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Thermostable Chitosanase from *Bacillus* sp. Strain CK4: Its Purification, Characterization, and Reaction Patterns

Ho-Geun YOON, Hee-Yun KIM,* Hye-Kyung KIM,** Bum-Shik HONG, Dong-Hoon SHIN, and Hong-Yon CHO†

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
**Department of Food Additives, Korea Food and Administration, Seoul 122-020, Korea*
***Department of Food and Biotechnology, Hanseo University, Chungnam 352-800, Korea*

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A thermostable chitosanase, purified 156-fold to homogeneity in an overall yield of 12.4%, has a molecular weight of about 29,000 ± 2,000, and is composed of monomer. The enzyme degraded soluble chitosan, colloidal chitosan, and glycol chitosan, but did not degrade chitin or other β-linked polymers. The enzyme activity was increased about 2.5-fold by the addition of 10 mM Co^{2+} and 1.4-fold by Mn^{2+}. However, Cu^{2+} ion strongly inhibited the enzyme. Optimum temperature and pH were 60°C and 6.5, respectively. The enzyme was stable after heat treatment at 80°C for 30 min or 70°C for 60 min and fairly stable in protein denaturants as well. Chitosan was hydrolyzed to $(GlcN)_4$ as a major product, by incubation with the purified enzyme. The effects of ammonium sulfate and organic solvents on the action pattern of the thermostable chitosanase were investigated. The amounts of $(GlcN)_3$–$(GlcN)_6$ were increased about 30% (w/w) in DAC 99 soluble chitosan containing 10% ammonium sulfate, and $(GlcN)_1$ was not produced. The monophasic reaction system consisted of DAC 72 soluble chitosan in 10% EtOH also showed no formation of $(GlcN)_1$, however, the yield of $(GlcN)_3$ ~ $(GlcN)_6$ was lower than DAC 99 soluble chitosan-10% ammonium sulfate. The optimal concentration of ammonium sulfate to be added was 20%. At this concentration, the amount of hexamer was increased by over 12% compared to the water-salt free system.

Key words: Thermostable chitosanase; *Bacillus* sp. strain CK4; Chitosan oligosaccharide

Chitosan is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amido-D-glucose units with the latter usually exceeding 80%.[1] It occurs in nature in the mycelial and sporangiophore walls of fungi and exoskeletons of insects and crustacea.[2,3] Chitin and its deacetylated form, chitosan, have attracted significant interest in view of varied proposed novel applications.[4] Uses of these two functional polymers, especially chitosan, are readily seen over a broad range of scientific areas, including applications in biomedical, food, and various chemical industries.[5,6] However, chitosan has potential bioactivities such as anti-microbial,[7] anti-cholesterol synthesis,[8] anti-tumor by immunostimulating[9] and elicitor activity, its effects *in vivo* are still ambiguous due to a low uptake rate into the interior of the body. Therefore, converting chitosan to its oligosaccharides is necessary for more effect *in vivo*. On the other hand, because the purification procedure of chitosan oligosaccharides was not simple, its application was limited to pharmaceutical and high value-added industries due to the high unit cost of production. For extensive application of chitosan oligosaccharides, enzymatic hydrolysate is actually more efficient than the purified oligomer. Over the last decade, many chitosanolytic enzymes with different substrate specificities have been characterized,[10-14] and most of them catalyze the endo-type cleavage of chitosan with a narrow range of deacetylation degrees.[15,16] In addition, most of them are thermolabile chitosanases, while little information is available on thermostable chitosanases. Thermostable chitosanases active between 60°C and 100°C and specifically attacking the β-D-glucosaminidic bonds are of special interest.[17,18]

We have screened for bacteria producing a thermostable chitosanase and found an enzyme catalyzing hydrolysis of chitosan only in *Bacillus* sp. strain CK4 cells. We report here purification and characterization of the enzyme that is thermostable, and that has unique substrate specificity and modified reaction patterns in monophasic systems composed of salts and organic solvents.

† To whom correspondence should be addressed. Phone: 82-2-923-8731; Fax: 82-2-923-8733; E-mail: hycho@korea.ac.kr

Abbreviations: DAC, degree of deacetylation; (GlcN), β-D-glucosamine; AS, ammonium sulfate; EDTA, ethylaenediaminetetraacetic acid; PMSF, phenymethylsulfonyl fluoride

Materials and Methods

Chemicals and substrates. DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, and TSK-Gel HW-55F were from Tosoh (Tokyo, Japan). Chitin, chitosan, glycol chitin, and glycol chitosan were purchased from Sigma Co. (St. Louis, USA). Colloidal chitin was also prepared by the method of Lockwood *et al.*[19] Colloidal chitosan was prepared using the method of Uchida and Ohtakara.[20] Colloidal chitosan and soluble chitosan with a different degree of deacetylation were prepared using the method of Kurita *et al.*[21] Degree of deacetylation was calculated by using the colloidal titration method.[22] Chitosan, D-glucosamine, and laminarin were obtained from Sigma Co. (St. Louis, USA). Chitosan dimer, tetramer, and other chitosan oligosaccharides were purchased from Seikagaku Co. (Tokyo, Japan). All other reagents were of analytical grade.

Microorganism and cultivation. A thermophilic bacterium, *Bacillus* sp. strain CK4, which was selected as a potent thermostable chitosanase producer from a hot spring in Korea, was used for the production of the enzyme. The culture medium composed of 1.0% colloidal chitosan, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.1% yeast extract (pH 6.5). The microorganism was first-cultured in the medium containing 1.0% glycerol instead of chitosan. For the production of the enzyme, this culture of microorganisms (1%, v/v) was added in a 500 ml baffle flask with 100 ml of the culture medium and cultivated at 60°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 the enzyme solution in a final volume of 1 ml was incubated at 60°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 measured using the modified dinitrosalicyclic acid (DNS) method.[23] One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min. D-glucosamine was used as a standard. The protein concentration was measured using the method of Lowry *et al.*[24] with bovine serum albumin as a standard. For the purified enzyme, protein was measured by the absorbance at 280 nm. The absorption coefficient ($A^{1\%/1\,cm} = 2.83$) estimated by ultracentrifugal analysis with a specific refractive increment for solute protein of 1.837×10^{-3} was used.

Purification of the thermostable chitosanase. 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 80% saturation. After standing for 1 h, the precipitate was collected by centrifugation and dissolved in a minimum volume of the buffer (10 mM phosphate, pH 7.0). The enzyme solution was dialyzed against the same buffer.

DEAE-Toyopearl column chromatography. The dialyzed solution was put on a DEAE-Toyopearl 650M column (1.8 × 30 cm) that had been equilibrated with 10 mM phosphate buffer (pH 7.0). After the column was washed thoroughly with the buffer, a linear gradient elution was made with the buffer containing NaCl added from 0 to 0.5 M. The active fractions were pooled, concentrated by addition of ammonium sulfate to 80% saturation, and centrifuged. 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 put on a Butyl-Toyopearl 650M column (1.6 × 30 cm) equilibrated with the buffer containing ammonium sulfate (30% saturated) and washed with the same buffer. A linear gradient was done with the buffer containing ammonium sulfate added from 30 to 0% saturation. The active fractions were pooled and concentrated by ultrafiltration.

TSK-Gel filtration. The enzyme solution was put through 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, and concentrated by ultrafiltration, and stored at −70°C.

Molecular weight measurement. The molecular weight of the enzyme was measured by the Superose-FPLC method with standard proteins (Oriental Yeast, Osaka, Japan). A 0.1 ml sample of enzyme solution (1.2 mg/ml) was put on two tandem gel filtration columns of Superose 12 (1 × 30 cm) in a Pharmacia FPLC system. The elution was done at a flow rate of 0.3 ml/min with 50 mM potassium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% 2-mercaptoethanol. The molecular weight of the subunit was measured by sodium dodecyl sulfate (SDS)/10% polyacrylamide gel electrophoresis by

Table 1. Purification of Thermostable Chitosanase from *Bacillus* sp. Strain CK4

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification folds
Crude extract	8,410	6,820	0.81	100	—
$(NH_4)_2SO_4$ fractionation	5,890	6,160	1.04	90.1	1.28
DEAE-Toyopearl chromatography	262	2,370	9.06	34.8	11.2
Butyl-Toyopearl chromatography	30.9	1,510	48.9	22.1	60.4
TSK-Gel HW-55F gel filtration	6.8	847	126	12.4	156

the method of Laemmli.[25]

Measurement of isoelectric point. Isoelectric focusing on 7.5% polyacrylamide gels containing 2% ampholyte and 10% glycerol was done by the method of Righetti.[26] The isoelectric point (pI) was estimated by comparing the migration position on the gel with other known proteins (Bio-Rad Lab, Hercules, U.S.A.).

Analysis of hydrolysis product. The substrate, soluble chitosan, was dissolved in 10 mM potassium phosphate buffer (pH 6.5) to give a 2.0% solution. The enzyme (0.1 mg/ml) was added to 1.0 ml of the substrate solution, and the reaction mixture was incubated at 60°C. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and boiled for 10 min to stop the enzymatic reaction. To analyze the chitosan oligosaccharide with TLC, the supernatants prepared under the conditions described above were spotted on a silica gel plate (Kieselgel 60, Merk, Germany) and developed with *n*-propanol:30% ammonia water (2:1). The sugars on the TLC plate were stained by spraying 0.1% ninhydrin dissolved with 99% ethanol. HPLC analysis was done with TSK-gel NH_2-60 column (Tosoh 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.

Results and Discussions

Purification of thermostable chitosanase from Bacillus sp. strain CK4

When the thermophilic bacterium, *Bacillus* sp. strain CK4, was cultivated at 60°C, 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 2.3 units/ml in extracellular medium, after 60 h. The intracellular activity, however, was not detected until 84 h (data not shown). The enzyme purification procedures are summarized in Table 1. The chitosanase was purified about 156-fold to a specific activity of 126 unit/mg of protein against completely deacetylated chitosan. The molecular mass of the enzyme was estimated to be 31,000 Da by SDS-PAGE and about 29,000 Da by Superose 12 gel filtration, indicating that the enzyme is monomeric (Fig. 1).

Effects of pH, temperature, and metal ions on enzyme activity

The enzyme had its maximum reactivity at pH 6.5 when assayed with soluble chitosan as a substrate. When the enzyme was assayed at various temperatures, the maximum activity was found at 60°C. The rate of soluble chitosan hydrolysis was increased about 2.5-fold by the addition of 10 mM Co^{2+} and 1.4-fold by Mn^{2+}. However, Cu^{2+} ion in the assay solution strongly inhibited (about 50%) the enzyme. Other divalent metal ions (Ba^{2+}, Mg^{2+}, Zn^{2+}, Ag^{2+}, and Hg^{2+}) did not inhibit the enzyme. This result was similar to those for other chitosanolytic enzymes, however, this enzyme is distinct from other enzymes in the inhibition of Cu^{2+} ion. EDTA, PMSF, and several reagents blocking the SH group had no effect on the enzyme activity. This suggests that the metal ions, serine residue, and SH group are not essential for the catalytic action of the enzyme.

Stability of enzyme

The thermostability of the protein was examined by measuring remaining activity after incubation at various temperatures. The remaining activities after treatment of the enzyme at 80°C for 30 and 60 min were 80% and 63%, respectively. The enzyme (0.5 mg per ml of 50 mM potassium phosphate buffer, pH 6.5) 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 nearly lost after 60 min at 90°C (Fig. 2(A)). 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 retained about 75% of its activity after incubation with 5% SDS at 55°C for 1 h. The stability of the enzyme was not influenced by differences in incubation temperature between 37°C

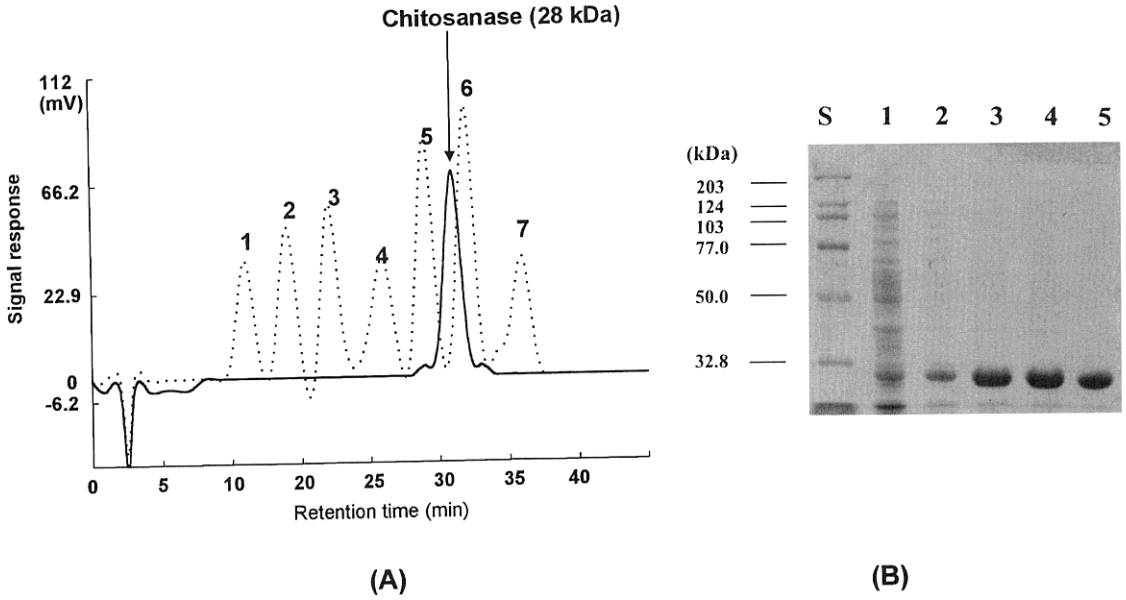

Fig. 1. Molecular Weight of Thermostable Chitosanase from *Bacillus* sp. Strain CK4.
(A) Molecular weight measurement by gel permeation chromatography. The protein standard in order to decreasing molecular weight: peak 1, ferritin (450,000); peak 2, catalase (240,000); peak 3, aldolase (158,0000); peak 4, bovine serum albumin (68,000); peak 5, hen egg albumin (45,000); peak 6, chymotrypsinogen (25,000); peak 7, cytochrome C (12,500) and the purified chitosanase is shown by bold line. (B) SDS-PAGE pattern of purified chitosanase. Lane 1, crude enzyme; lane 2, ammonium sulfate fractionation; lane 3, DEAE-Toyopearl 650M; lane 4, butyl-Toyopearl 650M; lane 5, TSK-Gel HW-55F column chromatography.

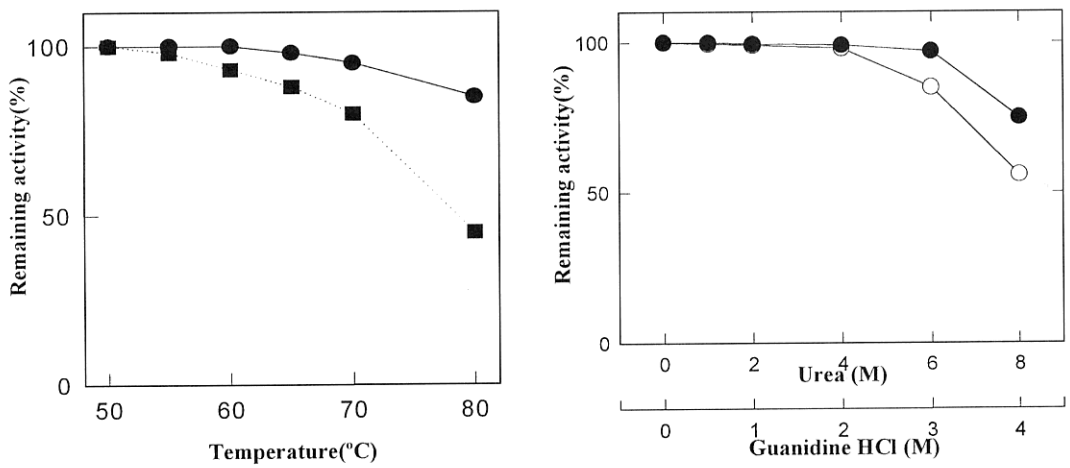

Fig. 2. Effects of Temperature, Urea, and Guanidine·HCl on Enzyme Stability.
After the enzyme was incubated at various temperatures for 30 (●) and 60 (■) min, or at 37°C for 30 min in the presence of various concentration of urea (●) and guanidine·HCl (○), the remaining activities were measured under the standard assay conditions.

and 60°C. 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. It is noteworthy that the enzyme is quite stable even in 8 M urea, which causes complete denaturation of ordinary proteins (Fig. 2(B)).

The positive effects of organic solvents on enzyme reactions normally done in an aqueous environment are several and include reversal of hydrolysis, alteration in catalytic specificity, increased thermostability, and improved product yield.[27] An enzyme stable in organic solvents has a great advantage, especially when the enzymes are used in industry. Therefore, we tested the effects of various organic solvents on the enzyme stability and enzyme reaction products. The results are shown Fig. 2. The enzyme retained more than 50% of the initial activity in 30% of the other water-miscible organic solvents, but the residual activities of chitosanase decreased when the nonpolarity of water-immiscible organic solvents increased (Fig. 3).

Substrate specificity

The activities of the purified chitosanase upon chitosan, chitosan derivatives, and other polysaccharides are presented in Table 2. The enzyme was reac-

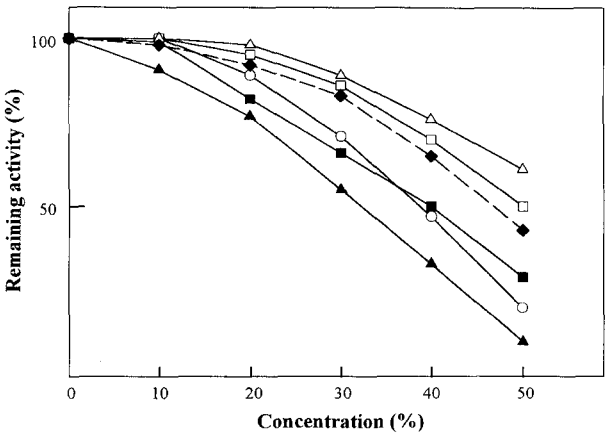

Fig. 3. Enzyme Stability in Organic Solvents.

After the enzyme was incubated in 10, 20, 30, 40, and 50% of each organic solvent at 55°C for 30 min on vigorous shaking at 400 rpm, the remaining activities were measured under the standard assay conditions. △, in ethanol; □, in metahnol; ◆, in acetone; ▲, in ethylacetate; ■, in chloroform; ○, in acetonitrile.

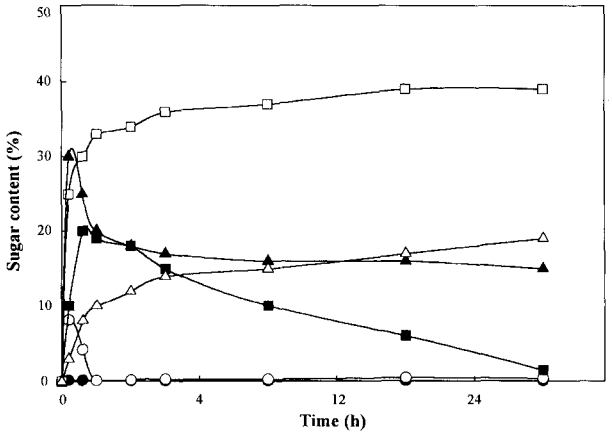

Fig. 4. Course of Soluble Chitosan Degradation by Thermostable Chitosanase.

$(GlcN)_n$ concentrations were calculated from peak areas from HPLC profiles. ●, $(GlcN)_1$; ○, $(GlcN)_2$; △, $(GlcN)_3$; □, $(GlcN)_4$; ▲, $(GlcN)_5$; ■, $(GlcN)_6$. Reaction conditions were 2.0% soluble chitosan in 50 mM potassium phosphate buffer, pH 6.5 at 60°C.

tive for soluble chitosan, colloidal chitosan, and glycol chitosan, but not chitin, cellulose, nor other polymers. The previously reported chitosanases classified in the group that hydrolyze only chitosan, also can act toward chitin and partially *O*-hydroxyethylated chitosan. The purified enzyme belongs to the enzyme group that is able to hydrolyze only chitosan. The enzyme *Enterobacter* sp. G-1 hydrolyzed colloidal chitin and colloidal chitosan (about 80% deacetylated), but did not hydrolyze colloidal chitosan (100% deacetylated).[28] The enzyme from *Fusarium solani* showed a preference for chitosan with 70% of deacetylation[29] and those from *Bacillus* sp. PI-7S[30] and *Pseudomonas* sp. H-14[31] were most active on

Table 2. Substrate Specificity of Thermostable Chitosanase

Substrate (1.0%)	Total activity (units)	Relative activity[a] (%)	V_{max} (unit/mg)	K_m (mg/ml)
Soluble chitosan				
DAC[b] 99%	73.7	100	130	0.91
DAC 83%	64.9	88.1	113	2.23
DAC 71%	52.3	71.2	92.3	3.54
DAC 53%	39.9	54.1	70.3	9.81
Colloidal chitosan				
DAC 99%	14.8	20.7	26.2	2.52
DAC 83%	25.1	34.0	44.2	3.79
DAC 71%	49.5	67.1	87.3	5.83
DAC 53%	64.4	87.4	114	1.92
Glycol chitosan	14.7	20.1	25.7	27.8
Chitin (Purified)	0	0	0	—
Glycol chitin	0	0	0	—

[a] The relative activity was expressed as percentage of the activity measured with soluble chitosan DAC 99%.

[b] Degree of deacetylation was calculated using the colloidal titration method.

approximately 100% deacetylated chitosan. These results suggested that the enzyme recognized *N*-acetyl groups at the cleavage site.

The substrate specificity of chitosanase on chitosan with different degrees of deacetylation (DAC), prepared by different procedure for *N*-acetylation, was examined. The relative activity increased when the DAC of soluble chitosan increased, but decreased when DAC of colloidal chitosan increased. This indicates that the physical form and DAC of substrate affects the rate of hydrolysis. However, no great difference was found among the hydrolysates of soluble chitosan and colloidal chitosan with different DAC (Table 2).

Hydrolysis Products of Soluble Chitosan

A change in the hydrolysis products from soluble chitosan was observed during incubation with the purified enzyme at 60°C for 36 h. At the initial stage, soluble chitosan was hydrolyzed to $(GlcN)_4$, $(GlcN)_5$, and $(GlcN)_6$ (80% of toatal products) and small amounts of the $(GlcN)_2$. After 12 h of incubation, the amount of $(GlcN)_5$ and $(GlcN)_6$ in the hydrolysate decreased, while $(GlcN)_3$ and $(GlcN)_4$ levels increased (Fig. 4). It is suggested that the mode of action of the enzyme is the endo-type. Endo-type chitosanases have been reported from several microorganisms and their degradation patterns on chitosan were similar. Although the product distribution was variable in each case, these enzymes, as previously reported, hydrolyzed chitosan into 1 ~ 6 oligomers using an endo-type catalytic action. However, the enzyme described here did produce a $(GlcN)_4$ as a major product of chitosan under the reaction conditions of an appreciably high temperature and long time.

Modification of hydrolysis patterns by ammonium sulfate

Table 3. Comparison of Oligosaccharides Produced by Thermostable Chitosanase in Reaction Mixture Containing Ammonium Sulfate and Ethanol

Hydrolysis system[c]	Oligosaccharide composition (%)[a]					
	$(GlcN^{b})_1$	$(GlcN)_2$	$(GlcN)_3$	$(GlcN)_4$	$(GlcN)_5$	$(GlcN)_6\leq$
Soluble DAC99	6.5	11.9	17.9	27.1	18.4	8.2
Soluble DAC99 + 10% $(NH_4)_2SO_4$	ND	8.8	18.1	35.2	29.5	9.8
Soluble DAC72 + 10% EtOH	ND	10.2	17.8	36.4	26.8	4.7
Soluble DAC99 + 20% $(NH_4)_2SO_4$	ND	3.1	15.6	29.8	22.7	20.4

[a]% of total. [b]D-Glucosamine. [c]Reaction mixture contained 2% chitosan, 50 mM potassium phosphate buffer (pH 6.5), 10% ammonium sulfate or ethanol, and 20 U chitosanase. Incubation was at 60°C for 12 hr and analysis was by HPLC. $(GlcN)_n$ product concentrations were calculated from peak areas in the HPLC profiles using the standards curves obtained from pure saccharide solutions.

The enzyme reaction pattern in salt or organic solvent-water mixtures investigated earlier,[32,33] suggested that modification of the substrate-binding site took place, because a relative increase in specific oligomers was observed. In our study, the most remarkable result is deacetylation degree (DAC) 99% soluble chitosan in 10% ammonium sulfate solution and DAC 72% in 10% EtOH solution, and no formation of $(GlcN)_1$ was observed (Fig. 5A). In a water system, the amount of $(GlcN)_1$ represented 10% of all formed oligosaccharide, but DAC 99% soluble chitosan in 10% ammonium sulfate-water mixture yield about 80% $(GlcN)_3\sim(GlcN)_6$ of all products with no formation of $(GlcN)_1$. DAC 72 soluble chitosan in 10 % EtOH-water mixture showed also no formation of $(GlcN)_1$, however, the yield of $(GlcN)_3\sim(GlcN)_6$ is lower than DAC 99% soluble chitosan + 10% ammonium sulfate-water (Table 3).

To examine the optimal concentration of ammonium sulfate to be added, the hydrolysis patterns of the enzyme were observed in a system at a 0–50% ammonium sulfate content. The amount of chitosan oligomer and D-glucosamine obtained in the reaction system with 0–50% ammonium sulfate after 12 h hydrolysis are shown in Fig. 5B. The 20% ammonium sulfate concentration in reaction mixture increased the content of $(GlcN)_3$–$(GlcN)_6$ nearby 30% (Fig. 6).

Throughout this article, on suggestion concerning the improvement of functional oligomer, $(GlcN)_3$–$(GlcN)_6$ concentration in a water-salt reaction system is that the enzyme conformational change might be altered for the substrate to be uniquely broken down. Another possibility is that the salts affect enzymatic activity by interacting with diffusible substrates or products of the reaction. Glycosidases can be used synthetically in two modes, reverse hydrolysis and transglycosylation. Transglycosylation is generally the method of choice, because the reverse hydrolases heavily favor hydrolysis and require a high concentration of starting substrates. With thermostable chitosanase from *Bacillus* sp. CK4, the possibility of transglycosylation is occurred in the ammonium sulfate solution, but it still remains unclear why $(GlcN)_1$ production is greatly

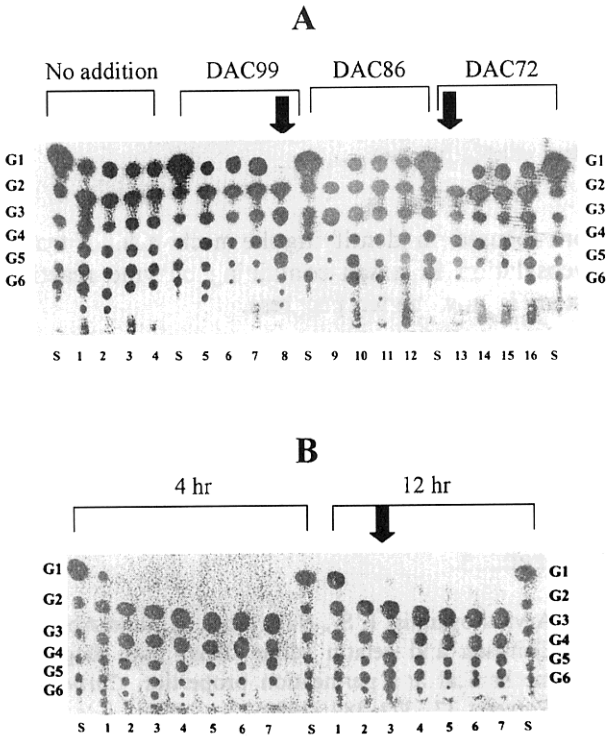

Fig. 5. TLC Profiles of Products Produced in the Hydrolysis of Soluble Chitosan with Various DAC containing 10% ethanol, methanol, acetone, and ammonium sulfate.

(A) The analysis sample was hydrolyzed at 60°C for 12 hr. Each of standards G1–G6 indicates monomer, dimer, trimer, tetramer, pentamer, and hexamer. S, Size marker; Lane 1, DAC99 soluble chitosan; lane 2, DAC83 soluble chitosan; lane 3, DAC71 soluble chitosan; lane 4, DAC53 soluble chitosan; lane 5, DAC99 soluble chitosan-10% ethanol; lane 6, DAC99 soluble chitosan-10% methanol; lane 7, DAC99 soluble chitosan-10% acetone; lane 8, DAC99 soluble chitosan-10% AS; lane 9, DAC86 soluble chitosan-10% ethanol; lane 10, DAC86 soluble chitosan-10% methanol; lane 11, DAC86 soluble chitosan-10% acetone; lane 12, DAC86 soluble chitosan-10% AS; lane 13, DAC72 soluble chitosan-10% ethanol; lane 14, DAC72 soluble chitosan-10% methanol; lane 15, DAC72 soluble chitosan-10% acetone; lane 16, DAC72 soluble chitosan-10% AS. (B) Effects of ammonium sulfate concentration on products produced in the hydrolysis of soluble chitosan with DAC99. Lane 1, 0% AS; lane 2, 10% AS; lane 3, 20% AS; lane 4, 30% AS; lane 5, 40% AS; lane 6, 45% AS; lane 7, 50% AS.

decreased on the enzymatic hydrolysis of chitosan in a water-ammonium sulfate reaction mixture. Further

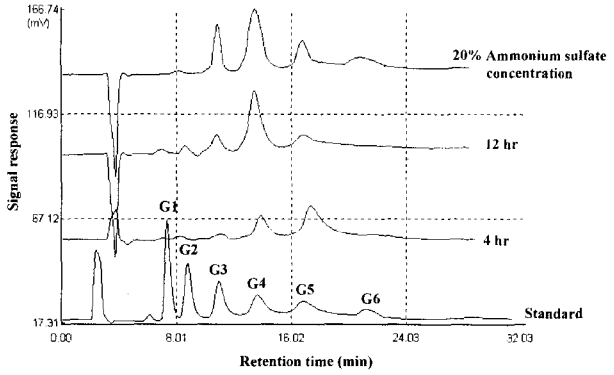

Fig. 6. The Hydrolysate Profiles of *Bacillus* sp. CK4 Chitosanase.

The enzyme (0.1 mg/ml) was added to 0.5 ml of 1% soluble chitosan dissolved in 10 mM potassium phophate buffer (pH 6.5). The products were analyzed on TSK-Gel NH_2 60 column for the chitosan oligosaccharide. Standard G1-G6 indicate standard $(GlcN)_n$ (n=1-6).

work focused on identifying the mechanism of transglycosylation in a salt-containing enzyme reaction system is now under progress.

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