Proc. KSAM Spring Meeting
April 28~28, 2000, Seoul, Korea

GENETIC AND BIOCHEMICAL ANALYSIS OF A THERMOSTABLE CHITOSANASE FROM *Bacillus* sp. CK4

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A thermostable chitosanase gene from the isolated strain, *Bacillus* sp. CK4, 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 in size. The deduced amino acid sequence of the chitosanase from *Bacillus* sp. CK4 exhibits 76.6%, 15.3%, and 14.2% similarities to those from *Bacillus subtilis, Bacillus ehemensis*, and *Bacillus circulans*, respectively. C-terminal homology analysis shows that *Bacillus* sp. CK4 belongs to the Cluster III group with *Bacillus subtilis*. The size of the gene was similar to that of a mesophile, *Bacillus subtilis* showing a higher preference for codons ending in G or C. The functional importance of a conserved region in a novel chitosanase from *Bacillus* sp. CK4 was investigated. Each of the three carboxylic amino acid residues were changed to E50D/Q, E62D/Q, and D66N/E by site-directed mutagenesis. The D66N/E mutants enzymes had remarkably decreased kinetic parameters such as V_{max} and k_{cat}, indicating that the Asp-66 residue was essential for catalysis. The thermostable chitosanase contains three cysteine residues at position 49, 72, and 211. Titration of the Cys residues with DTNB showed that none of them were involved in disulfide bond. The C49S and C72S mutant enzymes were as stable to thermal inactivation and denaturating agents as the wild-type enzyme. However the half-life of the C211S mutant enzyme was less than 60 min at 80℃, while that of the wild type enzyme was about 90 min. Moreover, the residual activity of C211S was substantially decreased by 8 M urea, and fully lost catalytic activity by 40% ethanol. These results show that the substitution of Cys with Ser at position 211 seems to affect the conformational stability of the chitosanase.

INTRODUCTION

Chitosanase (EC 3.2.1.99) hydrolyzes polymers of (1-4)-β-D-linked-glucosamine (GlcN) residues to chitosan oligomers. Over the last decade, some chitosanolytic enzymes with different substrate specificities have been characterized (5, 6, 8, 13, 16), and most of them catalyze the endo-type cleavage of chitosan with a narrow range of deacetylation degrees (6, 7, 14). Recently, chitosan and its partially degraded oligosaccharides have become important because of their potential applications as medical and agricultural agents (2). Thermostable chitosanases active between 60℃ and 100℃ and specifically attacking the β-D-glucosaminidic bonds are of special interest (52). Five chitosanases from mesophilic bacteria have been cloned and sequenced so far(1, 3, 11, 12, 14). Most of them belong to thermolabile chitosanase, whereas little information is available on thermostable chitosanase. Thermostability is presumably based on the protein structure. To elucidate the thermostable character of the enzyme, information on its molecular structure of the enzymes including entire amino acid sequence and three-dimensional structure is needed. Here we analyzed the homology of the 16S rRNA genes on the strains reported as the chitosanase producers, including *Bacillus* sp. CK4, to differentiate between them based on a phylogenetic analysis of the 16S rRNA genes. We performed the genetic analysis of a novel chitosanase gene from *Bacillus* sp. CK4. We also examined which residues were catalytically important in the primary structure of the thermostable chitosanase, and we discussed the difference in thermostability between the two chitosanases from *Bacillus subtilis* and *Bacillus* sp. CK4 on the basis of their primary structure.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The thermophilic bacterium, *Bacillus* sp. CK4, was isolated as a potent thermostable chitosanase producer in high temperature

RESULTS AND DISCUSSION

Strain properties and identification. The strain CK4 used in this study is one of the thermophilic bacterial strains isolated from high temperature environment (32, 33). Strain CK4 is a Gram-positive rod bacterium, motile by a polar flagellum; it is also obligate aerobic, catalase and esculin positive, and indole and oxidase negative. It doesn't require sodium ion for growth, and can not utilize galactose and arabinose, as opposed to most *Bacillus* species, which can utilize arabinose as a carbon source. Strain CK4 can also be distinguished from *Sporolactobacillus*, *Desulfotomaculum*, and *Sporosarcina* by high G+C content, growth at 55℃, andgas production from glucose. Although several characteristics such as growth temperature and carbon utilization were not consistent with most *Bacillus* species, the analysis of fatty acid composition in cell walls using a microbial identification system revealed that strain CK4 showed similar homology to them. 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*. The rRNA sequence of stain CK4 was compared to sequences available from Gene Bank. Fig. 1 shows a phylogenetic tree of the *Bacillus* species and other endo spore-forming bacteria. Strain CK4 and *B. 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. CK4.

Cloning of the chitosanase gene. The recombinant *E. coli* DH5α containing the chitosanase gene from the *Bacillus* sp. CK4 genomic DNA was screened as a colony forming an orange halo on glycol chitosan-Congo red agar medium. From approximately 10,000 ampicillin-resistant colonies, one colony exhibited the orange halo formed by the action of chitosanase. The DNA insert of the plasmid (designated as pKCO4) was analyzed by digestion with restriction enzymes. The resulting physical map showed that the plasmid insert size was 5.1 kb containing the restriction enzyme sites of *Pst*I, *Eco*RI, *Sac*II, *Eco*RV, and *Bgl*I .

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. There is a 5-base sequence, 5'-AAGGA-3', in six bases upstream from the ATG codon 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 that of the total *Bacillus* sp CK4 chromosomal DNA (42-48 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 *E. coli* promoter TATAAT (-10) and TTGACA (-35) *E. coli* promoter consensus sequence(Fig. 1). 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* ρ-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. CK4 (52-58 mol%) and is higher than that (44.8 mol%) of the chitosanase gene from mesophile *B. subtilis*. In particular, the *Bacillus* sp. CK4 thermostable gene shows a high preference for G or C residues at the third 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 DNA sequence, particularly in the wobble position of each codon, are thought to be one of the mechanisms of gene stabilization at high temperatures (9).

Comparison of deduced amino acid sequence of *cho*K gene with those of other chitosanases. The deduced amino acid sequence of thermostable chitosanase from *Bacillus* sp. CK4 was compared with the sequences of five bacterial chitosanases available. The six sequences were linearly aligned by clustal method as shown in Fig. 2A. The *cho*K sequence showed similarity of 76.6%, 18.2%, 16.8%, 15.3%, and 14.2% to the sequences of *Bacillus subtilis*, *Bacillus*

environment, and used as the source of chromosomal DNA to clone the enzyme gene. The tranformants were screened on CY medium (1.0% glycol chitosan, 0.1% yeast extract, 0.05% tryptone, 0.15% K_2HPO_4, and 0.05% KH_2PO_4; pH 7.0) with or without 2.0% agar, containing appropriate antibiotics (50 μg/ml). The plasmids pUC18, pUC19 (Pharmacia Biotech., Uppsala, Sweden), pBluescript II SK(-) and SK(+) (Stratagene, California, U.S.A.) were used as the cloning vectors. *Escherichia coli* DH5α {*sup*E44 Δ*lac*U169 (ϕ80*lac*ZΔM15) *hsd*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1} was used as the cloning host for recombinant plasmids. *Escherichia coli* BL21(DE3) {*has*S *gal*(λc*Its*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene1)} was used as the host for pET 28a(+) (Novagen, Inc., Madison, USA) to overproduce chitosanase. All recombinant strains were grown at 37℃ on Luria-Bertani(LB) medium containing 50 μg/ml ampicillin for the production of chitosanase.

DNA sequencing. The plasmid of the subclones was prepared for sequencing using a Wizard Plus SV DNA purification kit (Promega Co., Madison, U.S.A.). Dideoxy DNA sequencing was performed with an ALFexpress™ Autoread™ sequencing kit (Pharmacia Biotech., Uppsala, Sweden) as specified by the manufacturer. The DNA fragments were analyzed on an ALFexpress Autoread sequencer (Pharmacia Biotech., Uppsala, Sweden). Nucleotide and amino acid sequence analysis, including an open reading frame search, molecular weight calculation, and homology search, was performed using LAGERGENE (DNASTAR, Inc., Madison, U.S.A.).

Analysis of 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℃. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and boilled for 10 min in order to terminate the enzymatic reaction. In order to analyze the chitosan oligosaccharide with TLC, the supernatants prepared under the conditions as described above were spotted on silica gel plate (Kieselgel 60, Merk, Germany) and developed with n-propanol : 30% ammonia water (2:1). The sugars on the TLC plate were visualized by spraying 0.1% ninhydrin dissolved with 99% ethanol. HPLC analysis was carried out with TSK-gel NH_2-60 column (Toso Co., Tokyo, Japan). The products were eluted with an acetonitrile-water mixture (60:40) at a flow rate of 0.8 ml/min and detected with a refractive index (RI) detector. D-glucosamine, chitosan dimer, trimer, tetramer, pentamer, and hexamer (Seikagaku Co., Tokyo, Japan) were used as an authentic standard. $(GlcN)_n$ product concentrations were calculated from peak areas in the HPLC profiles using the standards curves obtained from pure saccharide solutions.

Site-directed mutagenesis and expression of fusion mutant proteins. The thermostable chitosanase mutants were constructed by a linear polymerase reaction using *Pfu* DNA polymerase. Plasmid pGEK4 carrying *cho*K (GeneBank accession number: AF160195), which encodes the mature wild-type thermostable chitosanase ligated as an *Bam*HI-*Eco*RI fragment adjacent to the *tac* promoter of the fusion-expression vector pGEX 4T-2, was used as the template for mutagenesis. The mutagenic oligoprimers (for forward and reverse replication) and the encoded amino acid substitutions are shown in Table I. After replication, the polymerase chain reaction mixture was treated with *Dpn*I restriction endonuclease to cleave the methylated template DNA; unmethylated DNA is not a substrate for *Dpn*I. Product DNA was ethanol-precipitated, redissolved in 10 mM Tris and 1 mM EDTA (pH 8.0), and then electrophorated into *E. coli* DH5α. The plasmid template was isolated from the resulting amp^r transformants and the mutated segments were sequenced to confirm the desired mutation. To overexpress the fusion proteins *E. coli* DH 5α containing the mutant fusion-expression vector was cultured in LB medium containing 50 μg/ml ampicillin at 37℃.

Nucleotide sequence accession number. The nucleotide sequences of *cho*K and 16S rRNA genes reported in this article have been assigned the Genebank accession numbers AF160195 and AF165188, respectively.

ehemensis, *Streptomyces* sp. N174, *Nocardioides* sp. N106, and *B. circulans* chitosanase, respectively. Linear alignment of two sequences, *Bacillus* sp. CK4 and *B. subtilis* chitosanase, revealed a marked similarity between the two enzymes (Fig. 2). The overall sequence homology is calculated as 76.6%, which is considerably high for degree of sequence homology between thermostable and thermolabile enzymes in interspecies, strongly suggesting that the two chitosanases may have very similar three-dimensional structures. The C-terminal sequence homologies among each chitosanase are calculated as 93% (*Bacillus* sp. CK4: *Bacillus subtilis*), 96% (*Bacillus circulans*: *Bacillus ehemensis*), and 95% (*Streptomyces* sp. N174: *Nocardioides* sp. N106). The 93% similarity between *Bacillus subtilis* and *Bacillus* sp. CK4 means that they belong to the same group, that is, cluster III (Fig. 2B). Since only five 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 (37-78 of the *Bacillus* sp. CK4 sequence), the *cho*K has no extensive similarity with other chitosanases in other parts. The *N*-terminal segments of the above six chitosanases have three amino acid residues in common, which were thought to be putative catalytic sites of chitosanase.

Analysis of hydrolysis products. The catalytic pattern of chitosanase was examined using soluble chitosan as the substrate. A change of hydrolysis products from soluble chitosan was observed during incubation with the recombinant purified enzyme at 55℃ for 12 hr. At the initial stage, soluble chitosan was hydrolysed to $(GlcN)_4$ ~ $(GlcN)_5$ (80% of total products) and small amounts of dimer and trimer. After 12 hr incubation, pentamer decreased in the hydrolysate, while dimer, trimer, and tetramer increased, and yet no monomer(Fig. 3). The hydrolysate profile of chitosanase was compared with other bacterial chitosanases. The chitosanase of *Bacillus* sp. PI-7S produced monomer~pentamer, and trimer as a main product. In the case of *Streptomyces* sp., the main product was monomer about 30% of total yield. Both enzyme produced monomer and dimer with a high rate about 40~60% of total product.

Enzyme purification and modification of cysteine with DTNB. To examine whether the three carboxylic and cysteine residues of thermostable chitosanase from Bacillus sp. CK4 have any functional role(s), they were replaced individually by site-directed mutagenesis as described under "MATERIALS AND METHODS". Each mutant enzyme and wild-type enzyme was purified to homogeneity as shown by SDS-PAGE. The purified enzymes were used to measure their thermal stability as well as resistance against denaturating agents and organic solvents and to determine their kinetic properties. Chitosanase activity was first estimated from activity staining after SDS-PAGE with the crude enzymes. Red haloes were absent in the lanes carrying the D66N and D66E mutant enzymes, and the intensities of red haloes of the other mutants were similar to that of the wild-type enzyme. Thermostable chitosanase from the thermophilic Bacillus is a monomer which contains three SH groups. The free sulfhydryl content of the enzyme was first quantified without denaturation, confirming whether any cysteine was exposed on the surface of the protein and then DTNB titration was performed with the denatured enzyme. Only two free SH groups were found, regardless of whether the reaction was carried out under native or denaturating conditions. The C211S mutant enzyme was titrated for two SH groups per subunit the wild-type, whereas the C49S and C72S mutant enzymes were titrated for only one cysteine residue. The titration of free cysteines in the mutated enzymes confirmed that only one of the cysteines, Cys-211 in C49S and C72S, was not reactive, while Cys-49 and Cys72 in C211S were reactive. Furthermore, treatment with 2-mercaptoethanol did not generate a free sulfhydryl, confirming that no disulfide bond had formed among the cysteine residues.

Thermal stability of mutants. The thermal stability of the three Cys mutant enzymes were examined. Fig. 4 shows the thermostability observed in the four enzymes after incubation at various temperatures for 60 min. While the half-life of the wild-type enzyme at 80℃ is about 90 min, that of the C211S mutant is less than 60 min, and those of the other mutants are around 85 to 95 min. It is well-established that the structural features responsible for the thermal stability of proteins often confer resistance to other denaturating agents (10). Therefore, we also assayed

the stability of the wild-type enzyme and the three Cys mutant enzymes in urea and ethanol. As shown in Fig. 5, C211S showed an 80% decrease in residual activity by 8 M urea, and fully lost catalytic activity by a 40% ethanol concentration. On the other hand, C49S and C76S showed similar denaturing patterns to the wild-type enzyme. Thus, it seems that Cys-211, which may occur in the interior of the protein, exerts a positive effect on the thermostability of the enzyme.

Kinetic properties of the enzymes. Kinetic parameters were examined under standard assay conditions that correspond to the optimum previously found for the wild-type enzyme. A comparison of these kinetic parameters is shown in Table 1. Two mutants, D66N/E had greatly lower *V*max values (99%) compared to that of the wild-type enzyme, whereas the mutant E50Q had an increased value (15%). The specificity constants (k_{cat}/K_m) of mutants D66N and D66E were 0.11% and 0.02% of the wild-type chitosanase, respectively. The substitution of Asp-66 with any amino acid residue drastically impaired the catalytic activity. Thus, Asp-66 seems to be essential for catalytic activity. Moreover, while specificity constant for the mutant E50Q enzyme was 58% higher than that for the wild-type enzyme, the specificity constant of the mutant E50D enzyme was nearly same as that of the wild-type enzyme. These results suggest that modification of the Glu-50 charged group to an uncharged group substantially enhances catalysis at the active site. It is estimated that the E50Q mutation brings about some increase in flexibility which makes the induced fit for the enzyme to the chitosan more tight since the higher activity is attributed to a smaller K_m value. In addition, modification of cysteine residues at 49 and 72 resulted in no change of catalytic ability. Comparison of the kinetic constants of C211S and wild-type enzymes revealed an approximately 16% decrease in the specificity constant in the C211S mutant enzyme, which is probably attributed to its thermal instability. This result supports that cysteine groups are little-involved in catalysis.

ACKNOWLEDGMENTS

This study was supported by a research grant from the Bioproducts Research Center of Yonsei University(Project No. 96-K3-04, 07-01-06-3).

REFERENCES

1. Akiyama, K., T. Fujita, K. Kuroshima, T. Sakane, A. Yokota, and R. Takata. 1999. J. Biosci. Bioeng. 87: 383-385.
2. Allan, G. G., J. R. Fox, and N. Kong. 1978. MIT Press., Cambridge, Mass.
3. Ando, A., K. Noguchi, M. Yanagi, H. Shinoyama, Y. Kagawa, H. Hirata, M. Yabuki, and T. Fujii. 1992. J. Gen. Appl. Microbiol. 38: 135-144.
4. David, J. L. 1991. John Wiley & Sons Ltd. 114-147.
5. Fenton, D. M., and D. E. Eveleigh. 1981. J. Gen. Microbiol. 126: 151-165.
6. Fukamizo, T., and R. Brzezinski. 1997. Cell. Biol. 75: 687-696.
7. Fukamizo, T., Y. Honda, S. Goto, I. Boucher, and R. Brzezinski. 1995. Biochem. J. 311: 377-383.
8. Hedges, A., and R. S. Wolfe. 1974. J. Bacteriol. 120: 844-853.
9. Kagawa. Y., H. Nojima, N. Nukiwa, M. Ishizuka, T. Nakajima, T. Yasuhara, T. Tanaka, and and T. Oshima. 1984. J. Biol. Chem. 259: 2956-2960
10. Mozhaev, V. V. (1993) *Trends Biotechnol.* 88-95.
11. Masson, J. Y., F. Dennis, and R. Brzezinski. 1994. Gene. 140: 103-107.
12. Masson, J. Y., I. Boucher, W. A. Neugebauer, D. Ramotar, and R. Brzezinski. 1995. Microbiology. 141: 2629-2635.
13. Ohtaka, A., H. Ogata, Y. Taketomi, and M. Mitsutomi. 1984. In Chitin, Chitosan and Related Enzymes. Academic Press, New York. 147-160.
14. Parro, V., M. S. Roman, I. Galindo, B. Purnelle, A. Bolotin, A. Sorokin, and R. P. Mellado. 1997. Microbiology. 143: 1321-1326.
15. Yoon, H. G., H. Y. Kim, H. K. Kim, K. H. Kim, H. J. Hwang, and H. Y. Cho. 1999. J. Microbiol. Biotechnol. 9: 631-636.
16. Yoon, H. G., S. C. Ha, Y. H. Lim, and H. Y. Cho. 1998. J. Microbiol. Biotechnol. 8: 449-454.

Table 1. Kinetic parameters of wild-type and mutant chitosanase from *Bacillus* sp. CK4

Enzyme	V_{max} (unit/mg)	K_m (mg/ml)	k_{cat} (min^{-1})	k_{cat}/K_m min^{-1}/(mg/ml)
Wild type	98.2	11.8	88.1	7.46
E50Q	113	8.62	101	11.8
E50D	98.9	9.92	88.7	8.94
E62Q	101	11.2	90.5	8.08
E62D	88.1	7.97	78.9	9.90
D66N	0.87	93.2	0.78	0.0084
D66E	0.21	89.4	0.19	0.0021
C49S	93.6	9.64	83.9	8.70
C72S	103	8.90	92.3	10.3
C211S	97.3	13.9	87.2	6.27

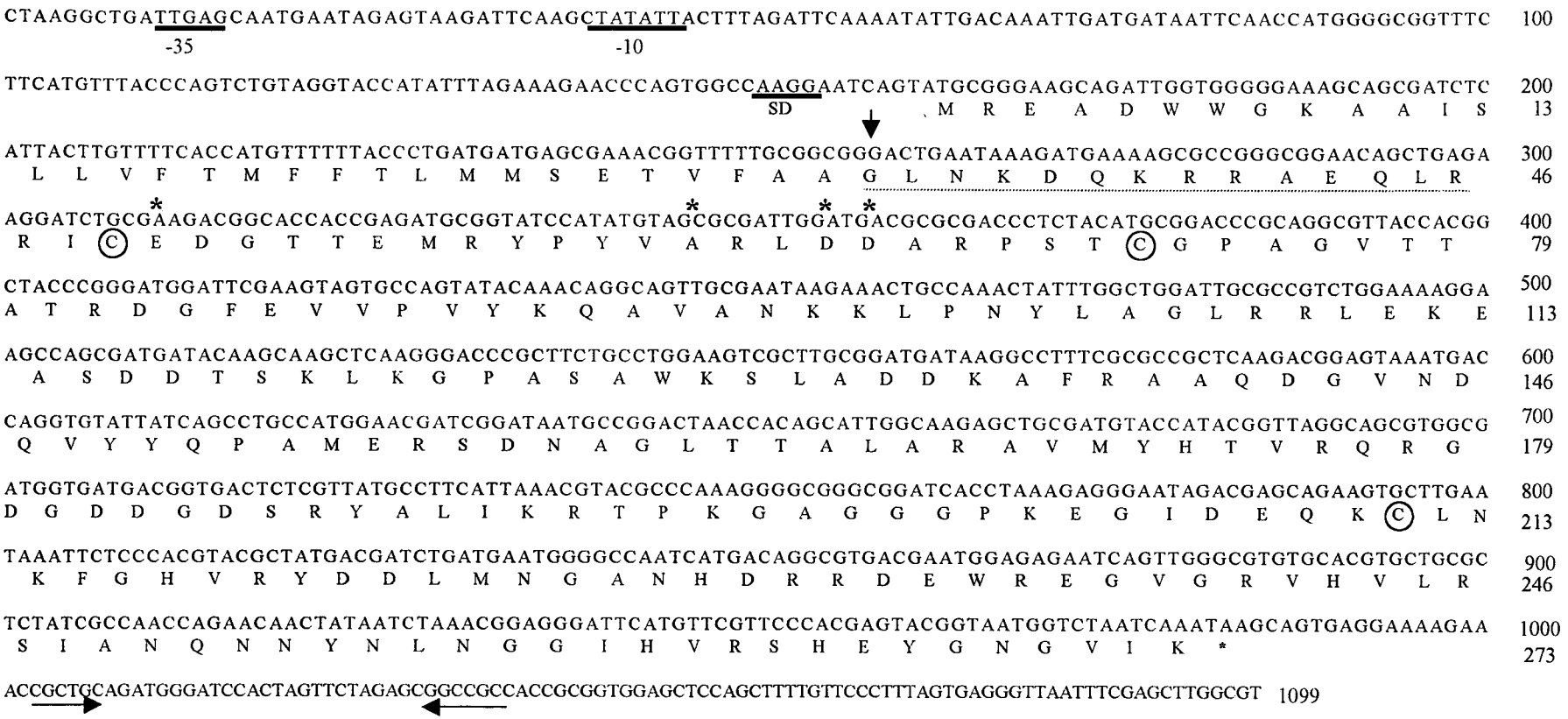

Fig. 1. Nucleotides sequence of the *cho*K gene and deduced amino acid sequence of the gene products. Coding region started at position 163 and was terminated at position 984. The –35 and -10 region of a putative promoter sequence and possible Shine-Dalgarno (SD) sequence for the ribosome binding site were underlined. In the 3'-flanking region from the coding sequence, a sequence capable of forming a stem and loop structure, which might be involved in transcription termination, was indicated by arrows. The *N*-terminal amino acids as determined by Edman degradation were also underlined by broken underline. The amino acid residues that seemed to be essential for chitosanase activity were asterisked. The cysteine residues, potential source of thermostability, were indicated by the circle.

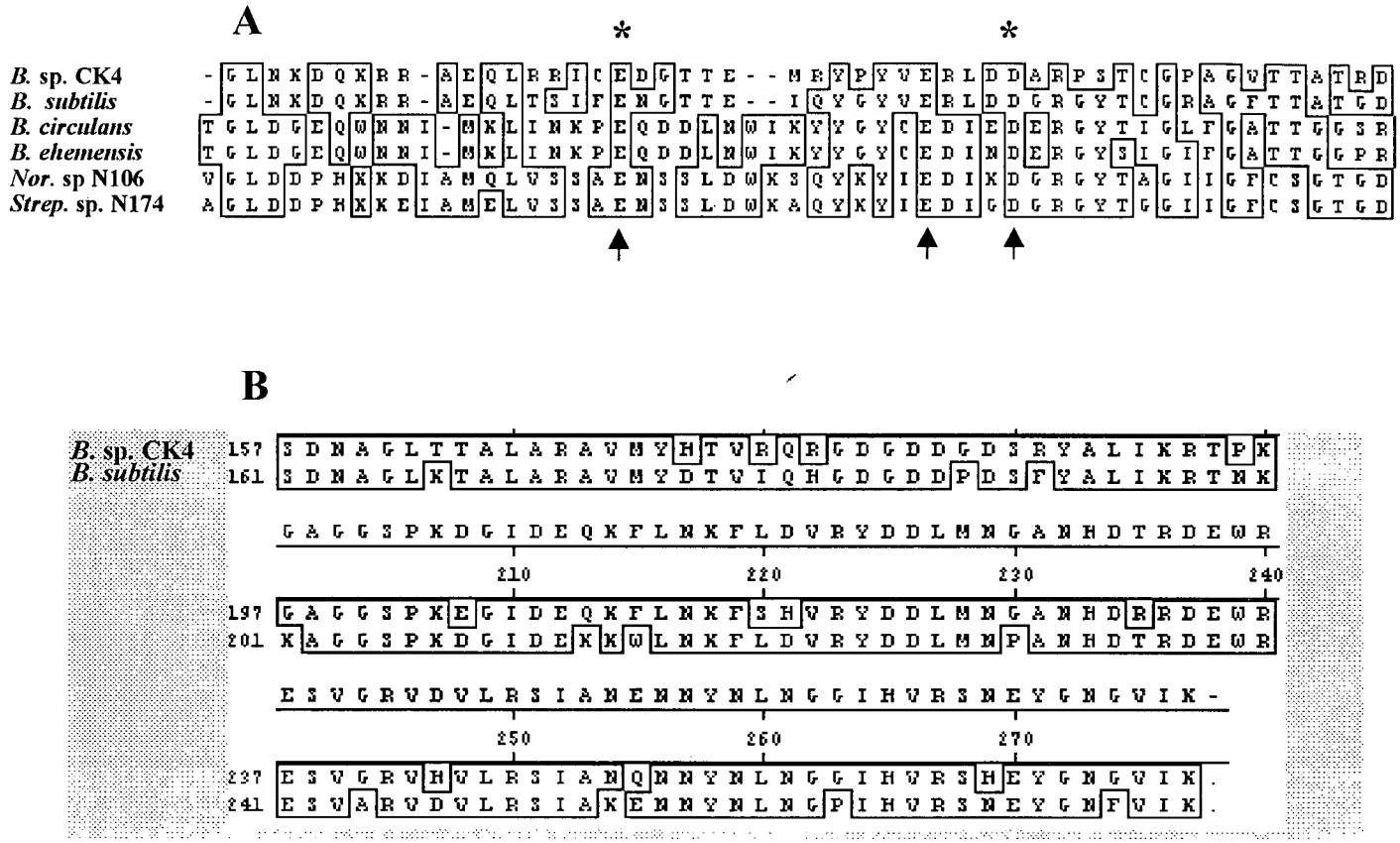

Fig. 2. A, Alignment of putative catalytic N-terminal segment. *, Essential catalytic residues of *Streptomyces* sp. N174; *the arrows*, the amino acid residues which seem to be essential for chitosanase activity. B, Amino acid sequence alignment of C-terminal region. Identical amino acids are boxed. All sequences are numbered from Met-1 of the peptide.

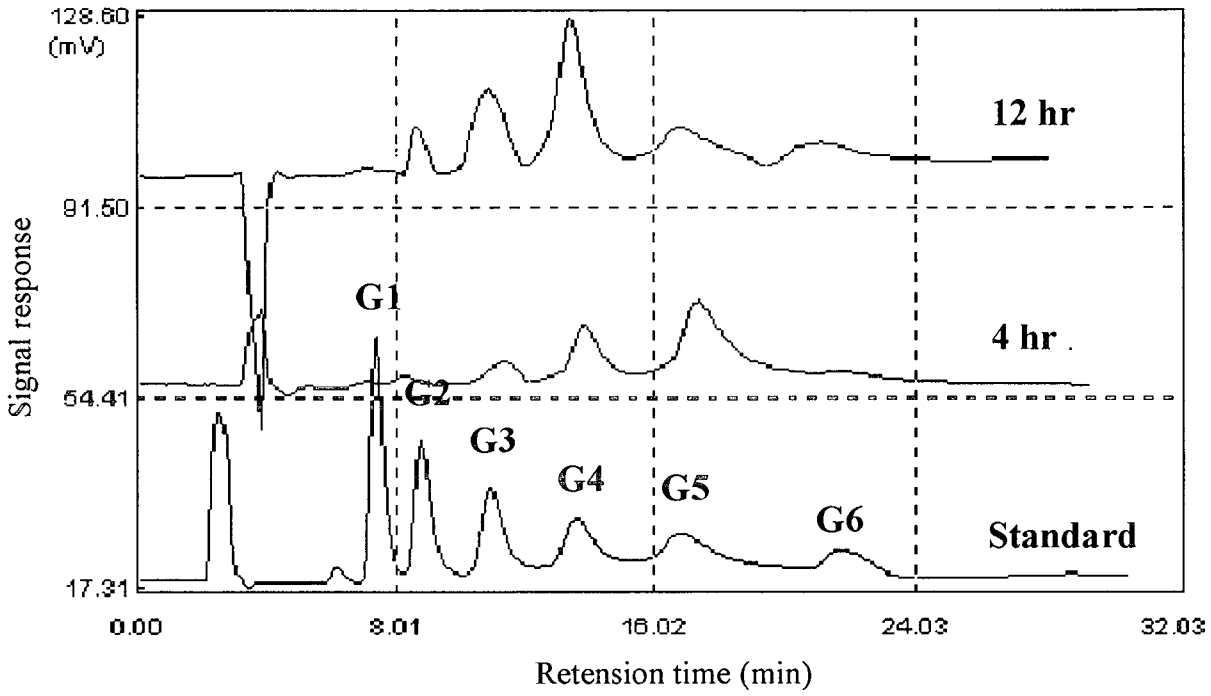

Fig. 3. The hydrolysate profiles of bacterial chitosanases. Samples were incubated at 37℃ for 12 hrs and analyzed on TSK-Gel $NH_2$60 column for the chitosan oligosaccharide. Standard G1-G6 indicate standard $(GlcN)_n$(n=1-6).

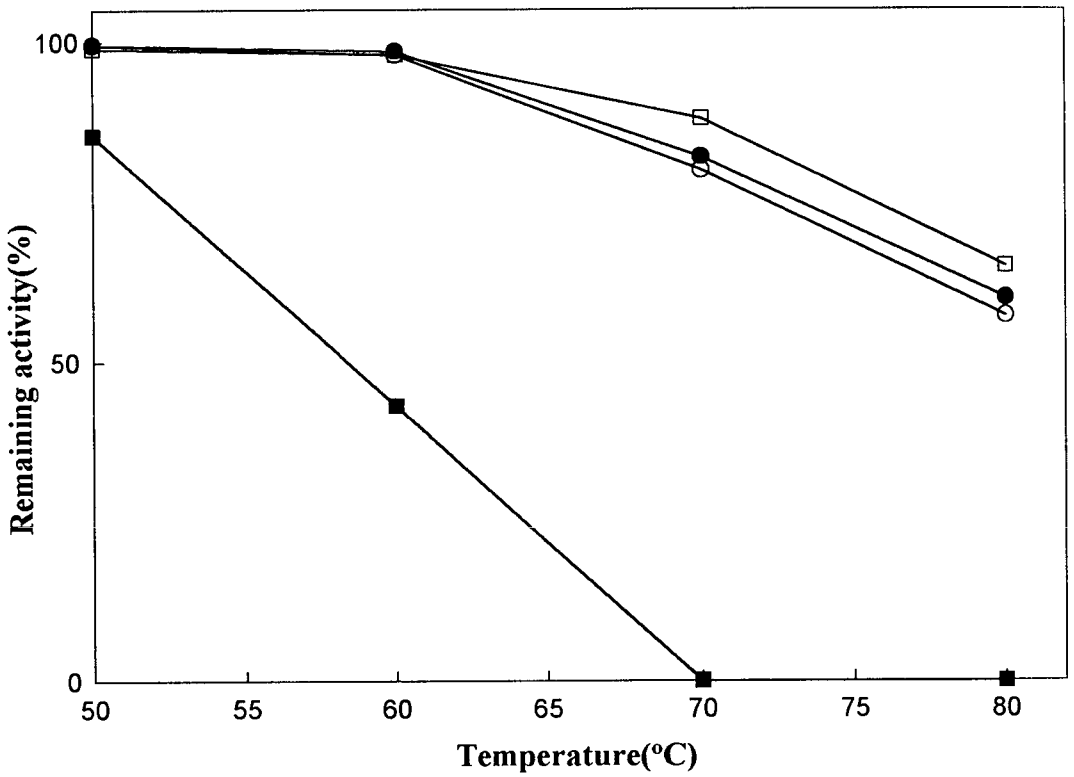

Fig. 4. Thermostability of chitosanases. The enzymes (1.2 mg/ml) were incubated in 50 mM potassium phosphate buffer (pH 7.0) at various temperature for 60 min, and the remaining activities were assayed. □ , wild-type enzyme ; ● , C49S; ○ , C76S; ■ , C211S.

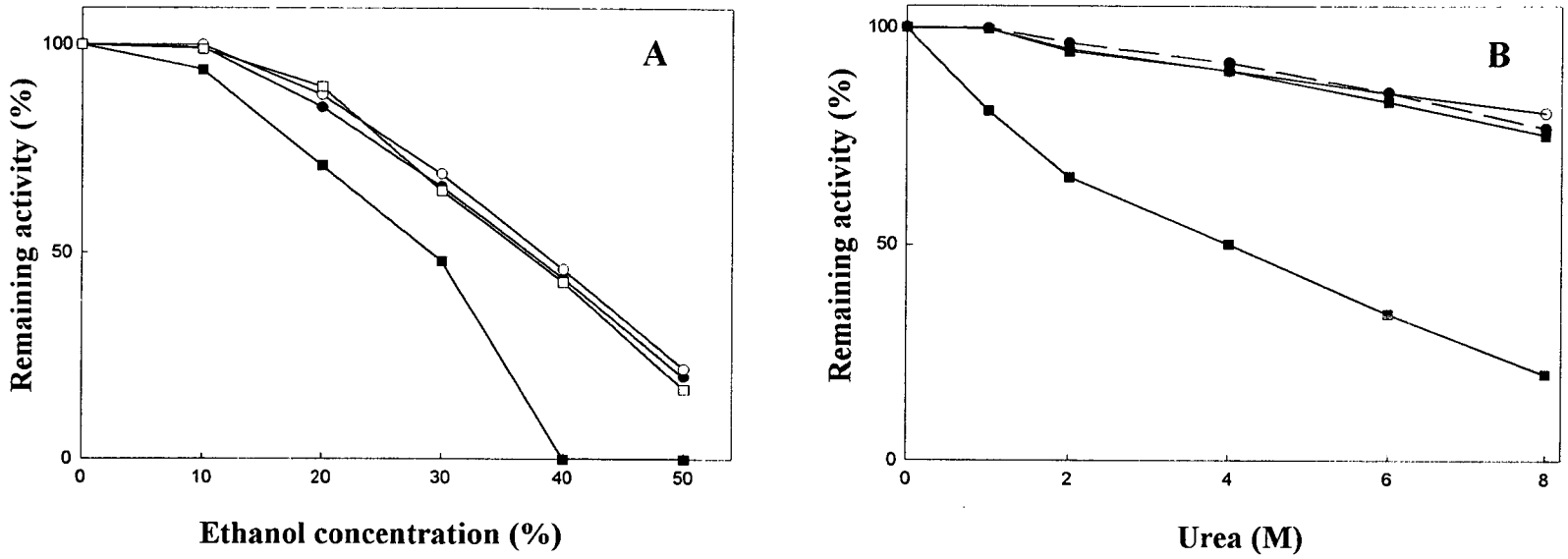

Fig. 5. Effect of ethanol and urea on stability of chitosanases. A, The enzymes (1.2 mg/ml) were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing various concentration of ethanol for 30 min, and then the remaining activity was assayed after ultrafiltration with Centricon-10 (Amicon Co.). B, The enzymes (1.2 mg/ml) were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing various concentration of urea for 30 min, and then the remaining activity was assayed after ultrafiltration with Centricon-10. □, wild-type enzyme; ●, C49S; ○, C76S; ■, C211S.