Thermostable Chitosanase from \textit{Bacillus} sp. Strain CK4: Cloning and Expression of the Gene and Characterization of the Enzyme

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A thermostable chitosanase gene from the environmental isolate \textit{Bacillus} sp. strain CK4, which was identified on the basis of phylogenetic analysis of the 16S rRNA gene sequence and phenotypic analysis, was cloned, and its complete DNA sequence was determined. The thermostable chitosanase gene was composed of an 822-bp open reading frame which encodes a protein of 242 amino acids and a signal peptide corresponding to a 30-kDa enzyme. The deduced amino acid sequence of the chitosanase from \textit{Bacillus} sp. strain CK4 exhibits 76.6, 15.3, and 14.2% similarities to those from \textit{Bacillus subtilis}, \textit{Bacillus ehemensis}, and \textit{Bacillus circulans}, respectively. C-terminal homology analysis shows that \textit{Bacillus} sp. strain CK4 belongs to cluster III with \textit{B. subtilis}. The gene was similar in size to that of the mesophile \textit{B. subtilis} but showed a higher preference for codons ending in \textit{G} or \textit{C}. The enzyme contains 2 additional cysteine residues at positions 49 and 211. The recombinant chitosanase has been purified to homogeneity by using only two steps with column chromatography. The half-life of the enzyme was 90 min at 80°C, which indicates its usefulness for industrial applications. The enzyme had a useful reactivity and a high specific activity for producing functional oligosaccharides as well, with trimers through hexamers as the major products.

Chitosan, a partly acetylated or nonacetylated counterpart (4-linked 2-amino-2-deoxy-\(\beta\)-D-glucopyranan) of chitin, is present in the mycelial and sporangiochorus walls of fungi and the exoskeletons of insects and crustacea (9, 27). It is usually obtained by the artificial deacetylation of chitin in the presence of alkali. Chitosan is a copolymer consisting of \(\beta\)-(1-\(\rightarrow\)4)-2-acetamido-\(\beta\)-D-glucose and \(\beta\)-(1-\(\rightarrow\)4)-2-amino-\(\beta\)-D-glucose, units, with the latter usually exceeding 80% (6). Chitosanase (EC 3.2.1.99) hydrolyzes polymers of (1-\(\rightarrow\)4)-\(\beta\)-D-glucosaminic bonds are of special interest (34, 35). Several chitosanases and predicted possible amino acid residues related to catalytic activity and thermostability.

**MATERIALS AND METHODS**

Materials. Chitin, chitosan, glycol chitin, and glycol chitosan were purchased from Sigma Chemical Co. (St. Louis, Mo.). Colloidal chitosan was prepared by the method of Uchida and Ohtakara (31). Colloidal chitin was also prepared by Hsu and Lockwood's methods (14). Partially N-acetylated chitosan (25 to 83% acetylated) prepared from practical-grade chitin was purchased from Sigma Chemical Co. Chitosanase of a \textit{Bacillus} sp. strain PI-7 was obtained from PIAS Inc. (Osaka, Japan). Chitosanase of a Streptomyces sp. was purchased from Sigma Chemical Co. Other reagents were of analytical grade.

Bacterial strains, plasmids, and culture conditions. The thermophilic bacterium \textit{Bacillus} sp. strain CK4 was isolated as a potent thermostable chitosanase producer from a hot spring in Korea and was used as the source of chromosomal DNA to clone the enzyme gene. The transformants were screened on CY medium (1.0% glycol chitin, 0.1% yeast extract, 0.05% tryptone, 0.15% \(K_2\)HPO\(_4\), and 0.05% \(KH_2\)PO\(_4\), pH 7.0) with or without 2.0% agar, containing appropriate antibiotic (50 \(\mu\)g/ml). The plasmids pUC18, pUC19 (Pharmacia Biotech, Uppsala, Sweden), and pBluescript II SK (-) and SK (+) (Strategene, La Jolla, Calif.) were used as the cloning vectors. \textit{Escherichia coli} DH5\(\alpha\) [supE44 ΔlacU169 (600 lacZΔM15) hsdR17 recA1 endA1 gyr96 thi-1 relA1] was used as the cloning host for recombinant plasmids. \textit{E. coli} BL21 (DE3) [pLysS glyA leu2 thi-1 Δind-1 Sam7 ninS lacU5-T7 gene 1] was used as the host for PET 28a (+) (Novagen, Inc., Madison, Wis.) to overproduce chitosanase. All recombinant strains were grown at 37°C on Luria-Bertani (LB) medium containing 50 \(\mu\)g ampicillin/ml for the production of chitosanase.

Analysis of biochemical and physiological properties of strain CK4. The morphological characteristics of strain CK4 were determined by using \textit{Bergey's Manual of Systematic Bacteriology} (13) and the method of Priest et al. (29).

Physiological tests were carried out by using the \textit{Bacillus} Biochemical Card of the Vitek system and API 50CHB (both from Biomerieux, Inc., St. Louis, Mo.). Fatty acid composition was analyzed by the microbial identification system (Sherlock; MIDI Co., Newark, N.J.), and the G + C content was determined by high-performance liquid chromatography (HPLC) by the method of Kumura et al. (18). Performance liquid chromatography (HPLC) by the method of Kumura et al. (18).

**ACKNOWLEDGMENT**

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Enzyme assay and protein determination. The reaction mixture containing 250 μl of 1.0% soluble chitosan, 50 μl of 1.0 M potassium phosphate buffer (pH 7.5), and the enzyme solution in a final volume of 1 ml was incubated at 55°C for 30 min with shaking. The reaction was stopped by heating at 100°C for 10 min, followed by centrifugation. The amount of reducing sugar in the supernatant was determined using the modified dinitrosalicylic acid (DNS) method (24). One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min. β-Glucosamine was used as a standard. The protein concentration was determined by using the Lowry method (20) with bovine serum albumin as a standard.

A modifier hydrolysis product. The substrate, soluble chitosan, was dissolved in 10 mM potassium phosphate buffer (pH 7.5) to give a 0.5% solution. The enzyme (0.1 mg/ml) was added to 1.0 ml of the substrate solution, and the reaction mixture was incubated at 55°C. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and boiled for 10 min in order to terminate the enzymatic reaction. In order to analyze the chitosan oligosaccharide by thin-layer chromatography (TLC), the supernatants prepared under the conditions described above were spotted onto silica gel plate (Kieselgel 60; Merck, Darmstadt, Germany) and developed in propan-30% ammonia water (2:1). The sugars on the TLC plates were visualized by spraying 0.1% ninhydrin dissolved with 99% ethanol. HPLC analysis was carried out with a TSK-Gel NH2-60 column (Toso Co., Tokyo, Japan). The products were eluted with an acetonitrile-water mixture (60:40) at a flow rate of 1 ml/min and detected with a refractive index (RI) detector. β-Glucosamine and a chitosan dimer, trimer, tetramer, pentamer, and hexamer (Seikagaku Co., Tokyo, Japan) were used as authentic standards. (GlcN)4 product concentrations were calculated from peak areas in the HPLC profiles using the standard curves obtained from pure saccharide solutions.

N-terminal amino acid sequence analysis. The purified thermotolerant chitosana (about 0.1 mmol of protein in 10 mM potassium phosphate buffer [pH 7.0]) was directly subjected for automated Edman degradation with an Applied Biosystems 470A gas-liquid phase protein sequencer. The phenylthiohydantoin (PTH) amino acid derivatives were separated and identified using an on-line PTH analyzer, model 120A (Applied Biosystems), with a PTH C4 column.

RESULTS

Strain properties and identification. The strain used in this study, strain CK4, is one of the thermophilic bacterial strains isolated from a hot spring in Korea (34, 35). Strain CK4 is a gram-positive rod-shaped bacterium, motile by a polar flagellum; it is also obligatorily aerobic, catalase and esculin positive, and indole and oxidase negative. It does not require iron ions for growth, and it cannot utilize galactose and arabinose, but it can utilize almost all glucose and galactose, which are used as a carbon source (13). Strain CK4 can also be distinguished from Sporolactobacillus, Desulfotomaculum, and Sponisorcina spp. by its high G+C content, growth at 55°C, and gas production from glucose. Although several characteristics, such as growth temperature and carbon utilization, were not consistent with those of most Bacillus species, the analysis of fatty acid composition in cell walls using a microbial identification system revealed that strain CK4 showed high levels of homology to Bacillus species (data not shown). We also determined the partial nucleotide sequence of the 16S rRNA gene from strain CK4, corresponding to the region between positions 50 and 1394 of the gene in E. coli (8). The rRNA sequence of strain CK4 was compared to sequences available from GenBank. Figure 1 shows a phylogenetic tree of the Bacillus species and other endospore-forming bacteria. Strain CK4 and Bacillus subtilis formed a robust clade but were not exactly identical with each other. Based on these data, we propose the assignment of our strain as Bacillus sp. strain CK4.

Cloning of the chitosanase gene. The recombinant E. coli DH5α containing the chitosanase gene from the Bacillus sp. strain CK4 genomic DNA was screened as a colony forming an orange halo on glycol chitosan Congo red agar medium. Of approximately 10,000 ampicillin-resistant colonies, 1 colony exhibited the orange halo formed by the action of chitosanase. The DNA insert of the plasmid (designated pKCO4) was an-
The DNA sequence of the 1.1-kb fragment contains an *AAGGA* ribosome binding site. Six bases upstream of the ATG codon, initiation site because its location was close to the possible translation codon ATG and ending with the termination codon TAA open reading frame of 822 nucleotides starting with the initiation codon was designated the region necessary for the production of chitosanase, and this fragment was constructed and the chitosanase activity was assayed. According to the physical map showed that the plasmid insert size was 5.1 kb, containing *PstI*, *EcoRI*, *SacII*, *EcoRV*, and *BglII* restriction enzyme sites.

To determine the location of the chitosanase gene in the 5.1-kb insert DNA, a series of deletion mutants of pKCO4 were constructed and the chitosanase activity was assayed. Deletions of a 2.9-kb region from left to right and 1.1 kb from right to left did not affect the expression of chitosanase activity. Accordingly, the 1.1-kb *EcoRI-PstI* fragment was identified as the region necessary for the production of chitosanase, and this fragment was designated *choK* (Fig. 2).

**Nucleotide sequencing of the thermostable chitosanase gene.** The DNA sequence of the 1.1-kb fragment contains an open reading frame of 822 nucleotides starting with the initiation codon ATG and ending with the termination codon TAA at position 984. The ATG codon was chosen as the translation initiation site because its location was close to the possible ribosome binding site. Six bases upstream of the ATG codon, there is a 5-base sequence, 5'-AAGGA-3', that is considerably complementary with the 3' end of 16S rRNA. The A+T content of the region upstream of the initiation codon is 61.8 mol%, which is higher than those of the total *Bacillus* sp. strain CK4 chromosomal DNA (42 to 48 mol%) and the reading frame of the thermostable chitosanase (48.4 mol%). This region contains a putative promoter that displays some sequence homology to the TATAAT (−10) and TTGACA (−35) of the *E. coli* promoter consensus sequence (Fig. 3). Downstream from the TAA stop codon, there is a G+C-rich region of dyad symmetry, capable of forming a stem-and-loop structure. However, the sequence is not followed by a stretch of T residues, unlike the *E. coli* *σ*-independent transcription terminators.

The G+C content of the coding region for the thermostable chitosanase is 52.6 mol%. This value is within the range of the genomic G+C content of *Bacillus* sp. strain CK4 (52 to 58 mol%) and is higher than that (44.8 mol%) of the chitosanase gene from the mesophile *B. subtilis*. In particular, the thermostable gene of *Bacillus* sp. strain CK4 shows a high preference for G or C residues at the 3rd base (the wobble position) of the codons; the G+C content at that position is 66.4 mol%, whereas it is 42.6 mol% in the *B. subtilis* gene. Changes from A/T to G/C in the DNA sequence, particularly in the wobble position of each codon, are thought to be one of the mechanisms of gene stabilization at high temperatures (16).

**Comparison of the deduced amino acid sequence of the choK gene product with those of other chitosanases.** The deduced amino acid sequence of the thermostable chitosanase from *Bacillus* sp. strain CK4 was compared with the sequences of eight available bacterial chitosanases. The nine sequences were linearly aligned by the Clustal method (Lagergene software) as shown in Fig. 4A. The ChoK sequence showed similarities of 76.6, 18.2, 16.8, 15.3, and 14.2% to the sequences of *B. subtilis*, *Bacillus ehemensis*, *Streptomyces* sp. strain N174, *Nocardioidea* sp. strain N106, and *Bacillus circulans* chitosanases, respectively. Linear alignment of two sequences, of the *Bacillus* sp. strain CK4 and *B. subtilis* chitosanases, revealed a marked similarity between the two enzymes (Fig. 4). The overall sequence homology is calculated as 76.6%, which is considerably high for interspecies sequence homology between thermostable and thermolabile enzymes, strongly suggesting that the two chitosanases may have very similar three-dimensional structures. C-terminal sequence homologies between pairs of chitosanases are calculated as 93% (*Bacillus* sp. strain CK4 and *B. subtilis*), 96% (*B. circulans* and *B. ehemensis*), and 95% (*Streptomyces* sp. strain N174 and *Nocardioidea* sp. strain N106). The 93% similarity between *B. subtilis* and *Bacillus* sp. strain CK4 means that they belong to the same group, that is, cluster III (Fig. 4B). Since only eight nucleotide sequences of bacterial chitosanases have been reported so far, the essential catalytic residues have not been studied clearly yet. Although some homologies were found in N-terminal segments (between positions 37 and 78 of the chitosanase gene in *Bacillus* sp. strain CK4), ChoK has no extensive similarity with other chitosanases in other parts (except for the *B. subtilis* chitosanase). The N-terminal segments of the nine chitosanases sequenced have 3 amino acid residues in common, which were thought to be putative catalytic sites of chitosanase.

**Overexpression of thermostable chitosanase and subcellular fractionation.** Plasmid pETCOK was transformed into
E. coli BL21(DE3) so that thermostable chitosanase could be overexpressed. A cell extract was prepared as described in Materials and Methods. Indeed, more than 50% of the soluble protein in the E. coli cell extract was estimated to be chitosanase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The activities of the enzyme from the periplasmic layer of this overproducer were compared with that of the extracellular enzyme from wild-type Bacillus sp. strain CK4. The activities of the enzymes produced by E. coli BL21(DE3)/pETCOK and E. coli DH5α/pKCO4 were about

FIG. 3. Nucleotide sequence of the choK gene and deduced amino acid sequence of the gene product. The coding region starts at position 163 and ends at position 984. The −35 and −10 regions of a putative promoter sequence and a possible Shine-Dalgarno (SD) sequence for the ribosome binding site are underlined. In the 3′-flanking region from the coding sequence, a sequence capable of forming a stem-and-loop structure, which may be involved in transcription termination, is indicated by arrows. The N-terminal amino acids determined by Edman degradation are also indicated (broken underline). The amino acid residues that seem to be essential for chitosanase activity are marked by asterisks. Cysteine residues, potential sources of thermostability, are circled.

E. coli BL21(DE3) so that thermostable chitosanase could be overexpressed. A cell extract was prepared as described in Materials and Methods. Indeed, more than 50% of the soluble protein in the E. coli cell extract was estimated to be chitosanase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The activities of the enzyme from the periplasmic layer of this overproducer were compared with that of the extracellular enzyme from wild-type Bacillus sp. strain CK4. The activities of the enzymes produced by E. coli BL21(DE3)/pETCOK and E. coli DH5α/pKCO4 were about
The optimal temperature and pH for chitosanase activity were examined. The enzyme was most active at 55°C and pH 7.5 under the standard assay conditions (data not shown). The thermostability of the expressed protein was examined by measuring the remaining activity after incubation at various temperatures. The remaining activities after treatment of the enzyme at 80°C for 30 and 60 min were 85 and 66%, respectively. The enzyme (0.5 mg per ml of 50 mM potassium phosphate buffer, pH 7.5) retained its full activity after treatment at 60°C for 30 min, and 92% initial activity remained even after incubation at 70°C for 30 min, although enzyme activity was completely lost after 60 min at 90°C (Fig. 6A). We also found that the enzyme is quite stable in a high concentration of chemical denaturants such as ethanol and SDS. For example, the enzyme was not inactivated at all when incubated with 50% ethanol at 55°C, and it retained about 81% of its activity after incubation with 5% SDS at 55°C for 1 h. The enzyme was resistant to urea and guanidine HCl as well; it retained full activity after incubation with 6 M urea or 2 M guanidine HCl at 37°C for 30 min (Fig. 6B). It is noteworthy that the enzyme is quite stable even in 8 M urea, which causes complete denaturation of ordinary proteins.

**Substrate specificity.** The activities of the purified chitosanase upon chitosan, chitosan derivatives, and other polysaccharides are presented in Table 1. Soluble chitosan, colloidal chitosan, and glycol chitosan served as good substrates. The $K_m$ values for soluble chitosan and colloidal chitosan were 0.8 and 8.7 mg/ml, respectively, and the $V_{\text{max}}$ values were 173 and 71.5 U/mg, respectively. Soluble chitosan was hydrolyzed 6.2 times faster than glycol chitosan. The enzyme was specific for chitosan but attacked neither chitin, cellulose, amylose, nor starch. The substrate specificity of chitosanase on chitosan with different degrees of deacetylation (DDA), prepared by different procedures for N-acetylation, was examined. The relative activity increased when the DDA of colloidal chitosan increased but decreased when the DDA of colloidal chitosan increased (Table 2). This indicates that the physical form and DDA of the substrate affect the rate of hydrolysis. However, no great difference was found among the hydrolysates of soluble chitosan and colloidal chitosan with different DDA (unpublished data).
Analysis of hydrolysis products. The catalytic pattern of chitosanase was examined by using soluble chitosan as the substrate. A change in the hydrolysis products from soluble chitosan was observed during incubation with the recombinant purified enzyme at 55°C for 12 h. At the initial stage, soluble chitosan was hydrolyzed to (GlcN)$_4$ to (GlcN)$_5$ (80% of total products) and small amounts of the dimer and trimer. After 12 h of incubation, the amount of the pentamer in the hydrolysate decreased, while dimer, trimer, and tetramer levels increased, but there was still no monomer (Fig. 7). The hydrolysate profile of the chitosanase of Bacillus sp. strain CK4 was compared with those of other bacterial chitosanases. The chitosanase of Bacillus sp. strain PI-7S produced oligosaccharides ranging from a monomer through a pentamer, with the trimer as the main product. In the case of Streptomyces sp., the main product was the monomer (about 30% of the total yield). Both enzymes produced a monomer and a dimer, with a high rate of about 40 to 60% of the total product.

**DISCUSSION**

We described here the characterization of a thermostable chitosanase-producing bacterium isolated from a hot spring in Korea. This strain was classified into the genus Bacillus by virtue of its morphological and physiological properties and by phylogenetic studies based on analysis of the 16S rRNA gene sequences. The 16S rRNA sequence of strain CK4 showed high similarity (95.7%) to that of *B. subtilis*. However, several characteristics such as growth temperature, carbon utilization, thermostable enzyme secretion, and chitinase production were not consistent between the two strains. Therefore, we classified strain CK4 as a new member of the *Bacillus* genus. A gene (*choK*) coding for chitosanase from *Bacillus* sp. strain CK4 was cloned, and the complete nucleotide sequence was characterized. The open reading frame of *choK* encodes a protein consisting of 242 amino acids, and the molecular size of the protein calculated from the open reading frame is 29,926 Da, which corresponds to that determined by SDS-PAGE and high-performance gel permeation chromatography. The thermostable chitosanase was purified to homogeneity from *E. coli* DH5α by using the GST fusion protein purification system and semipreparative HPLC. The expressed fusion protein was present as a form of insoluble inclusion body. A fraction of soluble recombinant GST-chitosanase was obtained when expression during the incubation of the recombinant strain was performed at 20°C. The production of soluble recombinant GST-chitosanase was dependent on induction with IPTG. The maximum yield of soluble material (about 20 mg/liter) was achieved upon induction with 0.2 mM IPTG. Computer analysis of the deduced amino acid sequence revealed that the C-terminal region of the enzyme had a high similarity with that of *B. subtilis*, but not with those of other groups. *Streptomyces* sp. strain N174 had two essential residues, Glu-22 and Asp-40, localized within the conserved N-terminal region for catalytic activity (7). In the case of glycosyl hydrolases, most catalytic amino acid residues are asparagine or glutamate residues conserved in regions sharing amino acid sequence similarities. N-terminal segments of all bacterial chitosanases had conserved Glu-22 and Asp-40, which were thought to be putative catalytic residues, like those in *Streptomyces* sp. strain N174 chitosanase.

The only significant difference between the chitosanases from *Bacillus* sp. strain CK4 and *B. subtilis* is thermostability. The two enzymes have a relatively low homology sequence in positions 86 to 110 of the *Bacillus* sp. strain CK4 enzyme. This portion might have a role in the thermostability of chitosanase. This is consistent with the idea that a considerable increase in the thermal resistance of proteins can be acquired by the addition of only a few intramolecular bonds such as hydrogen, ionic, and hydrophobic bonds (5). Compared with the amino acid sequences of other, thermolabile bacterial chitosanases, there were several conserved residues and/or regions in the primary structure of the thermostable chitosanase. It has been reported that the conserved residues and/or regions were important in the catalytic activity of the enzyme. Studies on the thermostability and heat inactivation of alanine dehydroge-

**TABLE 1. Substrate specificity of thermostable chitosanase from Bacillus sp. strain CK4**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total activity (U)</th>
<th>Relative activity (%)</th>
<th>$V_{max}$ (U/mg)</th>
<th>$K_m$ (mg/ml)</th>
</tr>
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<tr>
<td>Chitin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colloidal chitin</td>
<td>0</td>
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<td>0</td>
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<td>Glycol chitin</td>
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<td>0</td>
</tr>
<tr>
<td>Soluble chitosan</td>
<td>56.8</td>
<td>100</td>
<td>173</td>
<td>0.8</td>
</tr>
<tr>
<td>Colloidal chitosan</td>
<td>23.3</td>
<td>41</td>
<td>71.5</td>
<td>8.7</td>
</tr>
<tr>
<td>Glycol chitosan</td>
<td>9.7</td>
<td>17</td>
<td>27.9</td>
<td>23.9</td>
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</table>

*Expressed as a percentage of the activity measured with soluble chitosan.

*The purified chitin was purchased from Sigma.*
The substrate specificity of thermostable chitosanase on chitosan with various DDA

<table>
<thead>
<tr>
<th>Substrate (1.0%) and DDA (%)</th>
<th>Total activity (U)</th>
<th>Relative activity (%)</th>
<th>$V_{\text{max}}$ (U/mg)</th>
<th>$K_m$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble chitosan</td>
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<td></td>
</tr>
<tr>
<td>99</td>
<td>56.8</td>
<td>100</td>
<td>173</td>
<td>0.8</td>
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<td>83</td>
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<td>53</td>
<td>25.0</td>
<td>44.1</td>
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<td>Colloidal chitosan</td>
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<td>53</td>
<td>40.2</td>
<td>70.7</td>
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</table>

*DDA were calculated by using the colloidal titration method (36).*

*Expressed as a percentage of the activity measured with soluble chitosan with a DDA of 99%.

The catalytic role of the Asp-66 residue (Fig. 4) is identified by some recently obtained data (H. G. Yoon, H. Y. Kim, Y. H. Lim, H. K. Kim, D. H. Shin, B. S. Hong, and H. Y. Cho, unpublished data). In the site-directed mutagenesis experiment, Asp-66 was proposed as a catalytic residue, corroborating the conclusion drawn from the present work. On the other hand, glutamate residues (Glu-50 and Glu-62) are not found to play an important role in catalysis, seemingly essential for the Streptomyces sp. strain N174 chitosanase. Furthermore, it was found that Cys-211, which occur in the interior of the protein and form a disulfide bond with either Cys-49 or Cys-72, exerting a positive effect on thermostability (19, 30, 33).

The optimum temperature for recombinant chitosanase activity is in the range of the optimal growth temperature of Bacillus sp. strain CK4 (55°C), and the purified chitosanase shows high thermostability in this temperature range compared to other bacterial chitosanases. The purified enzyme belongs to the enzyme group that is able to hydrolyze only chitosan. The previously reported chitosanases classified into the group hydrolyzing only chitosan also can catalyze colloidal chitin and can partially catalyze O-hydroxyethylated chitosan as well. However, this new enzyme is distinct from other enzymes in the substrate specificity of colloidal chitin degradation. The substrate specificity of this enzyme is very high compared with that of other enzymes in the group. Among the hydrolysis products of colloidal chitosan, (GlcN)$_n$ was detected as the major product, with high levels of the trimer, pentamer, and hexamer, but no monomer (Fig. 7). This suggests that the mode of action of the enzyme is of the endo type. Endo-type chitosanases from several microorganisms have been reported, and their degrading patterns on chitosan are similar. Although the amounts of oligomers were variable in each case, these enzymes were previously reported to hydrolyze chitosan into oligomers of 1 to 6 units by an endo-type catalytic action. However, the thermostable enzyme described here produces functional oligomers, trimers through hexamers, with a high rate of about 80% of total yield at temperatures under 55°C for 12 h. The reaction pattern of this chitosanase, with its thermostability, makes the enzyme a good candidate for biotechnological applications in the industrial production of functional chitoooligosaccharides.

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