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New Thermostable Chitosanase from *Bacillus* sp.: Purification and Characterization

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Abstract A thermostable chitosanase was purified from *Bacillus* sp. KFB-C108, by fractionation of 30 to 70% saturation with ammonium sulfate, DEAE-Toyopearl chromatography, Butyl-Toyopearl chromatography, and TSK-Gel HW-55F gel filtration. The purified enzyme showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the molecular weight was estimated to be 48 kDa. The enzyme degraded soluble chitosan and colloidal chitosan, but did not degrade glycol chitosan, chitin, and the other compounds investigated. There was no effect on the chitosanase activity by treatment with chelating agents, alkylating agents, and various metals investigated, and only cobalt ions inhibited the activity. Optimum temperature and pH were 55°C and 6.5, respectively. The enzyme was stable after heat treatment at 80°C for 10 min or 70°C for 30 min and fairly stable in several organic solvents as well. Chitosan was hydrolyzed to $(GlcN)_4$ as a major product by incubation with the enzyme.

Key words: Chitosanase, thermostable enzyme, *Bacillus*, purification

Chitosan, the partly acetylated or nonacetylated counterpart (4-linked 2-amino-2-deoxy-β-D-glucopyranan) of chitin, is present in mycelial and sporangiophore walls of fungi and exoskeletons of insects and crustacea [6, 12, 17]. It is usually obtained by the artificial deacetylation of chitin. Recently, chitosan and its partially degraded oligosaccharides are becoming important because of potential applications as medical and agricultural agents. Chitosanases (EC 3.2.1.99), which hydrolyze chitosan to glucosamine oligomers, have been purified from several microorganisms [3, 4, 11, 13].

The purified chitosanases are classified into several groups by their substrate specificity; the first group hydrolyzes only chitosan [13, 16, 20], the second group hydrolyzes both chitosan and carboxymethycellulose [1, 10], and the third group hydrolyzes both chitosan and chitin [9, 15, 19]. However, their physiological roles and catalytic actions have not yet been clarified.

We have screened bacteria producing a thermostable chitosanase and found an enzyme catalyzing hydrolysis of only soluble and colloidal chitosan in *Bacillus* sp. KFB-C108 cells. The enzyme hydrolyzed neither glycol chitosan, chitin, nor carboxymethylcellulose. We report here purification and characterization of the enzyme that is thermostable, and that has stability in organic solvents and unique substrate specificity.

MATERIALS AND METHODS

Materials

DEAE-Toyopearl 650 M, Butyl-Toyopearl 650 M, TSK-Gel Toyopearl HW-55F were purchased from TOSOH (Tokyo, Japan). Chitin, chitosan, and glycol chitosan were from Sigma Co. (St. Louis, MO, U.S.A.). Colloidal chitosan was prepared by the method of Yabuki *et al.* [18]. Colloidal chitin was prepared by Lockwood's method [5]. All other chemicals were analytical-grade reagents.

Microorganism and Cultivation

A thermophilic bacterium, *Bacillus* sp. KFB-C108, which was selected as a potent thermostable chitosanase producer from soil at 70°C, was used for the production of the enzyme. The medium is composed of 1.2% colloidal chitosan, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract (pH 6.5).

The microorganism was precultured in the medium containing 1.2% glycerol instead of chitosan. For the production of the enzyme the precultured microorganism (1%, v/v) was added in a 500 ml baffle flask with 100 ml

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of the medium and cultured at 55°C for 40 h with agitation at 150 rpm.

Enzyme and Protein Assay

The reaction mixture containing 250 μl of 1.0% soluble chitosan, 50 μl of 1.0 M potassium phosphate buffer (pH 6.5), and 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 and the reaction mixture was centrifuged. The amount of reducing sugars in the supernatant was determined by the DNS method [2]. One unit of enzyme was defined as the amount of enzyme required for producing 1 μmol of reducing sugar (D-glucosamine) per hour. Protein concentration was determined by the Lowry method [8] with bovine serum albumin as a standard.

Purification of the Enzyme

After cultivation, the cells were removed by centrifugation at 14,000×g for 30 min. 0.1 mM Phenylmethylsulfonyl fluoride and 0.05 mM 1-tosylamido-2-phenylethylchloromethyl ketone were added to the supernatant before use as the crude enzyme extract solution.

Ammonium sulfate fractionation. Powdered ammonium sulfate was added to the crude enzyme extract solution to obtain 30% saturation. After 30 min, the supernatant was collected by centrifugation at 14,000×g for 10 min and additional ammonium sulfate was added to 70% saturation. After standing for 1 h, the precipitate was collected by centrifugation and dissolved with a minimum volume of the buffer (10 mM phosphate). The enzyme solution was dialyzed against the phosphate buffer.

DEAE-Toyopearl column chromatography. The dialyzed solution was applied to a DEAE-Toyopearl 650 M column (1.8×30 cm) that had been equilibrated with 10 mM phosphate buffer (pH 7.5). After the column was washed thoroughly with the buffer, a linear gradient elution was performed with the buffer supplemented with KCl by increasing the concentrations from 0 to 0.3 M. The active fractions were pooled, concentrated by addition of ammonium sulfate to 70% saturation, followed by centrifugation. The precipitate was dissolved with phosphate buffer to 30% saturation of ammonium sulfate.

Butyl-Toyopearl column chromatography. The enzyme solution saturated with ammonium sulfate (30%) was applied to a Butyl-Toyopearl 650 M column (1.6×30 cm) equilibrated with the buffer containing ammonium sulfate (30% saturated) and washed with the same buffer. A linear gradient was performed with the buffer supplemented with ammonium sulfate by decreasing the concentration from 30 to 0% saturation. The active fractions were pooled and concentrated by ultrafiltration.

TSK-Gel filtration. The enzyme solution was subjected to gel filtration with a TSK-Gel HW-55F column (1.5×70 cm) equilibrated with the buffer containing 50 mM KCl. The enzyme was eluted with the same buffer at a flow rate of 10 ml/h. The active fractions were pooled, dialyzed, concentrated by ultrafiltration, and stored at -70°C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 10% polyacrylamide gel containing 0.1% SDS by the Laemmli method [7].

Measurement of Molecular Weight

The molecular weight of the enzyme was determined by TSK-Gel permeation chromatography. The following molecular weight markers obtained from Boehringer Mannheim (Germany) were used as standards: triose phosphate isomerase (MW 26.6 kDa), glutamate dehydrogenase (MW 55.6 kDa), fructose-6-phosphate kinase (MW 85.2 kDa), and β-galactosidase (MW 116.2 kDa).

Analysis of the Products of the Enzymatic Hydrolysis

The reaction mixture, 700 μl of enzyme solution (5 mg/ml), 250 μl of 20% chitosan, and 50 μl of 1.0 M potassium phosphate buffer (pH 6.5) 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 (Tosoh Co., Japan), and eluted with an acetonitrile-water mixture (60:40). The eluate was detected by a refractive index (RI) detector. D-glucosamine, chitosan dimer, trimer, tetramer, and pentamer were used as authentic standards.

RESULTS AND DISCUSSION

Purification of Chitosanase

When the thermophilic bacterium, *Bacillus* sp. KFB-C108, was cultivated at 55°C, extracellular and intracellular chitosanase activities were detected. As shown in Fig. 1, the cell growth reached its maximum after 40 h. The enzyme activity appeared in the late exponential phase of cell growth and increased rapidly to the maximum value in the stationary phase, about 1.3 units/ml in extracellular medium, after 60 h. The intracellular activity, however, was not detected until 84 h.

Chitosanase was purified about 50-fold with an overall yield of 15% (Table 1). The purified enzyme was homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Molecular Weight

The molecular weight of the enzyme was calculated to be about 48,000 Da by gel filtration (Fig. 2A). The

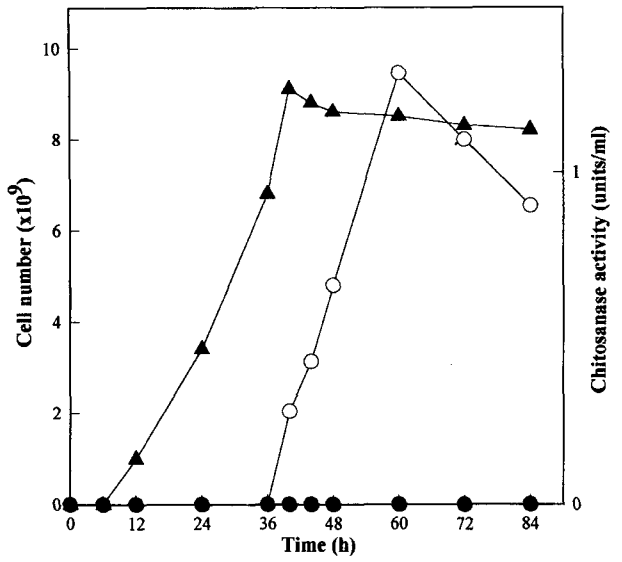

Fig. 1. Activities of extracellular (○) and intracellular (●) chitosanase during the growth (▲) of *Bacillus* sp. KFB-C108.

enzyme showed a single protein band with a molecular weight of about 48,000 Da upon SDS-PAGE (Fig. 2B). These data suggest that the enzyme is composed of a subunit.

Substrate Specificity

The activities of the purified chitosanase upon chitosan, chitosan derivatives, and other polysaccharides are presented in Table 2. Soluble chitosan and colloidal chitosan served as good substrates. The K_m values for soluble chitosan and colloidal chitosan were 3.8 and 9.1 mg/ml, respectively, and the V_{max} values were 101 and 27 unit/mg, respectively. Soluble chitosan was hydrolyzed 3 times faster than colloidal chitosan, indicating that the physical form of the substrate affects the rate of hydrolysis. The enzyme was specific for chitosan, but attacked neither chitin, cellulose, amylose, nor starch. The purified enzyme belongs to the enzyme group that is

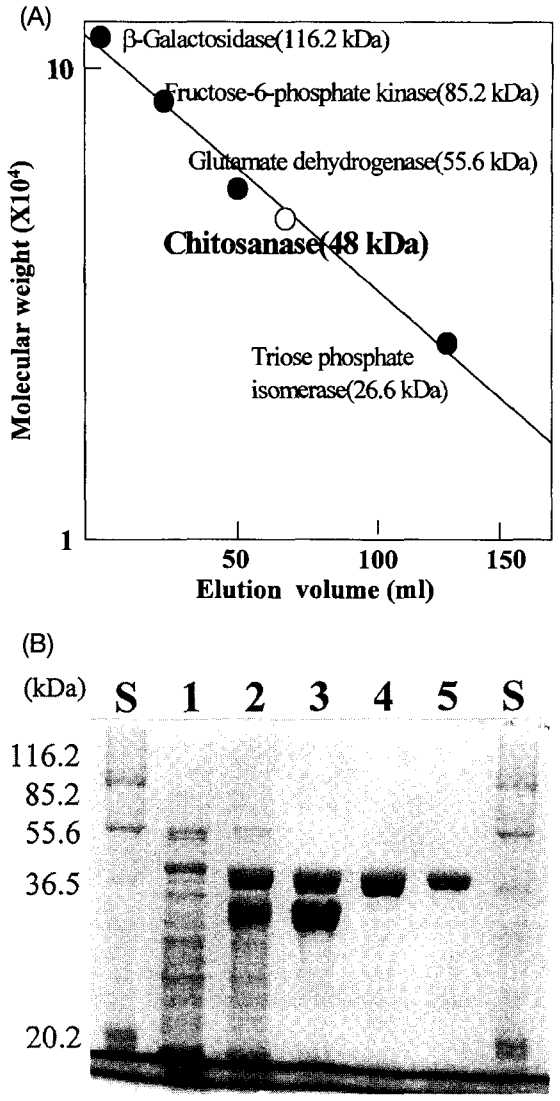

Fig. 2. Gel filtration (A) and SDS-PAGE (B) analysis of molecular weight of chitosanase from *Bacillus* sp. KFB-C108.
Lane S, size marker; lane 1, crude enzyme; lane 2, after ammonium sulfate fractionation; lane 3, after DEAE-Toyopearl 650 M; lane 4, after Butyl-Toyopearl 650 M; lane 5, after TSK-Gel HW-55F.

Table 1. Purification of thermostable chitosanase from *Bacillus* sp. KFB-C108.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yields (%)	Purification folds
Crude enzyme	3,100	6,750	2.2	100	--
Ammonium sulfate fractionation (30~70%)	1,260	5,400	4.3	80	2.0
DEAE-Toyopearl chromatography	60.2	2,900	48.2	43	22.0
Butyl-Toyopearl chromatography	21.7	2,030	93.5	30	43.0
TSK-Gel HW-55F gel filtration	9.0	989	110.0	15	50.0

Table 2. Substrate specificity of thermostable chitosanase from *Bacillus* sp. KFB-C108.

Substrate (1.0 %)	Relative activity (%)	V_{max} (unit/mg)	K_m (mg/ml)
Soluble chitosan	100	101	3.8
Colloidal chitosan	35	27	9.1
Glycol chitosan	0	0	-
Chitin	0	0	-
Glycol chitin	0	0	-
Colloidal chitin	0	0	-
Cellulose	0	0	-
CMC*	0	0	-
Laminarin	0	0	-
Amylose	0	0	-
Starch	0	0	-

*Carboxymethylcellulose; -, not applicable.

able to hydrolyze only chitosan. The previously reported chitosanases classified in the group hydrolyzing only chitosan, also can catalyze glycol chitosan and partially that of *O*-hydroxyethylated chitosan as well. However, this new enzyme is distinct from other enzymes in the substrate specificity of glycol chitosan degradation. Therefore, the substrate specificity of the enzyme is very high compared with that of other enzymes in the group. The enzyme *Enterobacter* sp. G-1 hydrolyzed colloidal chitin, colloidal chitosan (about 80% deacetylated), but did not hydrolyze colloidal chitosan (100% deacetylated) [19]. The enzyme from *Fusarium solani* showed a preference for chitosan with 70% of deacetylation [14] and those from *Bacillus* sp. PI-7S [13] and *Pseudomonas* sp. H-14 [20] were most active on approximately 100% deacetylated chitosan. These results suggested that the enzyme recognized *N*-acetyl groups at the cleavage site. Thus, the effect of the level of acetylation in the chitosan on the catalytic action of the enzyme from *Bacillus* sp. KFB-C108 is understudied.

Effects of Temperature and pH on the Enzyme Activity and Stability

The optimal temperature and pH for chitosanase activity were examined. The enzyme was most active at 55°C and pH 6.5 under the standard assay condition (data not shown).

To determine the heat stability of the enzyme, the enzyme solution in 20 mM potassium phosphate buffer, pH 6.5, was incubated at various temperatures for 10 and 30 min. After addition of soluble chitosan to the enzyme solution, the assay was performed as described in Materials and Methods. The result is shown in Fig. 3A. The remaining activities after treatment of the enzyme at 80°C for 10 and 30 min were 70 and 45%, respectively. 90% of initial activity was retained even after incubation at 60°C for 30 min. The enzyme activity was relatively stable up to 70°C. In order to determine the effect of pH on the stability of the enzyme, chitosanase was incubated in buffers of various pHs at 55°C for 30 min. After adjusting the solution to pH 6.5, the remaining activity was determined. The result is shown in Fig. 3B. The remaining activity was over 80% at pH 6.0 to 11.0. These results indicate that the enzyme is relatively stable in acidic and basic conditions.

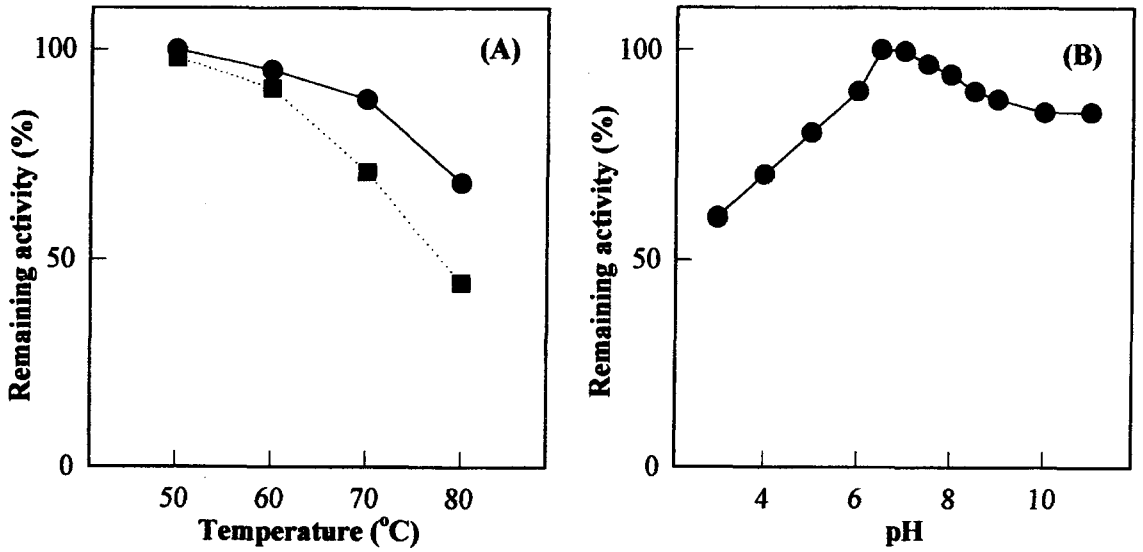

Fig. 3. Effects of temperature and pH on the stability of the purified chitosanase.

A. After the enzyme was incubated at 50, 60, 70, and 80°C for 10 (●) and 30 (■) min, remaining activities were measured for determining the thermostability of the enzyme. B. The purified enzyme was incubated in various pH range buffers at 55°C for 30 min. After adjusting the reaction solutions to pH 6.5, the remaining activities were measured to determine the pH stability of the enzyme.

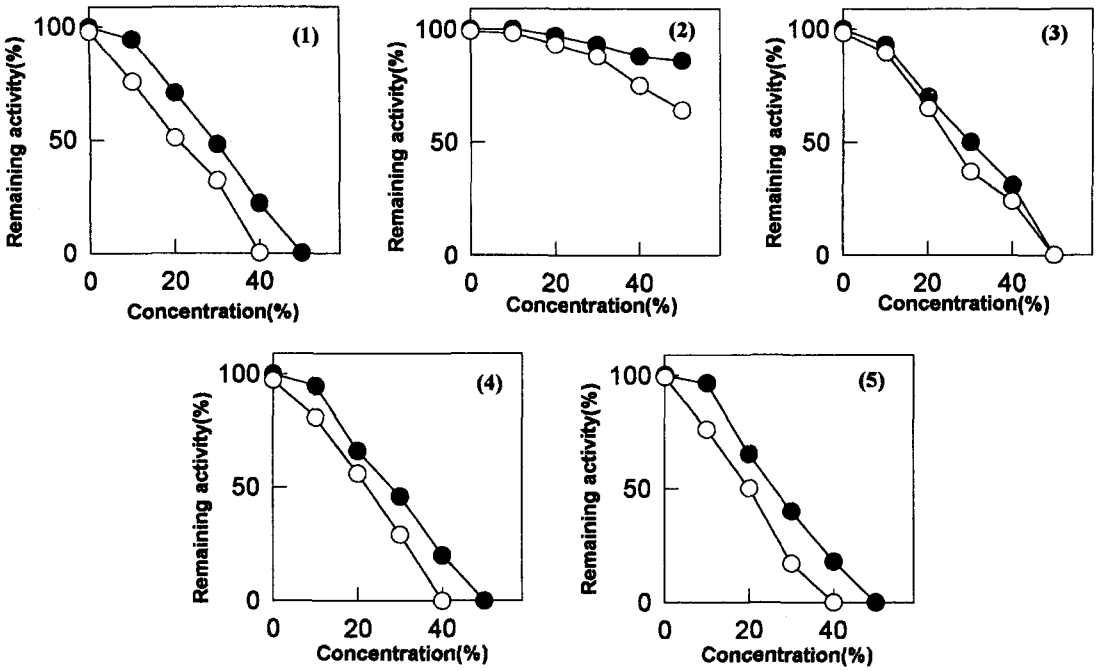

Fig. 4. Enzyme stability in organic solvents.

After the enzyme was incubated in 10, 20, 30, 40, and 50% of each organic solvent at 37°C (●) and 55°C (○) for 30 min, the remaining activities were measured under the standard assay conditions. (1), in ethanol; (2), in acetone; (3), in methanol; (4), in ethyl acetate; (5), in dimethylsulfoxide.

Enzyme Stability in Organic Solvents

Stable enzymes in organic solvents have a great advantage, specially when the enzymes are used in industry. We investigated the effect of organic solvents on the stability of the chitosanase activity. The result is shown in Fig. 4. The remaining activities of the enzyme were 86 and 64% of the initial activity after incubation in 50% acetone at 37°C and 55°C for 30 min, respectively. The activities of the enzyme retained more than 50% of the initial activity in 20% of the other organic solvents investigated. This means that the enzyme is very stable in organic solvents and this property would be useful in industrial applications.

Effects of Metal Ions and Chemical Reagents

To determine the effect of metal ions, each of various ions was added to the enzyme solution at a final concentration of 1 mM (Table 3). The enzyme activity was assayed after preincubation of the enzyme solution containing each ion at room temperature for 30 min. Only cobalt ions inhibited the enzyme activity. EDTA, PMSF, and several reagents blocking the SH group had no effect on the activity. This suggests that the metal ions, serine residue, and SH group are not essential for the catalytic action of the enzyme.

Table 3. Effect of metal ions and organic compounds on thermostable chitosanase activity from *Bacillus* sp. KFB-C108.

Chemicals (1 mM)	Relative activity (%)
None	100
$HgCl_2$	93.0
$CaCl_2$	124
$MnCl_2$	96.0
$Pb(CH_3COO)_2$	125
$NiCl_2$	83.0
$FeSO_4$	133
$ZnCl_2$	80.0
$CoCl_2$	52.0
$CuSO_4$	118
$MgSO_4$	119
Na-thiosulfate	113
Na-sulfite	118
Na-citrate	114
Cysteine	113
Ascorbic acid	120
EDTA[a]	85.0
SDS[b]	80.0
Sodium azide	100
p-Chloromercuribenzoic acid	100
PMSF[c]	98.0
N-Ethylmaleimide	100
β-Mercaptoethanol	99.2
Iodoacetate	98.7

[a]Ethylenediaminetetraacetate.
[b]Sodium dodecyl sulfate.
[c]Phenylmethylsufonyl fluoride.

Hydrolysis Products of Colloidal Chitosan

The catalytic pattern of chitosanase was examined using colloidal chitosan as the substrate. In the hydrolysis of colloidal chitosan with the purified enzyme, tetramer as a major product and small amounts of trimer and

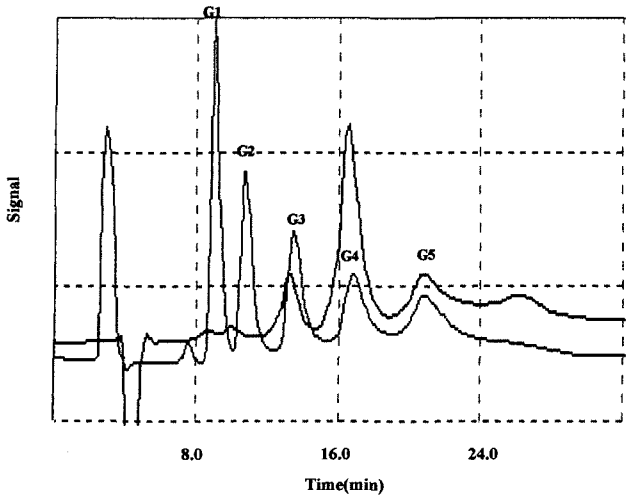

Fig. 5. HPLC profile of the products produced in the hydrolysis of colloidal chitosan by chitosanase from *Bacillus* sp. KFB-C108.
The analysis sample was hydrolyzed at 55°C for 12 h. Each of standards G1-5 indicates monomers, dimers, trimers, tetramers, and pentamers.

pentamer were produced, but not monomers and dimers (Fig. 5). It suggests that the mode of action of the enzyme is the endo-type. Endo-type chitosanases have been reported from several microorganisms [3, 4, 11-13] and their degrading patterns on chitosan were similar. Although the amount of oligomers was variable in each case, these enzymes previously reported hydrolyzed chitosan to 2-6 oligomers by endo-type catalytic action. However, the enzyme described here did not produce dimers as a degrading product of chitosan at all.

In conclusion, chitosanase from a thermophilic bacterium, *Bacillus* sp. KFB-C108 is quite thermostable. The enzyme shows stability in several organic solvents as well. The enzyme shows high substrate specificity and a characteristic feature of catalytic action producing tetramers as a major product. Thus, the enzyme is useful for comparative study of stability of the enzyme, for clarifying catalytic action, and for application in industry.

Acknowledgments

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