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Cloning and Expression of Thermostable Chitosanase Gene from *Bacillus* sp. KFB-C108

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Abstract The thermostable endo-chitosanase gene from the isolated strain *Bacillus* sp. KFB-C108 was identified on the basis of a phylogenetic analysis of the 16S rRNA gene sequence, and was cloned into plasmid pUC18 using *E. coli* DH5α as the host strain. Positive clones carrying recombinant plasmids (pKCHO I and pKCHO II) containing chitosanase activity were selected using the direct activity staining method. Detailed physical maps showed the two plasmid inserts were identical except that the KCHO II insert (2.6 kb) was 1.8 kb smaller than that of the KCHO I. The recombinant plasmids were analyzed to determine the essential region for chitosanase activity, and a 1.3-kb fragment (KCHO-6) was subcloned into pTrc99A using the *Eco*RI and *Bam*HI sites to construct pTrc99A/KCHO-6(pTrEB13). The resulting plasmid exerted high chitosanase activity upon transformation of *E. coli* DH5α cells, overproducing about 20 times more in the cloned cells than in the wild-type cells. The cloned chitosanase protein exhibited the same molecular weight and catalytic activity similar to those of *Bacillus* sp. KFB-C108. The cloned enzyme was an endo-type that produced a chitosan tetramer as the major reaction product; however, it produced no monomers or dimers.

Key words: Cloning, expression, thermostable chitosanase

Chitosan, a partly acetylated or nonacetylated counterpart (4-linked 2-amino-2-deoxy-β-D-glucopyranan) of chitin, is present in the mycelial and sporangiophore walls of fungi, and exoskeletons of insects and crustacea [4, 10, 19]. It is usually obtained by artificial deacetylation of chitin. Currently, chitosan and its partially degraded oligosaccharides are becoming important because of their potential applications as medical and agricultural agents [1, 29]. Chitosanases (EC 3.2.1.99), which hydrolyze chitosan to glucosamine oligomers, have been purified from several microorganisms [2, 4, 7, 16, 28], and most of them catalyze the endo-type cleavage of chitosan with a narrow range of deacetylation degrees [5, 6, 17, 18, 20, 22, 24, 28]. Chitosanases can be classified into at least three groups according to their cleavage specificity; the first group can split GlcNAc-GlcN [5, 6, 18] or GlcN-GlcN [26], the second group can only split GlcN-GlcN, and the third group hydrolyzes GlcN-GlcNAc or GlcN-GlcN [21, 28]. However, chitosanases cannot in general split the linkage of GlcNAc-GlcNAc. In most cases, these chitosanses are inducible by the substrate chitosan, and play a role in degradation and utilization of exogenous chitosan. Recently, thermolabile chitosanase genes from several bacteria such as *Bacillus circulans* MH-K1 [2], *Streptomyces* sp. N174 [12], and *Norcadia* sp. N106 [13] have been cloned and sequenced. However, the thermostable enzyme and its gene have not yet been characterized in detail.

The purification and properties of the thermostable enzyme from strain KFB-C108 have already been reported [26]. This new enzyme hydrolyzes chitosan into 3~6 oligomers by an endo-type catalytic action, and produces no dimer or monomer as a degrading product of the chitosan. However, the mechanism of hydrolysis and the catalytic system involved in the enzyme reaction are still unclear. Since pure protein is available for detailed mechanistic studies, and as an extension to our previous work on the structural analysis of this enzyme and its application in industrial chitooligosaccharide production, we undertook to clone the chitosanase gene and to overexpress it. This paper describes the identification of the isolated strain on the basis of a phylogenetic analysis of the 16S rRNA gene, and the cloning and expression of the thermostable chitosanase gene from the thermostable gram-positive bacterium, KFB-C108.

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MATERIALS AND METHODS

Materials

Colloidal chitosan was prepared using the Yabuki *et al.* method [25]. Chitosan, D-glucosamine, lysozyme, and RNase were obtained from Sigma Co. (St. Louis, U.S.A.). Chitosan dimer, tetramer, and other chitosan oligosaccharides were purchased from Seikagaku Co. (Tokyo, Japan). The vectors used (pUC18, pTrc99A, and pKK223-3) were obtained from Pharmacia Biotech (Uppsala, Sweden). The restriction enzymes and T4 DNA ligase were purchased from Promega Co. (Madison, U.S.A.). Thermostable chitosanase from *Bacillus* sp. KFB-C108 was prepared as described previously [26] and the active fraction separated by second Butyl-Toyopearl column chromatogaphy was used as the purified enzyme. All other reagents were of analytical grade.

Microorganism and Cultivation

The thermophilic *Bacillus* sp. KFB-C108 was selected as a potent thermostable chitosanase producer from soil at 55°C [26], and used as the source of chromosomal DNA. *Escherichia coli* DH5α was used as the recipient strain for the recombinant plasmids. *E. coli* was grown at 37°C on an LB medium for selection of the transformants.

Isolation and Cloning of DNA

Chromosomal DNA was prepared from *Bacillus* sp. KFB-C108 using Marmur's method [14]. The DNA was partially digested with *Sau*3AI and electrophoresed on a 1.0% agarose gel. Fragments corresponding to the size of 2~10 kb were collected using a Prep-A Gene DNA Purification kit (BioRad, Hercules, U.S.A.). The plasmid pUC18 was cleaved at the *Bam*HI site and treated with calf intestinal alkaline phosphatase. The *Sau*3AI fragments from the chromosomal DNA were ligated into the dephosphorylated *Bam*HI site of the pUC18. Transformed cells were grown on a chitosan-Congo red agar medium containing ampicillin at 37°C. The colonies of the enzyme-positive transformants developed clear orange haloes on the red background of the medium, because the chitosan was hydrolyzed by the enzyme freed from the cells due to partial autolysis.

Analysis of Recombinant Plasmids

The recombinant plasmid was digested with *Bam*HI and the insert DNA was isolated using agarose gel electrophoresis. The insert DNA was used for both restriction mapping and subcloning. Various lengths of the DNA fragments derived from the 4.4 kb region of KCHO I and the 2.6 kb region of KCHO II were unidirectionally detected from each side. The deletion mutants of pKCHO I and II obtained were introduced into *E. coli* DH5α. The chitosanase activity of each transformant was assayed [27]. Plasmid DNAs from the recombinants were prepared using an alkaline lysis procedure [8].

Subcellular Fractionation of Chitosanase

The enzyme product from the *E. coli* transformant was subcellularly fractionated [9]. A 2-ml sample of the culture broth was sonicated three times at 20 kHz for 20 sec in an ice-water bath. This sonicated lysate was analyzed for the whole chitosanase activity. Another sample (5 ml) of the culture broth was centrifuged at 7,000 ×g for 10 min at 4°C. The supernatant was analyzed for the extracellular enzyme activity. The precipitate was suspended in 5 ml of a 25% (w/v) sucrose-1 mM ethylenediaminetetraacetic acid-10 mM Tris-HCl buffer (pH 8.0). A 1 ml sample of the suspension was diluted three-fold with water and sonicated as described above. This sonicated lysate was analyzed for intracellular chitosanase activity. The remaining suspension (4 ml) was incubated at 30°C for 1 h after the addition of egg white lysozyme (0.3 mg). A 1-ml sample of the spheroplast suspension was diluted three-fold with a 25% (w/v) sucrose-30 mM $MgSO_4$-10 mM Tris-HCl buffer (pH 7.3) and centrifuged. The supernatant was analyzed for periplasmic enzyme activity. The precipitate was washed with a buffer of high osmotic strength, suspended in 0.5 mM $MgCl_2$, and sonicated briefly. This sonicated lysate was then analyzed for cytoplasmic enzyme activity.

Enzyme and Protein Assays

The reaction mixture containing 250 µl of 1.0% chitosan, 50 µl of a 1.0 M potassium phosphate buffer (pH 6.5), and the enzyme solution in a final volume of 1 ml was incubated at 55°C for 30 min in a shaking water bath. The reaction was stopped by heating at 100°C for 10 min, followed by centrifugation. The amount of reducing sugars in the supernatant was determined by the dinitrosalicyclic acid (DNS) method and also with a modified version of Miller's method [15]. One unit of the enzyme was defined as the amount of the enzyme required to produce 1 µmol of reducing sugar per min. D-glucosamine was used as the standard. The protein concentration was determined by the Lowry method [11] with bovine serum albumin as the standard.

Analysis of Enzymatic Hydrolysis Products

The reaction mixture containing 700 µl of the enzyme solution (5 mg/ml), 250 µl of 5.0% colloidal chitosan, and 50 µl of a 1.0 M potassium phosphate buffer (pH 7.0) containing 0.01% NaN_3, was incubated at 55°C for 12 h. The reaction was stopped by heating at 100°C for 10 min, followed by centrifugation. The products in the supernatant were analyzed by HPLC with a TSK-gel NH_2-60 column (Tosho Co., Japan) and eluted with an acetonitrile-water mixture (60:40). The eluted chitosan oligosaccharide was detected with a refractive index (RI) detector. D-glucosamine, chitosan dimer, trimer, tetramer, pentamer, and hexamer were used as the authentic standards.

Southern Blot Analysis

The restricted partial digestion of the chromosomal DNA was separated in 1.0% agarose gels and blotted onto Hybond-N membranes (Amersham) according to the manufacturer's recommendation. Detection was carried out with nonradioactive DNA probes. The Probe, a 1.3-kb *Eco*RI-*Bam*HI fragment, isolated from the plasmid pKCHO II (Fig. 2), was labeled using an Amersham ECL nucleotide detection kit.

Nucleotide Sequence Determinaion of 16S rRNA Gene

A polymerase chain reaction was performed to amplify the 16S rRNA coding region, using two oligonucleotide primers, 5'-GGCTGCAGAACACATGCAAGTCGAAC-GGT-3' (position 50 to 70 relative to *E. coli* 16S rRNA) and 5'-GGCTTAAGTGTTCCGGGCCCTTGCATAAG-3' (position 1374 to 1394 relative to *E. coli* 16S rRNA), and then the PCR product with the predicted size was cloned into the pBluescript II SK(+). The plasmids of the subclones were 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., Sweden) as specified by the manufacturer. The DNA fragments were analyzed on an ALFexpress Autoread Sequencer (Pharmacia Biotech., Sweden). The homology search and construction of a phylogenetic tree were performed using LAGERGENE (DNASTAR, Inc., U.S.A.).

Results and Discussion

Classification of Strain KFB-C108

The strain KFB-C108 used in this study is one of the thermophilic bacterial strains isolated from a high temperature environment and stocked in the authors' laboratory. The morphological and physiological characteristics of this strain are summarized in Table 1. An analysis of the fatty acid composition in the cell wall using a microbial identification system (Sherlock system, MIDI Co., U.S.A.) revealed that KFB-C108 showed a similar homology to most *Bacillus* species, although several characteristics such as growth temperature were not consistent with this genera. Therefore, the partial nucleotide sequence of the 16S rRNA gene from strain KFB-C108 was determined, the sequence being corresponding to the region between positions 50 and 1394 of the gene in *E. coli* [3]. A comparison of this sequence with those registered in nucleotide sequence databases indicated that it had a high homology to the 16S rRNA gene of *Bacillus* sp., however, it was not identical to any type of the strains (Fig. 1). Based on both the morphological and physiological properties, the strain KFB-C108 can be identified as a member of the genus *Bacillus*.

Table 1. Characteristics of strain KFB-C108.

Traits		Characteristics
Gram stain		+
Shape		Rod (0.6×2.5) μm
Motility		+
Spore		+
Strict aerobes		-
Hydrolysis of casein		+
starch		+
gelatin		+
Catalase		+
Oxidase		-
VP test		-
Nitrate reduced to nitrite		-
Utilization of citrate		-
propionate		-
Growth at 40°C		+
55°C		+
60°C		-
SI (Similarity Index)[a] to	*Bacillus subtilis*	0.78
	Bacillus circulans	0.80
	Bacillus cereus	0.65
	Bacillus coagulans	0.70
GC Content[b]		36.1%

[a]SI was estimated using a microbial identification system (Sherlock, MIDI Co., U.S.A.). The perfect score is 1.0.
[b]GC content was estimated by T_m.

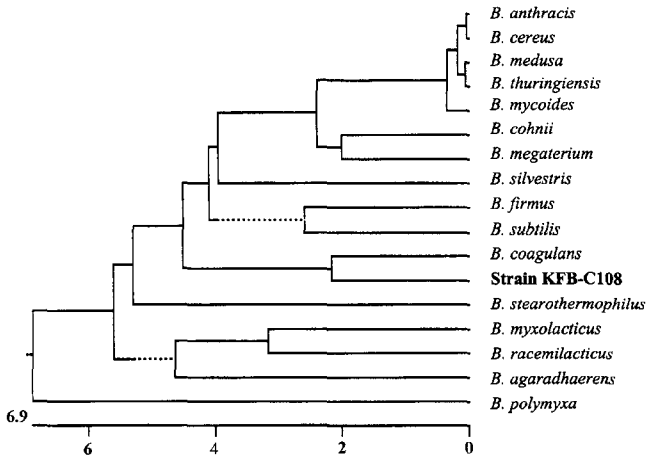

Fig. 1. The phylogenetic tree of 16S rRNA genes of genus, *Bacillus*.

The sequences for comparison with that of the 16S rRNA genes of *Bacillus* sp. were obtained from the GenBank database. The origin and the accession numbers of the sequences are as follows: *B. anthracis*, X5509; *B. cereus*, D16266; *B. medusa*, X60628; *B. thuringiensis*, D16281; *B. mycoides*, Z84583; *B. cohnii*, AF140014; *B. megaterium*, D16273; *B. silvestris*, AJ006086; *B. firmus*, D16268; *B. subtilis*, AB018595; *B. stearothermophilus*, AJ005760; *B. myxolacticus*, D16274; *B. racemilacticus*, D16279; *B. agaradhaerens*, X76445; *B. polymyxa*, D16276. The phylogenetic tree was constructed, using Lagergene (DNASTAR, Inc., U.S.A.), by the clustal method with a weighted residue weight table. The numbers on the base line refer to the divergence between species.

Cloning of the Chitosanase Gene from *Bacillus* sp. KFB-C108

Recombinant *E. coli* DH5α containing the clone of the *Bacillus* sp. KFB-C108 genomic DNA was screened for cloning of the chitosanase gene. The transformant possessing the chitosanase gene was selected by formation of an orange halo on the glycol chitosan-Congo red agar medium containing ampicillin. From about 8,000 ampicillin-resistant colonies, two colonies were found to exhibit orange haloes, which suggested that the cloned fragments were derived from the chitosanase gene of *Bacillus* sp. KFB-C108. The DNA inserts of the plasmids (designated KCHO I and KCHO II) were characterized by digestion with restriction enzymes, and detailed physical maps showed that the two plasmid inserts were identical except that the pKCHO II insert (2.6 kb) was 1.8 kb smaller than that of the pKCHO I (Fig. 2A).

To determine the location of the chitosanase gene in the 4.4-kb insert DNA, a series of deletion mutants of pKCHO I were constructed and the chitosanase activity was assayed. Deletion of a 3.1-kb region from left to right did not affect the expression of the chitosanase activity, however, deletion of 1.3 kb from right to left (KCHO-6) caused the loss of chitosanase activity. Accordingly, the 1.3-kb *Eco*RI-*Bam*HI fragment was identified as the region necessary for the production of chitosanase (Fig. 3). The *Eco*RI-*Bam*HI fragment was subcloned using pUC18 and pUC19. The transformants carrying pUC18/KCHO-6(pEB13) or pUC19/KCHO-6(pBE07) showed high chitosanase activity with colloidal chitosan as substrate, indicating that the transcription of the gene occurred by using its own promoter.

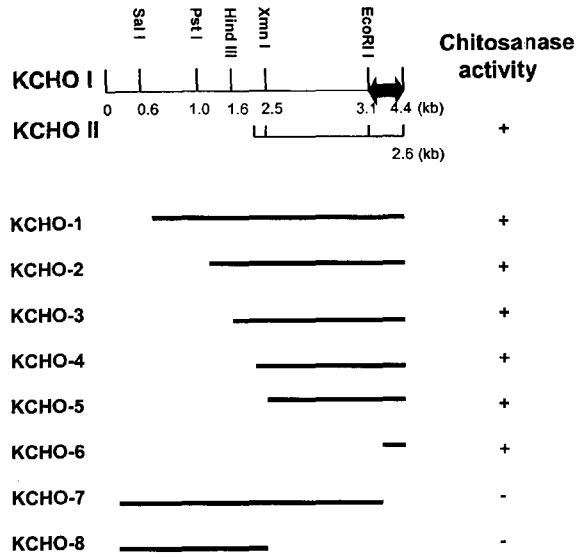

Fig. 3. Deletion analysis of KCHO I and KCHO II.
The transformants carrying the plasmids with appropriate deletions were transferred to an LB agar plate containing 0.5% glycol chitosan, 0.1% Congo red and ampicillin. The chitosanase activity was judged by the formation of orange haloes. The arrow indicates the necessary region for the expression of chitosanase. +, Detected chitosanase activity; -, No detected chitosanase activity.

The chromosomal DNA of *Bacillus* sp. KFB-C108, partially digested, was analyzed using a Southern blot hybridization with a 1.3-kb *Eco*RI-*Bam*Hi fragment isolated from the plasmid pKCHO II as a probe (Fig. 2(B)). This result revealed that the *Eco*RI-*Bam*HI fragment in the two plasmids was derived from *Bacillus* sp. KFB-C108 as the DNA donor.

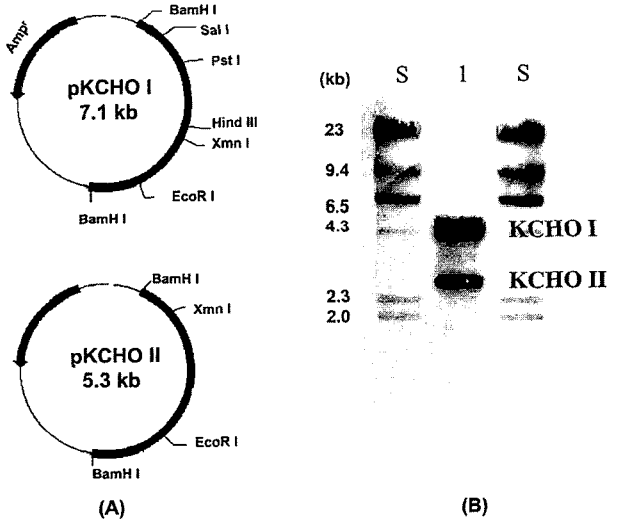

Fig. 2. Restriction map (A) and Southern blot hybridization (B) of KCHO I and KCHO II.
The probe, 1.3-kb *Eco*RI and *Bam*HI fragment, was labeled using an Amersham ECL nucleotide detection kit. Lanes S, Lamda-DNA digested with *Hin*dIII; Lane 1, Genomic DNA/*Sau*3AI partial digestion.

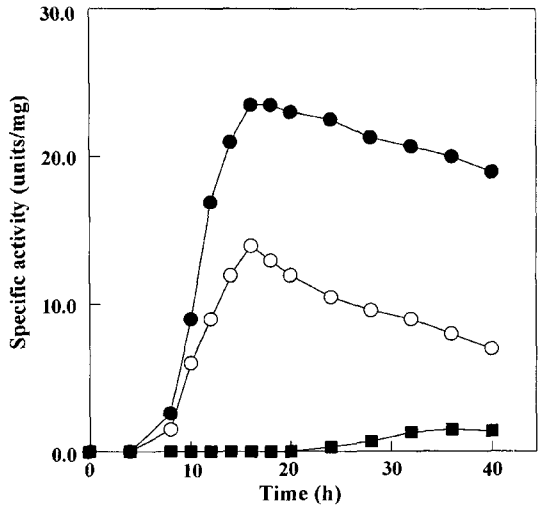

Fig. 4. Overexpression of the chitosanase gene using pTrc99A/KCHO-6 in *E. coli* DH5α.
Specific activities of periplasmic fractions from recombinant cells, pEB13 (-○-) and pTrEB13 (-●-); Extracellular fraction from *Bacillus* sp. KFB-C108 (-■-).

Construction of an Over-Producer of Chitosanase

To increase the enzyme productivity, the 1.3 kb gene fragment KCHO-6 was ligated into the pTrc99A expression vector and the over-producer pTrc99A/KCHO-6 (pTrEB13) was constructed. The specific activities of the enzyme from the periplasmic layer of this over-producer were compared with those of the extracellular enzyme from the wild-type *Bacillus* sp.. The specific activities of the enzyme produced by *E. coli* DH5α carrying pTrEB13 and *E. coli* DH5α carrying pEB13 were about 20- and 9-fold higher than those of the enzyme from *Bacillus* sp. KFB-C108, respectively (Fig. 4). An SDS-PAGE analysis indicated that the molecular weight of the recombinant enzyme was identical with that of chitosanase from the native strain (Fig. 5). This result suggests that KCHO-6 may correspond to the intact gene product not generated by proteolytic processing.

The location of the cloned chitosanase in *E. coli* was determined by separating bacterial proteins into extracellular, periplasmic, and cytoplasmic fractions. The chitosanase activity was located mainly in the periplasmic fraction for up to 18 h of cultivation. Low chitosanase activity was also detected in both the culture supernatant and the cytoplasmic fraction. These results indicate that the cloned chitosanase is mainly translocated into the periplasm of *E. coli* (Table 2).

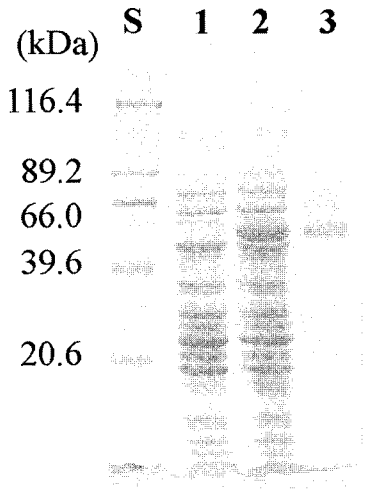

Fig. 5. SDS-PAGE analysis of chitosanase protein from *E. coli* DH5α carrying pTrEB13.
Lane S, Size marker; Lane 1, Extracellular fraction of *E. coli* DH5α carrying pTrc99A; Lane 2, Intracellular fraction of *E. coli* DH5α carrying pTrEB13; Lane 3, Purified chitosanase from *Bacillus*. sp KFB-C108 in the previous work.

Table 2. Subcellular fractionations of chitosanase activity.

Plasmid[a]	Chitosanase activity (units/ml)[b]			
	Extracellular	Intracellular	Periplasmic	Cytoplasmic
pEB13	ND[c]	6.8	5.2	2.4
pTrEB13	ND[c]	26	24	1.9

[a]*E. coli* DH5α carrying pUC18/CHO-6/(designated as pEB13) or pTrc99A/CHO-6(designated as pTrEB13) was grown in an LB medium at 37°C for 12 h.
[b]Chitosanase was subcellularly fractionated as described in Materials and Methods. The activity in each fraction is represented as units/ml of equivalent culture medium.
[c]ND, not detected.

Hydrolysis Products of Colloidal Chitosan

A change of hydrolysis products from colloidal chitosan was observed during incubation with the recombinant crude enzyme at 55°C for 12 h. The hydrolysis products, $(GlcN)_4$, was detected as the major product and small amounts of trimer and pentamer were produced, however, no monomer or dimer (Fig. 6). It is suggested that the mode of action of the enzyme is the endo-type. The reaction product and mode of action of the cloned enzyme was the same as that of the enzyme from the native strain [26]. Endo-type chitosanases have been reported from several microorganisms [9-18] and their degrading patterns on chitosan were similar. Although the amount of oligomers was varied in each case, as previously reported, these enzymes hydrolyzed chitosan into 1-6 oligomers using an endo-type catalytic action. However, the enzyme described in the present study did not produce a dimer or monomer as a degrading product of chitosan under the reaction conditions of considerably high temperature and long period of time.

In conclusion, the chitosanase from *Bacillus* sp. KFB-C108 is quite thermostable, and also shows stability in several organic solvents [26]. Futhermore, the enzyme from the recombinant *E. coli* shows remarkable characteristic features of a catalytic action that produces a tetramer as the major product. Recently, several bacteria such as *Bacillus circulans* MH-K1 [2], *Streptomyces* sp. N174 [12], and *Nocardioides* sp. N106 [13] have been cloned and sequenced, yet the thermostable enzyme and its gene have

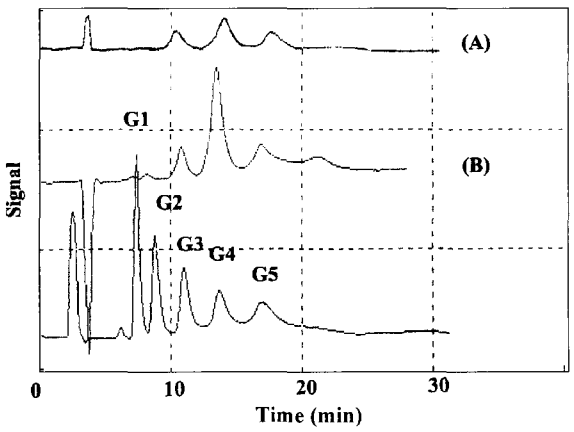

Fig. 6. HPLC of products in the hydrolysate of colloidal chitosan by chitosanases from native strain, *Bacillus* sp. KFB-C108 (A) and *E. coli* DH5α carrying pTrEB13 (B).
Hydrolysis was performed for 12 h. Standard G1-5 indicate $(ClcNAc)_n$ (n=1-5).

not yet been characterized in detail. Thus, the thermostable gene of this enzyme will be useful for a comparative study of the enzyme stability, clarification of its catalytic action, and its application in industry.

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