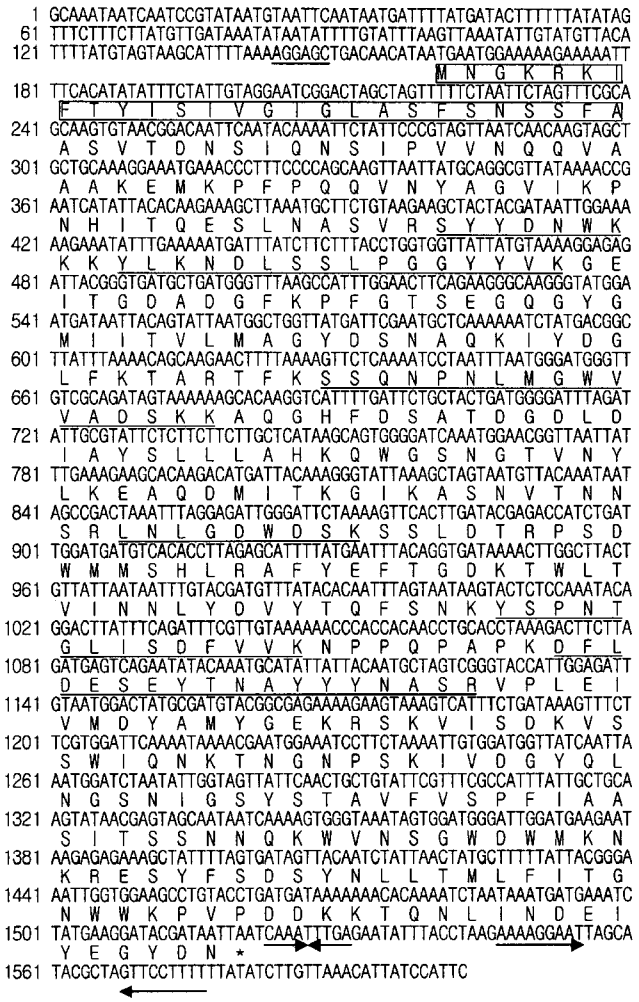


Fig. 6. Open reading frame of *B. cereus* H-1 chitosanase gene (*choA*). The putative signal sequence from computer analysis is represented as an open box. The ribosome binding site (RBS) are marked by thick underline, while the palindromic (TCAAATTTGA) and inverted repeated sequences (AAAAGGAA, TTCCTTTT) at transcriptional termination region are indicated by inverted arrows. The peptides underlined correspond to the MALDI-TOF MS analyses results shown in Fig. 3B.

revealed that it harbored promoter and terminator region around 1.3 kb of coding region of *ChoA* gene (Fig. 6). The 1.6 kb PCR product containing the chitosanase gene fragment was cloned into pGEM T-easy vector and subsequently subcloned into *EcoR*I site of pUC18. The expression of cloned 1.6 kb *choA* gene in *E. coli* was confirmed by its cleared halo around the colony on glycol-chitosan plate (data not shown) and chitosanase activity staining (Fig. 3B). The expressed recombinant ChoA protein of *E. coli* showed an identical 42 kDa chitosanase activity band with that of wild type *Bacillus* chitosanase (Fig. 3B). This suggests that the chitosanase gene of H-1 strain was successfully cloned into *E. coli*. Eventhough 3-D structure prediction analysis employing NCBI BLAST FASTA program suggests that it belongs to family 46 microbial endo-chitosanase with chitosanase-cellulase activity [1, 7, 8, 21], the N-terminal amino acid sequences of *choA* resembled the typical family 8 microbial chitosanase as shown in A-A-K-E-M-K-P-F-P-Q-Q-V-N-Y-A sequences. Consequently, ChoA are seemed to belong to family 8 microbial chitosanases. *B. cereus* was well known as pathogenic strain due to its contaminants on foods [7, 9]. So far, several *B. cereus* chitosanases were purified and characterized but its DNA sequence was not determined [7, 8]. The cloning of new chitosanase gene from *B. cereus* H-1 could provide the details about the gene analysis of *B. cereus* chitosanases.

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국문초록

Bacillus cereus H-1으로부터 Chitosanase의 분리와 특성연구 및 유전자 클로닝

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새롭게 분리된 *Bacillus cereus* H-1으로부터 크기가 45-kDa인 chitosanase를 정제하여 특성을 파악하였고 1.3-kb의 chitosanase 유전자(*choA*)를 대장균에 클로닝하여 발현시켰다. H-1의 chitosanase 단백질(ChoA)은 ammonium sulfate 침전과 CM-sephadex칼럼 크로마토그래피에 의해 정제하였다. 최적 pH는 약 7이었고 pH 안정성은 50°C에서 4-11로 나타났다. 최적 온도는 약 50°C였으며 효소 활성은 45°C 아래에서 비교적 안정하였다. H-1 chitosanase는 soluble 또는 glycol chitosan 뿐만아니라 carboxymethyl cellulose(CMC)에 대한 활성도 나타내었다. 정제된 ChoA의 MALDI-

TOF MS 분석에 기초하여 이미 알려진 다른 *Bacillus chitosanases*와의 데이터베이스 검색을 통해 전체 아미노산 서열을 밝혀내었다. Chitosanase gene에 해당하는 1.6 kb의 PCR 산물을 얻었으며 그의 DNA 서열을 결정하였다. *choA*의 추정 아미노산은 *Bacillus* sp. No 7-M 과 *Bacillus* sp. KCTC0377BP의 아미노산과 98%의 유사성을 나타내었다. 재조합 ChoA 단백질은 *E. coli* DH5α에서 원 균주와 동일한 크기로 발현되었다. N말단의 추정아미노산서열을 다른 chitosanase의 서열과 비교해 볼때 ChoA는 chitosanase-cellulase 활성을 갖는 family 8에 속하는 미생물 endo-chitosanase로 추정되었다.

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