J. Microbiol. Biotechnol. (1998), **8**(6), 568–574

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY
© Korean Society for Applied Microbiology

Purification and Characteristics of Two Types of Chitosanases from *Aspergillus fumigatus* KH-94

KIM, SOON-YOUNG*, DONG-HWA SHON[1], AND KE-HO LEE

Department of Food Science & Technology, College of Agricultural Life Sciences, Seoul National University, Suwon, Kyonggido 441-744, Korea
[1]Food Chemistry and Biochemistry Division, Korea Food Research Institute, Seongnam, Kyonggido 463-420, Korea

Received: May 4, 1998

Abstract Two types of chitosanases produced from *Aspergillus fumigatus* KH-94 were purified by ion exchange and gel permeation chromatography. Molecular weights of the enzymes are 22.5 kDa (chitosanase I) and 108 kDa (chitosanase II). pI, optimum pH, and temperature of chitosanase I are 7.3, 5.5, and 70~80°C, respectively, and those of chitosanase II are 4.8, 4.5~5.5, and 50~60°C, respectively. Activities of both chitosanases were increased by Mn^{2+} but inhibited by Cu^{2+} and Hg^{2+}. Chitosanase I has endo-splitting activity that hydrolyzes chitopentaose, chitohexaose, and chitosan to chitobiose, chitotriose, and chitotetraose, whereas chitosanase II has exo-splitting activity that hydrolyzes chitobiose and chitosan to glucosamine. Chitosanase II was found to have transglycosylation activity also in the reaction of 2% more chitooligosaccharides as a substrate and at the initial reaction. The higher degree of deacetylation, the stronger activities of chitosanase I and II toward chitosans. Both chitosanases could hydrolyze chitosan and glycol chitosan but not chitin, cellulose, and carboxymethyl cellulose. To produce higher degree of polymerization of chitooligosaccharides, chitosanase I was used and yielded 80% of recovery.

Key words: Chitosanase, *Aspergillus fumigatus* KH-94, chitooligosaccharides

Chitosan is a D-glucosamine polymer which is produced industrially by deacetylation of chitin. Chitooligosaccharides produced by enzymatic hydrolysis of chitosan are used as food additives, pharmaceuticals, and elicitors in plant cell culture etc. In particular, it has been reported that chitooligosaccharides above the degree of polymerization (DP) of 5 have physiological activities such as antitumor [14], immuno-potentiating [6], and antimicrobial activity [13] and they could be made from chitosan by treatment of microbial chitosanase (EC 3. 2. 1. 132) [17, 18]. However, most chitosanases from the isolated microorganisms intended to make dimers, trimers and tetramers rather than oligomers of above DP 5, so the utility of enzymes was not good [4]. Therefore, for the effective production of chitosan oligomers above DP 5, novel chitosanases are required.

In this research to obtain a strong chitosanase originated from food microorganisms, screening was done from Korean traditional koji (Nuruk) and *Aspergillus fumigatus* KH-94 was obtained. This report describes the purification and properties of two types of chitosanases from the strain and the effective production of higher chitosan oligomers above DP 5 by one of the chitosanases.

*Corresponding author
Phone: 82-331-290-2588; Fax: 82-331-293-4789;
E-mail: ksych@unitel.co.kr

MATERIALS AND METHODS

Chemicals

Chitosan and glucosamine were obtained from Sigma Co., U.S.A. and chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose were from Seikagaku Co, Japan. All other chemicals were commercial special-grade products. To prepare partially acetylated chitosan, reacetylation was performed according to Yabuki *et al.* [17]. The degree of deacetylation of the chitosan was measured by the colloidal titration method [5].

Microorganism and Cultivation

A fungal strain KH-94 having strong chitosanase activity was isolated from Nuruk which was collected from local areas, using chitosan-agar plates containing 0.5% chitosan, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, and 1.5% agar; pH 5.5. It was identified as *Aspergillus fumigatus* and named KH-94. The fungus was cultivated in 500-ml Erlenmeyer flasks containing 200 ml of a medium consisting of 1.5%

chitosan, 1% tryptone, 0.5% glucose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$ in 1% lactic acid solution at pH 6.2 adjusted with 1 M NaOH, and incubated for 72 h at 40°C on a rotary shaker in a rate of 150 rpm.

Chitosanase Assay

Chitosanase activity was assayed using soluble chitosan as a substrate. Soluble chitosan 1% (w/v) was prepared as follows. One g of chitosan was suspended in 70 ml of deionized water and 1 ml lactic acid was added and dissolved by stirring and then adjusted to pH 5.5 with 1 M NaOH. The final volume was raised to 100 ml by adding deionized water. The assay mixture consisted of 0.1 ml of enzyme solution, 0.4 ml of 50 mM acetate buffer (pH 5.5), and 0.5 ml of 1% soluble chitosan. After incubation at 50°C for 20 min, the reaction was terminated by addition of 0.2 ml of 1 M NaOH. The mixture was centrifuged at 5,000×g for 10 min and the supernatant was used for chitosanase activity, which was determined by the Rondle & Morgan method [10].

Analytical Methods

Protein was measured by the Bradford method [2], with bovine serum albumin as a standard protein, and monitored at 280 nm (A_{280}). For the identification of chitooligosaccharides, thin layer chromatography was used with silicagel 60F TLC plate (Merck Co., U.S.A.). The developing solvent was composed of n-propanol:ammonia water (30%) (2:1, v/v) and sugars were detected with ninhydrin solution (0.2% in ethanol) spray. To analyze chitosanase products, HPLC (Samsung electronic device, Korea) was done with RI detector by permeation on TSKgel NH_2-60 (4.6×250 mm, Toyo Co., Japan) which was eluted with acetonitrile:distilled water (6:4) at a flow rate of 1 ml/min. For estimation of molecular weight, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using a 10% polyacrylamide gel according to the procedure of Bollag [1]. Isoelectrofocusing was carried out with a Phast system (Pharmacia LKB, Sweden).

Purification of Chitosanases

After cultivation, the mycelia were removed through filtration. Solid ammonium sulfate was added to the culutre filtrate to reach 75% saturation. After standing overnight, the precipitate was collected by centrifugation at 12,000×g for 20 min and dialyzed against 50 mM acetate buffer (pH 5.6).

On a Q-Sepharose (Sigma Co.) column (3×10 cm) and an S-Sepharose (Sigma Co.) column (3×7 cm) equilibrated with 50 mM acetate buffer (pH 5.6) at a flow rate of 7 ml/min, enzyme elution was attained with a stepwise gradient of NaCl (0.1~0.4 M); 7 ml per fraction were collected. The fractions containing enzyme activity were pooled and concentrated with polyethylene glycol (MW 6,000).

For the gel fractionation, the enzyme was eluted with 50 mM acetate buffer (pH 5.6), 150 mM NaCl on a Sephadex G-150 (Sigma Co.) column (1×90 cm).

As a last step of enzyme purification, FPLC (Fast protein liquid chromatography) was carried out on a Mono-S HR 5/5 (Pharmacia LKB, Sweden) and a Mono-Q HR 5/5 (Pharmacia LKB) column with 50 mM acetate buffer (pH 5.6). The active fraction was chromatographed with a linear gradient of NaCl (0~0.4 M) at a flow rate of 1 ml/min.

Effects of Temperature and pH on the Chitosanase Activity

Chitosanase activities were assayed at various temperatures (40~90°C) at pH 5.6 under standard conditions. The activities were also measured at different pHs (3.5~8.5) using 10 mM formic acid buffer (pH 3.5~5.5), 10 mM acetate buffer (pH 5.5~6.5), and 10 mM malic acid buffer (pH 6.5~8.5) at the optimum temperature.

Effects of Temperature and pH on the Chitosanase Stability

Chitosanase solutions were preincubated for 30 min at various temperatures (50~80°C) and pHs (3.5~8.5) at room temperature without the substrate. The remaining activity was assayed at the optimum pH and temperature.

Effect of Metal Ions

Chitosanases were preincubated with various 1 mM metal ions dissolved in each optimum buffer and the activities were assayed under a standard condition at the optimum pH and temperature.

Production of Higher Chitosan Oligomers

To produce chitooligosaccharides above DP 5 from chitosan using chitosanase I, it was treated with 3% soluble chitosan (pH 5.5). The reaction was terminated by addition of 1 M NaOH, diluted to 1/10, and the turbidity was measured at 600 nm. Gel fractionation of produced chitosan hydrolysates was performed on a Biogel P-4 column (1.2×100 cm). Oligomer fractions were detected by Moore's method [7] and the composition was analyzed on a TLC plate.

RESULTS

Purification of Chitosanases

There have been many reports on bacterial chitosanases but not on the purification of chitosanases from *Aspergillus* sp. The enzyme was precipitated from culture

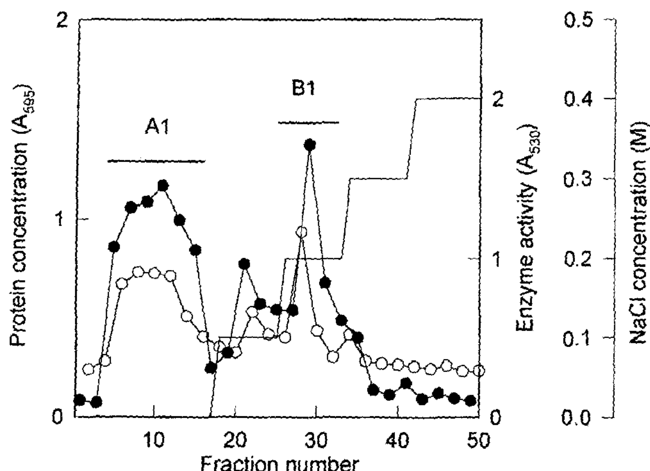

Fig. 1. Separation of chitosanases from crude enzyme preparation using Q-Sepharose.

The eluates from numbers 3 to 17 were collected and separated as A1 and from numbers 27 to 33 were collected and separated as B1. -○-, protein concentration; -●-, enzyme activity; —, NaCl concentration.

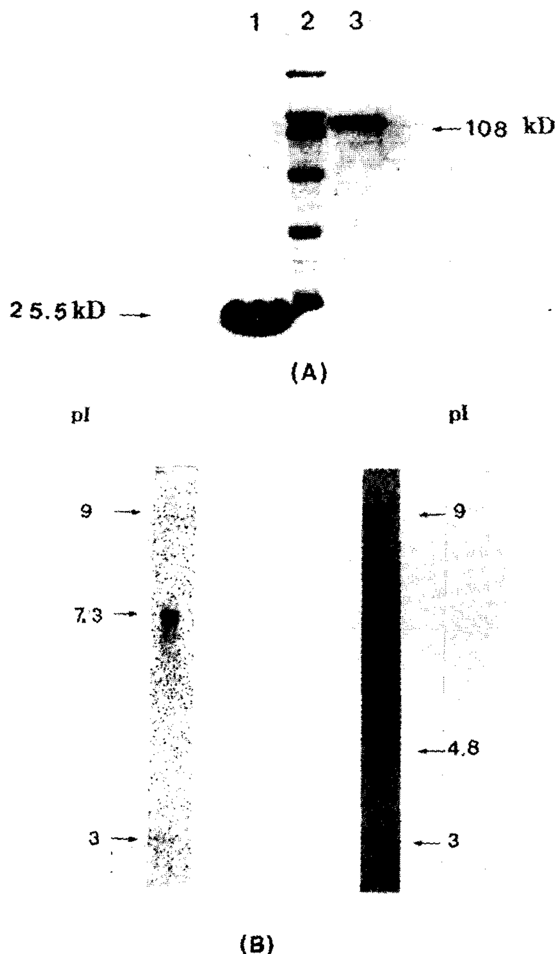

Fig. 2. (A) SDS-PAGE patterns of purified chitosanase I and II. (B) Isoelectric focusing patterns of purified chitosanase I and II.

(A) Lane 1, chitosanase I; lane 2, marker; lane 3, chitosanase II. (B) (a) chitosanase I; (b) chitosanase II.

filtrate by ammonium sulfate, and separated in an anionic exchange column using Q-Sepharose (Fig. 1). A1 fractions were further purified in a cationic exchange column (S-Sepharose) and finally a Mono-S column and named chitosanase I (data not shown). B1 fractions were further purified in a Sephadex G-100 and subsequently a Q-Sepharose, and finally a Mono-Q column, and named chitosanase II (data not shown). The each final preparation showed a single band on the SDS-PAGE (Fig. 2). Molecular sizes of chitosanase I and II were estimated to be 25.5 kDa and 108 kDa and the isoelectric point was about 7.3 and 4.8, respectively.

General Properties of Chitosanases

The effects of pH and temperature on the enzyme activity are shown in Fig. 3. The optimum pHs of chitosanase I and II were 5.5 and 4.5~5.5 and the optimum temperatures were 70~80°C and 50~60°C, respectively. The effects of pH and temperature on the enzyme stability are shown in Fig. 4. Chitosanase I was stable at the pH range of 5.5~6.5 and chitosanase II was stable at the wide pH range of 4.5~8.5. Chitosanase I was stable at a temperature lower

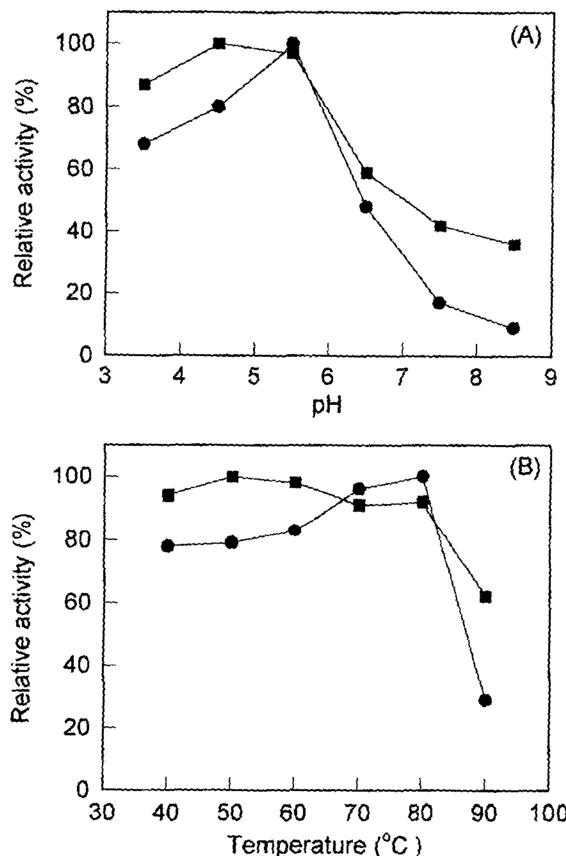

Fig. 3. Effect of pH (A) and temperature (B) on the activities of the chitosanase I and II.

-●-, chitosanase I; -■- chitosanase II.

than 70°C and dramatically inactivated at 80°C but chitosanase II was stable at a temperature lower than 50°C and inactivated slowly over 50°C. The effects of metal ions on chitosanases are presented in Table 1. Both Cu^{++} and Hg^{++} inhibited the activities of chitosanase I and II about 80% and 50%, respectively, and Mn^{++} remarkably increased the activities of chitosanase I and II about 80% and 60%, respectively.

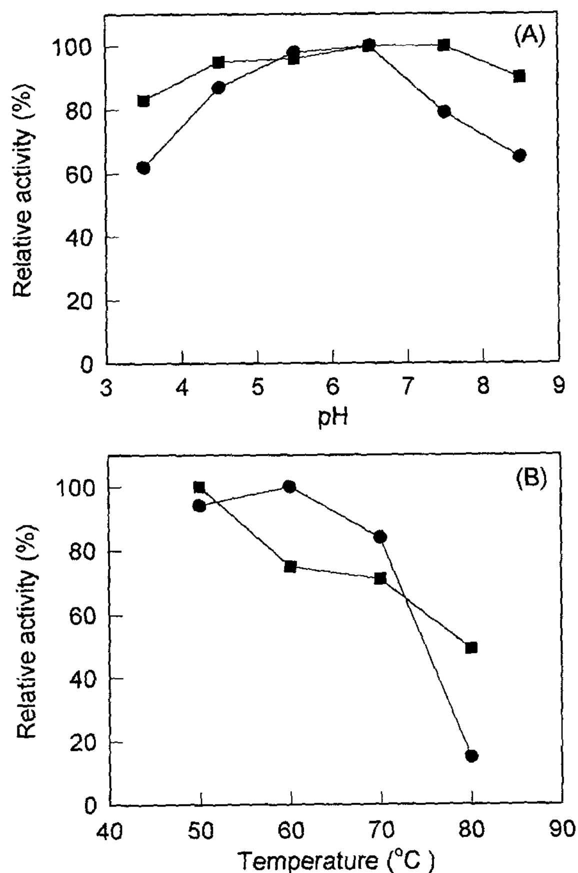

Fig. 4. Effect of pH (A) and temperature (B) on the stabilities of chitosanase I and II.

-●-, chitosanase I; -■- chitosanase II.

Table 1. Effect of metal ions on the activity of chitosanase I and II.

Reagent*	Relative activity (%)	
	Chitosanase I	Chitosanase II
None	100	100
$MnCl_2$	182	162
$CaCl_2$	124	120
$MgCl_2$	98	99
Na_2MoO_4	89	85
$BaCl_2$	84	102
$FeCl_2$	69	67
$ZnCl_2$	67	95
$CuCl_2$	21	52
$HgCl_2$	16	53
EDTA	101	93

*1 mM metal ions were tested.

Hydrolysis Action of Chitosanases

Both chitosanases acted specifically on chitosan, chitooligosaccharides, and partially acetylated chitosans. As shown in Table 2, the higher the degree of deacetylation (DDA) of chitosan, the lower the activity of chitosanases and the activities were low to the glycol chitosan. But the activity was not detected to colloidal chitin, carboxymethyl cellulose (CMC), or cellulose. The hydrolysis products of chitosan and chitooligosaccharides by chitosanase I and II were analyzed by thin layer chromatography (TLC) and the results are shown in Fig. 5. Chitosan hydrolysates by chitosanase I were composed of chitotriose, chitotetraose, chitopentaose, chitohexaose, and so on. Chitosanase I hardly hydrolyzed chitotetraose, chitotriose, and chitobiose, but it partially hydrolyzed chitopentaose to chitotriose

Table 2. Substrate specificity of chitosanases.

Substrate (0.5%)	Relative activity (%)	
	Chitosanase I	Chitosanase II
97% DDA[1] chitosan	100.0	100.0
80% DDA chitosan	63.5	76.4
64% DDA chitosan	52.2	67.3
55% DDA chitosan	50.9	48.8
Glycol chitosan	34.3	31.9
Colloidal chitin[2]	0	0
CMC[3]	0	0
Cellulose[3]	0	0

The reactions were done under the standard assay condition using several substrates instead of 97% DDA chitosan. [1]Degree of deacetylation. [2]Activity was determined by the method of Imoto & Yagishita (1971) [13]. [3]Activity was determined by the DNS (dinitrosalicylic acid) method.

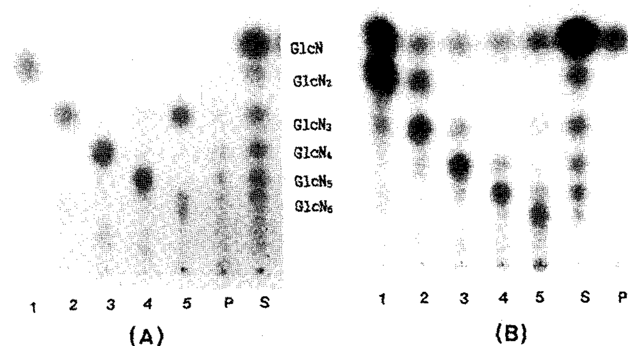

Fig. 5. Thin layer chromatograms of chitooligosaccharides after treatment of chitosanase I (A) and II (B).

Lane 1, Chitobiose; Lane 2, chitotriose; Lane 3, chitotetraose; Lane 4, chitopentaose; Lane 5, chitohexaose; Lane P, chitosan; Lane S, standard chitooligosaccharides. The reaction mixture of 10 µl (10 mU) enzyme in 50 mM acetate buffer and 50 µl of 2% substrate was incubated at 50°C for 5 min for partial hydrolysis.

Table 3. The substrate specificity and cleavage pattern of the chitosanase I on chitooligosaccharides.

Substrate	Reaction	Ratio of produced GlcN-oligomers (mol%)
$(GlcN)_4$ (4)	-	-[1]
$(GlcN)_5$ (5)	5 → 2+3	94
$(GlcN)_6$ (6)	6 → 3+3	90
	6 → 2+4	10

The reaction mixture consisted of 10 μl (15 mU) enzyme in 50 mM acetate buffer and 50 μl of 10 mM of each substrate; chitotetraose, chitopentaose, and chitohexaose. It was incubated at 50°C for 30 min. [1]Not detected.

and chitobiose and it hydrolyzed chitohexaose to chitotriose. However, when hydrolysis products of chitohexaose by chitosanase I were analyzed by HPLC, there were chitotetraose and chitobiose as well as chitotriose as the predominant product (Table 3).

Chitosanase II hydrolyzed chitosan and chitooligosaccharides to glucosamine so that chitosanase II was classified as an exo-type hydrolysing enzyme (Fig. 5). However, when the digesting-profiles of chitooligosaccharides under various conditions were monitored by HPLC, an unusual action of chitosanase II was found. As shown in Fig. 6, typically, chitosanase II made higher DP of chitooligosaccharides than that of substrate at the initial reaction (1 min) of chitotetraose and chitohexaose but not in the prolonged reaction (15 min) and at a lower concentration (10 mM) of chitotetraose. Therefore, it was concluded that chitosanase II had transglycosylation activity in more than 2% chitooligosaccharide at the initial reaction (1 min).

Referring to those results, one unit of chitosanase I activity was defined as the amount of enzyme which produced 1 μmol of $(GlcN)_3$ from 10 mM $(GlcN)_6$ as substrate per min and one unit of chitosanase II activity was defined as the amount of enzyme which produced 1 μmol of GlcN from 10 mM $(GlcN)_2$ as substrate per min under standard conditions. According to these definitions the specific activities of the purified chitosanase I and II were 152 U/mg and 297 U/mg each.

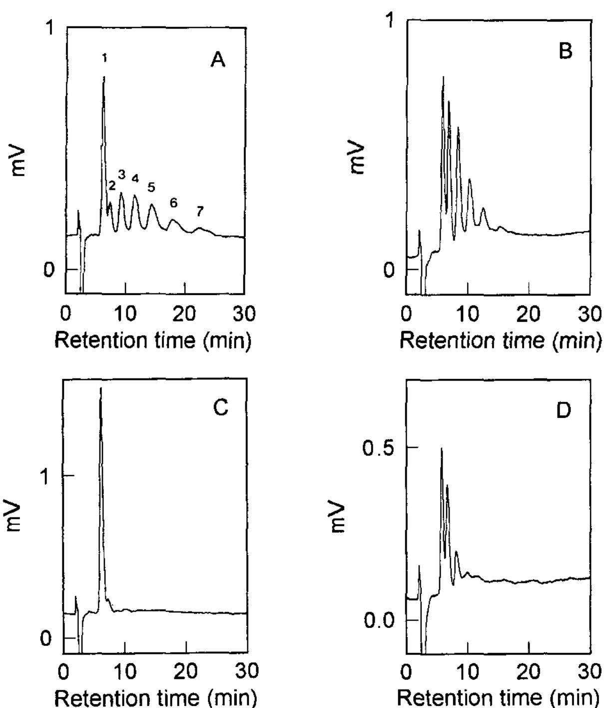

Fig. 6. HPLC chromatograms of the chitooligosaccharide hydrolysates by chitosanase II.

The reaction mixture consisted of 50 μl each of substrate and 10 μl of enzyme (35 mU) in 50 mM acetate buffer. The peak numbers: 1, glucosamine; 2, GlcN; 3, $GlcN_2$; 4, $GlcN_3$; 5, $GlcN_4$; 6, $GlcN_5$; 7, $GlcN_6$. The conditions are as follows: (A) substrate, 20 mM chitohexaose; reaction time, 1 min (B) substrate, 30 mM chitotetraose; reaction time, 1 min (C) substrate, 30 mM chitotetraose; reaction time, 15 min (D) substrate, 10 mM chitotetraose; reaction time, 1 min.

Production of Chitooligosaccharides

Since chitooligosaccharides of above DP 5 are physiologically active and water soluble that they are useful for applications, we produced them from chitosan by use of the chitosanase I from *A. fumigatus* KH-94. Where the reaction was monitored by the turbidimetric method, it was found that the degree of hydrolysis is dependent on time and 30 min is enough for the hydrolysis (Fig. 5). After 30 min reaction, the hydrolysate was freeze dried and then chromatographed on a Biogel P4 column. Fractions from 62 to 90, which showed a positive ninhydrin reaction, were further anayzed by TLC (Fig. 8). When they were collected, the yield of chitosan oligomers

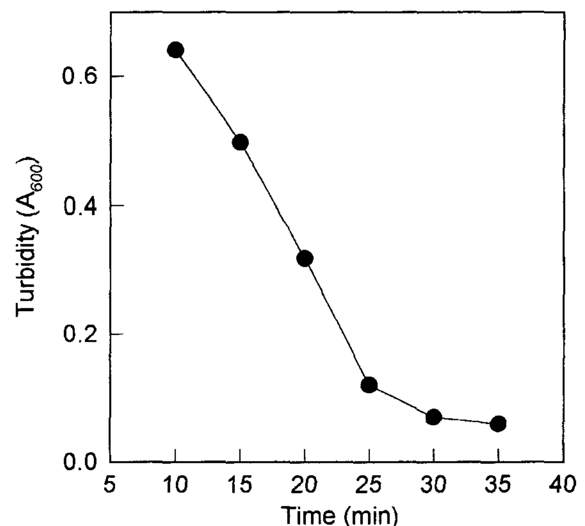

Fig. 7. Determination of hydrolysis time for the production of higher chitosan oligomers.

The reaction mixture consisted of 1 ml of chitosanase I (2.5 U) and 50 ml of 3% soluble chitosan (97% DDA) at 50°C.

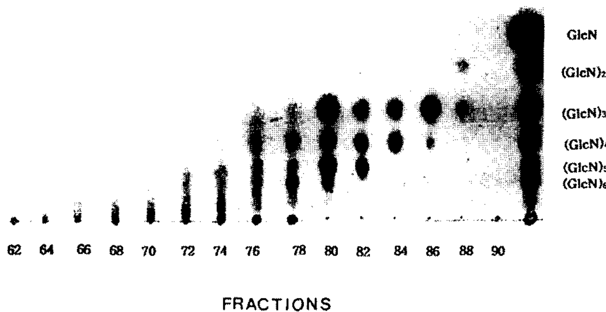

Fig. 8. Thin layer chromatograms of chitosan hydrolysate.

Freeze dried hydrolysate was applied to a Biogel P4 column (90×1.5 cm). Elution was carried out at room temperature with distilled water. 2 ml fractions were collected and products were detected with ninhydrin solution reagent.

from chitosan was about 80%. 20% of chitosan hydrolysate was not hydrolyzed fraction which was precipitated after neutralization to stop the hydrolysis. TLC results showed that the hydrolysate was composed of about 50% of DP 3~6 of chitooligosaccharides and 50% of above DP 7 of chitosan oligomers which were water soluble.

DISCUSSION

There were two types of chitosanases from *A. fumigatus* KH-94 which was screened and selected from Nuruk. One is an endo-splitting enzyme, chitosanase I, that needs a minimum DP of 5 as a substrate. Chitosanase of *Pseudomonas* sp. H-14 [18] and *Norcardia orientalis* [11] acted on the DP 4 as a substrate in an endo-splitting manner. The other is exo-splitting enzyme, chitosanase II. Chitosanases were generally endotype enzymes except for the case of Nanjo *et al.* [9] and molecular weight of chitosanase II (108 kDa) is similar to exo-β-D-glucosaminidase (97 kDa) of *N. orientalis* [9].

To produce N-acetylchitooligosaccharides of above DP 5, transglycosylation activity was studied with chitinase by Usui *et al.* [16] but until now the transglycosylation of chitosanase has not been reported. The best evidence of transglycosylation is given in Fig. 6, where an intermediate chitoheptaose was made from chitohexaose and chitopentaose and chitohexaose were made from chitotetraose at high substrate concentrations. The production of higher DP of chitooligosaccharides than that of substrate could be explained as the result of transglycosylating activity. By the transglycosylation activity, chitosanase II could transfer a glucosamine unit from one substrate to another substrate, and then degrade it to glucosamine as a final product. But the intermediates were not observed at low substrate concentrations. The meaning of transglycosylation of chitosanase II in the side of *A. fumigatus* KH-94 physiology can be assumed for the efficient utilization of chitosan substrate by way of cooperative action of chitosanases I and II. That is, at a initial reaction of high substrate concentration, chitosanase II transglycosylate and make intermediates of above DP 5 which are more favorable to be hydrolyzed to a smaller DP of chitooligosaccharides by chitosanase I. Then, the smaller DP of chitooligosaccharides made by chitosanase I are hydrolyzed again by chitosanase II efficiently, as can be supported from the result of chitobiose hydrolysis rate in Fig. 5 (b). Chitobiose was hydrolyzed to glucosamine much faster than any other higher DP of chitooligosaccharides. Therefore, the fungus can utilize chitosan as a glucosamine by chitosanase II which hydrolyzes it fast using the transglycosylating character.

Muraki *et al.* reported the production of chitooligosaccharides of above DP 6 from chitosan by using not chitosanase but cellulase which originated from *Trichoderma viride*. However, the reaction took 6 hr and the products were not water soluble but acid soluble [8]. Compared with cellulase in the production of chitooligosaccharides, it was shown that chitosanase I reduced the viscosity of chitosan substrate rapidly due to its strong activity and the reaction required a lesser amount of chitosanase I than that of the cellulase which used in the research of Muraki *et al.* It is assumed that chitosanase I hydrolyzes chitosan in a random-splitting manner in the initial cleavage, and then hydrolyzes oligomers of above DP 4 which are produced by a primary hydrolysis into DP 2~4 in the final cleavage. In spite of the high chitosanase activity of chitosanase I, the purification of chitooligosaccharides was not satisfactory (Fig. 8). For the efficient recovery of chitooligosaccharides of above DP 5, the ultrafiltration method by Seino *et al.* [12] would be applicable in future experiments.

REFERENCES

1. Bollag, D. M., M. D. Rozycki, and S. J. Edelstein. 1996. *Protein Methods*, pp. 107–154. 2nd ed. John Wiley & Sons. New York.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Imoto, T. and K. Yagishita. 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* **35:** 1154–1156.
4. Izume, M., S. Nagal, H. Kawagishi, M. Mitsutomi, and A. Ohtakara. 1992. Action pattern of *Bacillus* sp. No.7-M

chitosanase on partially *N*-acetylated chitosan. *Biosci. Biotech. Biochem.* **56:** 448–453.
5. Knorr, D. and J. Klein. 1986. Production and conversion of chitosan with cultures of *Mucor rouxii* or *Phycomyces blakesleeanus*. *Biotechnol. Lett.* **8:** 691–694.
6. Mikami, T., A. Tokoro, K. Suzuki, S. Suzuki, and M. Suzuki. 1988. Interferon inducing activity of chitooligosaccharides. *Tohoku Yakka Daigaku Kenkyu Nempo* **35:** 177–182.
7. Moore, S. 1968. Amino acid analysis: Aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *J. Biol. Chem.* **243:** 6281–6283.
8. Muraki, E., F. Yaku, and H. Kojuma, 1993. Preparation and crystallization of D-glucosamine oligosaccharides with dp 6-8. *Carbohydr. Res.* **239:** 227–237.
9. Nanjo, F., R. Katsumi, and K. Sakai. 1990. Purification and characterization of an exo-β-D-glucosaminidase, a novel type of enzyme, from *Norcardia orientalis*. *J. Biol. Chem.* **265:** 10088–10094.
10. Rondle, C. J. M. and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. *Biochem. J.* **61:** 586–589.
11. Sakai, K., R. Katsumi, A. Isobe, and F. Nanjo. 1991. Purification and hydrolytic action of a chitosanase from *Norcadia orientalis*. *Biochim. Biophys. Acta* **1079:** 65–72.
12. Seino, H., K. Tsukuda, and Y. Shimasue. 1991. Properties and action pattern of a chitosanase from *Bacillus* sp. PI-7S. *Agric. Biol. Chem.* **55:** 2421–2423.
13. Suzuki, K., A. Tokoro, Y. Okawa, S. Suzuki, and M. Suzuki. 1985. Enhancing effect of *N*-acetylchitoligosaccharides on the active oxygen-generating and microbicidal activities of peritoneal exudate cells in mice. *Chem. Pharm. Bull.* **33:** 886–888.
14. Tokoro, A., N. Tatewaki, K. Suzuki, T. Mikami, S. Suzuki, and M. Suzuki. 1988. Growth-inhibitory effect of hexa-*N*-acetylchitohexaose and chitohexaose against Meth-A solid tumor. *Chem. Pharm. Bull.* **36:** 784–790.
15. Uchida, Y., M. Izume, and A. Ohtakara. 1989. Purification and enzymatic properties of chitosanase from *Bacillus* sp. No. 7-M. *Bull. Fac. Agr. Saga Univ.* **66:** 105–116.
16. Usui, T., H. Matsui, and K. Isobe. 1990. Enzymic synthesis of useful chitooligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. *Carbohydr. Res.* **203:** 65–77.
17. Yabuki, M., A. Uchiyama, K. Suzuki, A. Ando, and T. Fujii. 1988. Purification and properties of chitosanase from *Bacillus circulans* MH-K1. *J. Gen. Appl. Microbiol.* **34:** 255–270.
18. Yoshihara, K., J. Hosokawa, T. Kubo, M. Nishiyama, and Y. Koba. 1992. Purification and properties of a chitosanase from *Pseudomaonas* sp. H-14. *Biosci. Biotech. Biochem.* **56:** 972–973.