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Purification and Characterization of a Chitinase from *Cytophaga* sp. HJ Isolated from Sea Sand

LEE, DONGMI, HEE-JUNG NOH, AND KANG MAN LEE*

The Research Institute of Pharmaceutical Sciences and College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

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Abstract An extracellular chitinase-producing bacterial strain induced by colloidal chitin was isolated from sea sand and was identified to be a member of the genus *Cytophaga*. The chitinase was purified successively by 30-60% ammonium sulfate fractionation, and DEAE-Bio gel A column, Octyl-Sepharose CL-4B column, and DEAE-Bio gel A column chromatographies. The enzyme had a molecular mass of 59.75 kDa, and the amino terminal amino acid sequence was ATPNAPVISW MPTDXXLQNXS. The enzyme acted better on colloidal chitin as a substrate than on chitosan. For colloidal chitin and chitosan (Degree of Acetylation, 15-25%), K_{cat} values were 0.60 U/mg and 0.08 U/mg, respectively. HPLC analysis of the enzymatic reaction products showed that the chitinase produced mostly *N*-acetyl-D-glucosamine and di-*N*-acetylchitobiose. The optimum temperature and pH for the enzyme were 50°C and 4.0, respectively. *N*-Bromosuccinimide and Hg^{2+} inhibited the chitinase activity as much as 90%, and Sb^{3+}, diethylpyrocarbonate, and Ag^{+} inhibited it by 50-70%.

Key words: *Cytophaga* sp. HJ, chitinase, chitin, *N*-acetylchitooligosaccharide

Chitin, a poly-β-(1→4)-*N*-acetyl-D-glucosamine (GlcNAc), which is a cellulose-like biopolymer, is identified as the second most abundant organic compound on earth [23].

Since chitin was first described as a component of mushrooms by Braconnot in 1811, much research on chitin was directed towards the study of its occurrence in living organisms, its degradation by bacteria, and its chemical content [14]. However, recently, research subjects were focused on applications of chitin derivatives and N-acetylchitooligosaccharides in the areas of medicine, agriculture, food industry, water treatment, and cosmetics [1, 9].

*Corresponding author
Phone: 82-2-3277-3041; Fax: 82-2-3277-2851;
E-mail: kmlee@mm.ewha.ac.kr

N-Acetylchitooligosaccharides are usually prepared by a partial hydrolysis of chitin with hydrochloric acid [17]. Unfortunately, this method is very time consuming for fractionation and it gives low yields of *N*-acetylchitooligosaccharides. Therefore, a chitin-hydrolyzing enzyme is in need for the production of *N*-acetylchitooligosaccharides in mild conditions.

Chitin is hydrolyzed to low-molecular-mass multimers of GlcNAc by an endochitinase (EC 3.2.1.14), which produces predominantly the soluble di-*N*-acetylchitobiose, and a chitobiase (EC 3.2.1.30) which hydrolyzes the intermediates to GlcNAc. Chitinases have been isolated from many microorganisms such as *Saccharomyces cerevisiae* [2], *Streptomyces erythraeus* [4], *Pseudomonas* sp. YHS-A2 [11], *Vibrio alginolyticus* [13], *Streptomyces olivaceoviridis* [16], *Bacillus stearothermophilus* [18], *Acinetobacter* sp. WC-17 [19], *Streptomyces kurssanovii* [21], *Bacillus licheniformis* [22], *Streptomyces thermoviolaceus* [24], *Alteromonas* sp. strain O-7 [25], *Aeromonas* sp. No. 10S-24 [26], *Trichoderma harzianum* [27], and *Aeromonas hydrophila* [29].

This report describes in detail the isolation of chitinase-producing bacterium, *Cytophaga* sp. HJ, derived from sea sand in Korea, and the purification and properties of a chitinase from the culture broth of the bacteria for the production of N-acetylchitooligosaccharides.

MATERIALS AND METHODS

Reagents

The mixture of *N*-acetylchitooligosaccharides was purchased from Seikagaku Co. (Tokyo, Japan). Chitosan (Degree of Acetylation [D.A.], 15-25%) was obtained from Showa Chemicals Inc. (Tokyo, Japan). Glycol chitosan was from Sigma Chemical Co. (St. Louis, U.S.A.). Colloidal chitin was prepared by the method described by Jeuniaux [7], using a commercially available chitin from crab shells (Sigma, St. Louis, U.S.A.). Chitosan (D.A., 7-8%) was

prepared by the method described by No and Meyers [15], while *N*-trimethyl chitosan was prepared by the method described by Domard *et al.* [3].

Screening of Bacterial Strains Hydrolyzing Chitin

The samples used in this experiment were obtained from sea sand of Kwanganli beach, Pusan, Korea. Five gram of sea sand was added into 50 ml of chitinase detection medium (5 g of colloidal chitin, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.1 mg of $FeSO_4 \cdot 7H_2O$, 0.01 mg of $ZnSO_4 \cdot 7H_2O$, and 0.01 mg of $MnCl_2 \cdot 4H_2O$ in 1 l, pH 7.0). After the samples were incubated at 30°C for 7 days on a rotary shaker (200 rpm), they were spread onto chitinase detection medium solidified with 2% agar. Clear halos were observed around 10 colonies after incubation for 7 days at 30°C. Among the 10 colonies, one colony showing a predominent chitinase activity was selected and identified by investigating its morphological, biochemical, and physiological characteristics according to *Bergey's Manual of Determinative Bacteriology* [5] and *Bergey's Manual of Systematic Bacteriology* [20]. The morphological characteristics were studied by using a scanning electron microscope (SEM).

Culture Conditions

The isolated strain was inoculated into 50 ml of seed culture medium (0.5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.1 mg of $FeSO_4 \cdot 7H_2O$, 0.01 mg of $ZnSO_4 \cdot 7H_2O$, 0.01 mg of $MnCl_2 \cdot 4H_2O$, 15 g of NaCl, 5 g of yeast extract, 5 g of peptone, and 2 g of glucose in 1 l, pH 7.0), and incubated at 20°C for 24 h on a rotary shaker (220 rpm). The cultures (50 ml) were inoculated into 1 l of main culture medium (seed culture medium supplemented with 0.5% colloidal chitin) and incubated for an additional 30 h under the same conditions.

Purification of Chitinase

All purification procedures were performed at 4°C. The culture supernatant (1 l) was collected by centrifugation. Crude proteins were obtained by 30-60% ammonium sulfate fractionation and dissolved in 20 ml of 10 mM potassium phosphate buffer (pH 7.0) (buffer A). The samples were dialyzed against buffer A and loaded onto a DEAE-Bio gel A (Bio-Rad, Richmond, U.S.A.) column (1.5×15 cm) equilibrated with buffer A. Washing the column with a linear gradient of buffer A containing 0 to 0.5 M NaCl in 400 ml resulted in elution of proteins with chitinase activity, and then the active fractions were pooled. After ultrafiltration (YM 30, Amicon Co., Denvers, U.S.A.) and dialysis against buffer A, the solution was made to 1 M of ammonium sulfate with a 4 M ammonium sulfate solution (buffer A) and it was loaded onto an Octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (2.5×11 cm) equilibrated with buffer A containing 1 M of ammonium sulfate. The active fraction eluted with buffer A (Fig. 3B, peak II) was concentrated by ultrafiltration (YM 30, Amicon Co., Denvers, U.S.A.), dialyzed against 25 mM Tris-HCl buffer (pH 8.0) (buffer B), and then loaded onto a DEAE-Bio gel A (Bio-Rad, Richmond, U.S.A.) column (1.5×15 cm) previously equilibrated with buffer B. Washing the column with a linear gradient of buffer B containing 0 to 1 M NaCl in 400 ml resulted in the elution of proteins with chitinase activity, and the active fractions were pooled.

Enzyme Assay

Chitinase activity was assayed by using 0.1% (w/v) colloidal chitin suspended in buffer A as a substrate. The enzyme solution (0.5 ml) was mixed with 1 ml of the substrate solution and the mixture was incubated at 20°C in a water bath for 30 min with shaking. The mixture was then boiled for 10 min, and the amount of GlcNAc released was determined by the modified method of Schales [6]. One unit of enzyme activity was defined as the amount that released 1 µmol of GlcNAc per min at 20°C.

Protein Concentration Determination

Protein concentration was determined by the method of Lowry *et al.* [12], with bovine serum albumin as the standard. During the column chromatography, protein content was determined by A_{280}.

SDS-PAGE and Native PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [10]. Protein samples were boiled for 5 min in a sample solubilization solution (pH 6.8) which was composed of 5% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.0625 M Tris-HCl. The samples were loaded onto 10% polyacrylamide gel containing 0.1% SDS, and they were electrophoresed at 50 mA. SDS-6H (Sigma, St. Louis, U.S.A.) was used as a molecular mass standard mixture which contained myosin (205 KDa), β-galactosidase (116 KDa), phosphorylase b (97.4 KDa), bovine albumin (66 KDa), egg albumin (45 KDa), and carbonic anhydrase (29 KDa). Native PAGE was carried out at a temperature of 4°C under the same condition as SDS-PAGE, except with no addition of SDS.

Molecular Mass Determination of Chitinase by MALDI-TOF-MS

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a HP G2025A system (Hewlett-Packard, Palo Alto, U.S.A.). Samples were prepared by 1:1 dilution with UV-absorbing matrix sinapinic acid (50 mM) followed by a rapid vacuum evaporation in the HP 2024A sample preparation accessory. Twenty eight laser shots were summed up for the spectrum.

Analysis of the Amino Terminal Amino Acid Sequence
The purified chitinase was electrophoresed under the same condition as mentioned earlier and was transferred to the PVDF membrane (Hybond™-PVDF, Amersham Life Science, Buckinghamshire, England). The N-terminal amino acid sequence of chitinase in the PVDF membrane was determined by using a Procise Protein Sequencing System (Applied Biosystems).

HPLC Analysis of the Enzymatic Reaction Products
Reaction products were analyzed by high-pressure liquid chromatography (HPLC) with a carbohydrate analysis column (3.9×300 mm, Waters Co., Milford, U.S.A.) with a solvent system of 72% acetonitrile and 28% water at flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Isolation and Identification of the Bacterial Strain Hydrolyzing Chitin
About 10 microbial colonies that formed clear halos were observed on chitinase detection agar medium plates, and we isolated the bacterial strain that formed the largest clear halo. The bacterial isolate was subjected to taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology* [20]. As shown in Table 1, the strain was gram-negative and rod-shaped. It was motile in SIM agar medium, but a motor organ could not be seen by SEM. Consequently, it was thought to be a gliding bacterium. Because the strain could reduce NO_3^- to NO_2^-, it was suggested that NO_3^- was used as a terminal electron acceptor. Based on these results, this strain was identified as a member of the genus *Cytophaga* family. The strain had similar characteristics as *Cytophaga uliginosa* [20] in almost all traits tested, however, it differed in that the strain could produce urease but not utilize agar and it was negative in the flexirubin reaction. As a result, this strain was designated as *Cytophaga* sp. HJ.

Table 1. Morphological, biochemical, and physiological characteristics of *Cytophaga* sp. HJ and *Cytophaga uliginosa* (ND: not determined, +: positive, - : negative).

	Cytophaga sp. HJ	*Cytophaga uliginosa*
Length of cells (μm)	1.2-1.5	1.2-4
Width of cells (μm)	0.4-0.5	0.4
Cell shape	rod	rod
Color of cell mass	bright yellow	golden yellow
Gram staining	-	-
Flexirubin reaction	-	+
Motility	+	+
Degradation of		
gelatin	+	+
casein	+	+
starch	+	+
agar	-	+
chitin	+	+
Lipase	-	-
Urease	+	-
H_2S produced	-	-
Indole produced	-	-
Catalase	+	+
Oxidase	+	+
Highest NaCl concentration tolerated (%)	6	ND
Citrate utilization	-	ND
NO_3^- reduced	+	+
Acid from glucose	-	+
Strict aerobe (a)/ facultative aerobe (f)	a	a
Endospore	-	ND

Chitinase Production by *Cytophaga* sp. HJ
Cytophaga sp. HJ secreted chitinase into its cultural medium when colloidal chitin was added (Fig. 1A). Chitinase activity increased in proportion to the degree of microbial growth and also to the amount of extracelluar proteins up

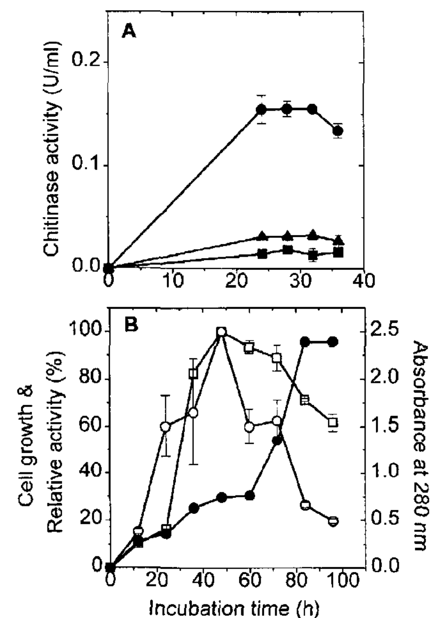

Fig. 1. Effects of chitin (A) and cell growth (B) on the chitinase production.
(A) Cells were grown in Nutrient Broth containing 1.5% NaCl (■), and seed culture medium supplemented with 0.5% colloidal chitin (●) or 0.5% non-colloidal chitin (▲) at 20°C by shaking. Chitinase was induced by colloidal chitin that was better than non-colloidal chitin. (B) Cells were grown in a main culture medium at 20°C by shaking. At regular intervals (12 h), relative activity (%, □), cell growth (%, ○), and the amount of extracellular proteins (●) were measured. Cell growth was determined by counting viable cells and the amount of extracellular proteins was determined by absorbance at 280 nm. The maximum values of chitinase activity and cell growth were 0.11 U/ml and 2.6×10^{10}, respectively. The data were the mean value of duplicate experimental results. Error bar indicates the range of standard deviation of two data points.

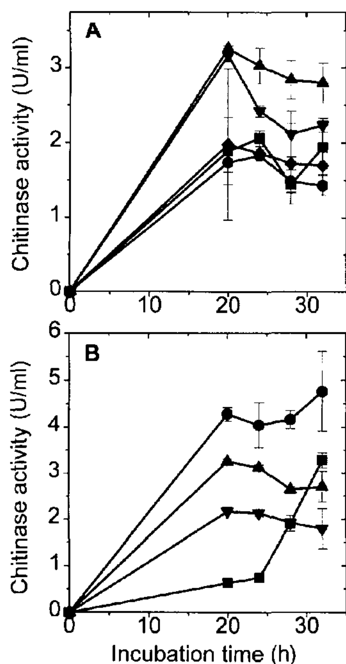

Fig. 2. Effects of pH (A) and temperature (B) on the chitinase production.

(A) Seed cultures of *Cytophaga* sp. HJ were inoculated (5%) into a main culture medium adjusted to various pHs and incubated at 20°C on a rotary shaker (220 rpm). The culture broths (0.2 ml) were sampled at regular intervals and the chitinase activities were measured. ■, pH 6.0; ●, pH 6.5; ▲, pH 7.0; ▼, pH 7.5; ◆, pH 8.0. (B) Seed cultures of *Cytophaga* sp. HJ were inoculated (5%) into a main culture medium adjusted to pH 7.0 and incubated at various temperatures on a rotary shaker (220 rpm). At regular intervals, the culture broths (0.2 ml) were sampled and the chitinase activities were measured. ■, 15°C; ●, 20°C; ▲, 25°C; ▼, 30°C. The data were the mean value of duplicate experimental results. Error bar indicates the range of standard deviation of two data points.

to 2 days of incubation (Fig. 1B), when extracellular proteins increased abruptly. This result indicated that a leakage of the intracelluar protein occurred due to cell lysis. Chitinase production rate was the highest when the strain was cultured at 20°C and pH level was 7.0 (Fig. 2).

Purification of Chitinase from *Cytophaga* sp. HJ

As shown in Table 2, chitinase was purified 2.7 fold with a recovery of 9.7%. The enzyme had a specific activity of 0.19 U/mg. Although the purification fold of the chitinase was low, the elution profiles of the protein and its activity from the final DEAE-Bio gel A column (Fig. 3C) showed that the enzyme was eluted as a single activity peak. In the process of Octyl-Sepharose CL-4B column chromatography (Fig. 3B), we excluded a small quantity of proteins (peak I) with chitinase activity due to some difficulties of applying the protein solutions to the next purification process. However, this protein (peak I) had a very high specific activity (7.4 U/mg). Therefore, the specific activity of chitinase (peak II) might have been reduced by removing the protein in peak I, and another chitinase might have been produced by this strain. Analysis of SDS-PAGE (Fig. 4) established that the protein was purified to homogeneity. The purified protein in a native PAGE showed some chitinolytic activity by hydrolyzing colloidal chitin on agar medium plates (Fig. 5). Molecular mass of chitinase was determined to be 59.4 kDa by SDS-PAGE. MALDI-TOF-MS gave rise to 3 peaks as shown in Fig. 6. The molecular mass of the major peak was 59.75 kDa.

Analysis of the Amino Terminal Amino Acid Sequence

The amino terminal amino acid sequence of chitinase was identified as ATPNAPVISWMPTDXXLQNXS in which X was not determined. As shown in Fig. 7, the purified chitinase showed a very low amino terminal amino acid sequence homology with chitinases derived from *Serratia marcescens* QMB 1466 (30% homology) [8], *Alteromonas* sp. strain O-7 (30% homology) [25], and *Streptomyces thermoviolaceus* OPC-520 (20% homology) [24]. However, it was clearly different from those of *Streptomyces olivaceoviridis* [16], *Aeromonas* sp. No. 10S-24 [26], and *Bacillus circulans* WL-12 [28]. Although the sequences are too short to have a definitive conclusion, the *Cytophaga* sp. HJ chitinase can be a novel protein, because no other protein with the same amino terminal amino acid sequences was found, in a database analyzed by NCBI's BLAST (Basic Local Alignment Search Tool) program.

Effects of pH and Temperature on the Chitinase Activity

The optimum pH and the optimum temperature for the chitinase activity were 4.0 and 50°C, respectively. Chitinase was stable at a pH range of 4.0-11.0 and also at below 50°C, retaining over 90% of its activity under these conditions (Fig. 8). Most of the chitinases reported in the literature [2, 4, 11, 13, 16, 18, 21-22, 24-27, 29] had high

Table 2. Purification of chitinase from *Cytophaga* sp. HJ.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification Fold
Culture filtrate	77	1077	0.07	100	1.0
30-60% ammonium sulfate precipitate	29	196	0.15	38	2.1
The first DEAE-Bio gel A column	23	110	0.21	30	3.0
Octyl-Sepharose CL-4B column	16	92	0.17	21	2.4
The second DEAE-Bio gel A column	7.5	39	0.19	9.7	2.7

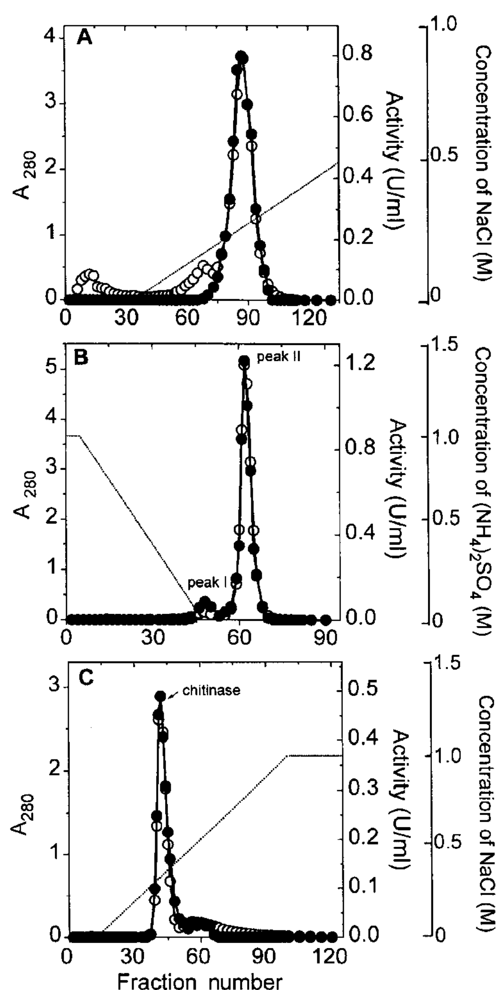

Fig. 3. Elution profiles of the protein and an activity in the column chromatography.

(A) The first DEAE-Bio gel A column chromatography. Proteins were eluted with a linear gradient of buffer A containing 0 to 0.5 M NaCl in 400 ml. (B) Octyl-Sepharose CL-4B column chromatography. After washing the column with a linear gradient of buffer A containing 1 to 0 M ammonium sulfate in 200 ml, proteins showing a chitinase activity (peaks I, II) were eluted with a buffer A in 200 ml. We excluded a small quantity of proteins included in a peak I because of difficulties of applying the protein solutions to the next purification process. (C) The second DEAE-Bio gel A column chromatography. Washing the column with a linear gradient of buffer B containing 0 to 1 M NaCl in 400 ml resulted in the elution of proteins with chitinase activity. The chitinase enzyme was eluted as a homogeneous protein. Symbols: ●, activity elution profile; ○, protein elution profile; ···, concentration gradient.

optimum temperatures. The optimum temperature for the chitinase isolated in the present study was identical with that of *Pseudomonas* sp. YHS-A2 [11], *Streptomyces kurssanovii* [21], and *Alteromonas* sp. strain O-7 [25]. Optimum temperatures of other chitinases were over 70°C. On the contrary, optimum pH varied depending on chitinases. Many chitinases including the present one showed a pH optimum in the acidic range, while *Streptomyces thermoviolaceus* OPC-520 [24] and *Alteromonas* sp. strain

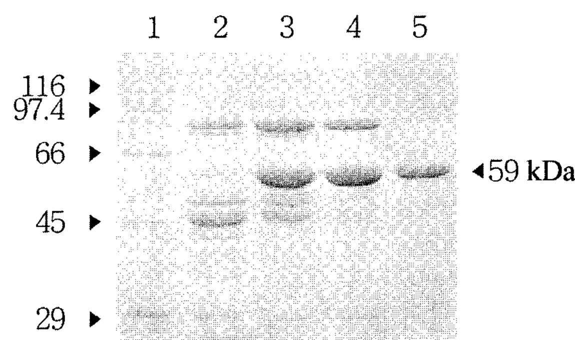

Fig. 4. SDS-PAGE of the chitinase during the protein purification steps.

After 10% polyacrylamide gel was electrophoresed, it was stained with Coomassie brilliant blue. Lane 1: Molecular size marker (in kilodaltons); Lane 2: 30-60% Ammonium sulfate precipitate; Lane 3: The first DEAE-Bio gel A column fraction; Lane 4: Octyl-Sepharose CL-4B column fraction; Lane 5: The second DEAE-Bio gel A column fraction.

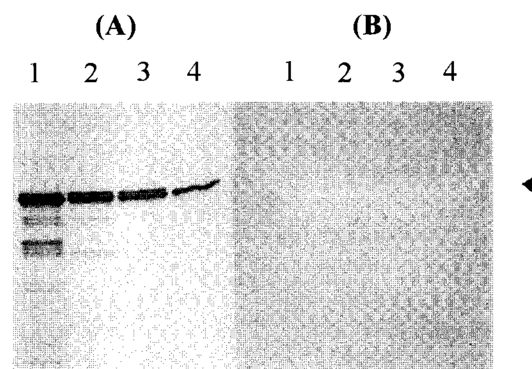

Fig. 5. Native PAGE (A) and chitin degradation patterns (B) of chitinase during protein purification steps.

After 10% of the native polyacrylamide gel was electrophoresed, it was set on the chitinase detection agar medium plates at 20°C for 24 h. Then, the gel was stained with Coomassie brilliant blue. Lane 1: 30-60% Ammonium sulfate precipitate; Lane 2: The first DEAE-Bio gel A column fraction; Lane 3: Octyl-Sepharose CL-4B column fraction; Lane 4: The second DEAE-Bio gel A column fraction.

O-7 [25] was in the basic range and *Pseudomonas* sp. YHS-A2 [11] was in the neutral range.

Effects of Metal Ions and Chemical Reagents on the Chitinase Activity

The purified chitinase was preincubated in the presence of various metal ions and chemical reagents at 4°C for 30 min at concentrations ranging from 1 μM to 1 mM, and its residual activity was assayed. The results are shown in Table 3. In the concentration of 1 mM, N-bromosuccinimide, Hg^{2+}, Sb^{3+}, diethylpyrocarbonate (DEP), and Ag^{+} inhibited the chitinase activity by 88%, 87%, 65%, 58%, and 47%, respectively. Other chemicals such as Ba^{2+}, Cu^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Pb^{2+}, Zn^{2+}, EDTA, and iodoacetic acid inhibited the enzyme by approximately 10-20%. On the contrary, Fe^{2+}, Ca^{2+}, and Co^{2+} activated the chitinase activity by 14%,

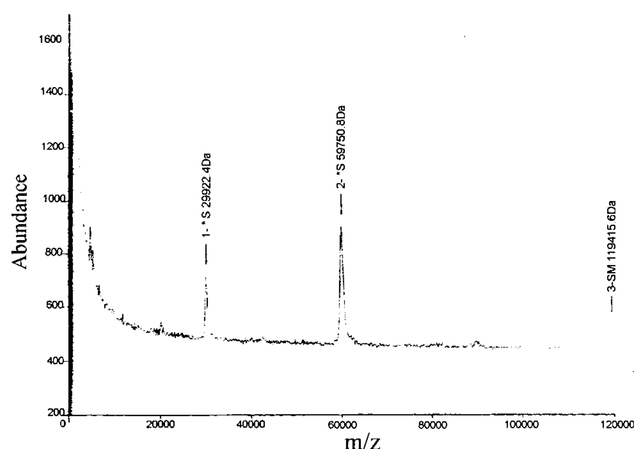

Fig. 6. Molecular mass determination of chitinase by MALDI-TOF-MS.

MALDI-TOF-MS was performed on a HP G2025A system. Samples were prepared by a 1:1 dilution with the UV-absorbing matrix sinapinic acid (50 mM) followed by a rapid vacuum evaporation in the HP 2024A sample preparation accessory. 28 Laser shots were summed up for the spectrum.

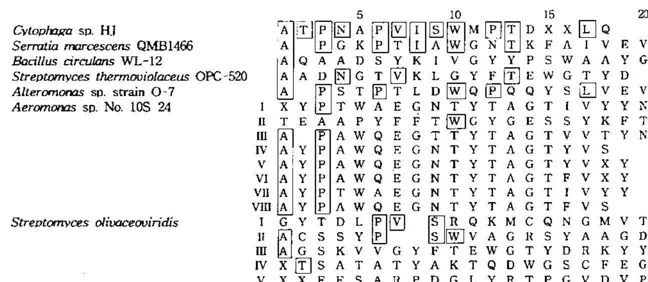

Fig. 7. Comparison of the amino terminal amino acid sequence of *Cytophaga* sp. HJ chitinase with other microbial chitinases.

The purified chitinase showed a very low sequence homology of amino terminal amino acid sequences with other chitinases. Amino acids having similar positions are boxed (X: Not determined).

33%, and 2.4%, respectively. Despite of the fact that the degree of activation was very low, the activating agents are expected to be possible cofactors because the chitinase had a tendency to be inhibited by the presence of both metal ions and chemical reagents; however, the agents showed no marked activating effect at other concentration levels (1 μM, 10 μM, and 100 μM). Based on these results, we can clearly state that sulfhydryl moiety, tryptophan, and histidine may be involved in the enzyme activity.

Substrate Specificity of Chitinase

The chitinase acted better on colloidal chitin than on chitosan and its derivatives (Fig. 9). The increasing amounts of GlcNAc during 4 h of reaction were 11.85 mg (colloidal chitin), 2.81 mg (chitosan, D.A., 1525%), 3.20 mg (chitosan, D.A., 78%), 0.45 mg (*N*-trimethyl chitosan), and 0.24 mg (glycol chitosan), respectively. In colloidal chitin and chitosans, a significant amount of GlcNAc was detected at zero time. This is possibly caused by contaminants in the

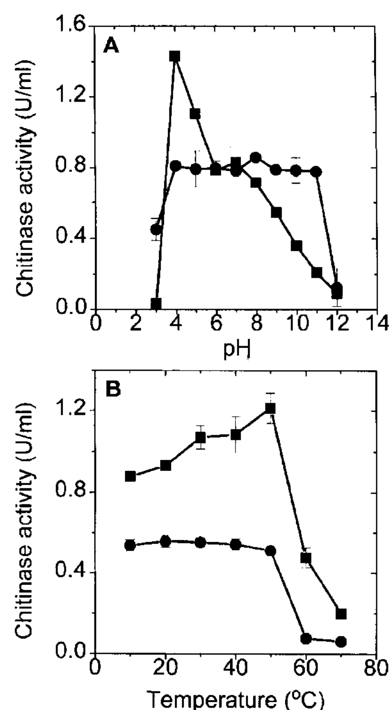

Fig. 8. Effects of pH (A) and temperature (B) on the chitinase activity and stability.

(A) Effect of pH on the chitinase activity was determined by varying pH of the reaction mixtures using 10 mM citric acid-NaOH buffer (pHs 3, 4), 10 mM sodium acetate buffer (pH 5), 10 mM potassium phosphate buffer (pHs 6, 7), 10 mM Tris-HCl buffer (pHs 8, 9), and 10 mM glycine-NaOH buffer (pHs 10, 11, 12). Chitinase stability was determined by incubating the purified enzyme with the above buffer solutions at 4°C for 24 h and assaying the residual activity of chitinase precipitated by adding a solid ammonium sulfate to 80%. (B) Effect of temperature on the chitinase activity was determined by incubating the mixtures at various temperatures. Chitinase stability was determined by assaying the residual activity of chitinase after maintaining the purified enzyme for an hour at various temperatures (buffer A). Symbols: ■, activity; ●, stability. The data were the mean value of duplicate experimental results. Error bar indicates the range of standard deviation of two data points.

enzyme or substrate solutions rather than products released by chitinase. Therefore, we concluded that the chitinase hydrolyzed the bond between GlcNAc and GlcNAc, while it was unable to hydrolyze the bond between glucosamine (GlcN) and GlcN, or between GlcN and GlcNAc. Kinetic studies were conducted and analyzed by the Lineweaver-Burk plot. For colloidal chitin and chitosan (D.A., 15-25%), K_m values (mg/ml) were 4.0 and 1.6, V_{max} (U/ml) were 2.4 and 0.13, and the catalytic efficiency (K_{cat}, U/mg) of chitinase, the ratio of V_{max}/K_m, were 0.60 and 0.08, respectively.

HPLC Analysis of the Enzymatic Reaction Products

The chitinase (18 mU) secreted by *Cytophaga* sp. HJ produced 34 μM of GlcNAc and 85 μM of $(GlcNAc)_2$ after 30 min of incubation. After 24 h of incubation, 90 μM of GlcNAc, 165 μM of $(GlcNAc)_2$, and 3.2 μM of $(GlcNAc)_3$ were produced (Fig. 10). HPLC analysis of the

Table 3. Effects of metal ions and chemical reagents on the chitinase activity.

Chemicals	Concentration	Residual activity (%)
No addition	-	100.0
$AgNO_3$	1 mM	53.4
$BaCO_3$	1 mM	98.2
$CaCl_2$	1 mM	103.3
	100 μM	92.0
	10 μM	94.0
	1 μM	90.0
$CoCl_2 \cdot 6H_2O$	1 mM	102.4
	100 μM	110.3
	10 μM	91.0
	1 μM	93.0
$CuSO_4$	1 mM	90.8
$FeSO \cdot 7H_2O$	1 mM	114.0
	100 μM	108.1
	10 μM	93.0
	1 μM	94.0
$HgCl_2$	1 mM	12.5
LiCl	1 mM	94.8
$MgCl_2 \cdot 6H_2O$	1 mM	93.6
$MnCl_2 \cdot 4H_2O$	1 mM	89.9
NaCl	1 mM	97.8
$Pb(NO_3)_2$	1 mM	82.3
$SbCl_3$	1 mM	34.7
$ZnCl_2$	1 mM	89.6
EDTA	1 mM	85.3
N-bromosuccinimide	1 mM	11.9
Diethylpyrocarbonate (DEP)	1 mM	41.8
Iodoacetic acid	1 mM	89.6

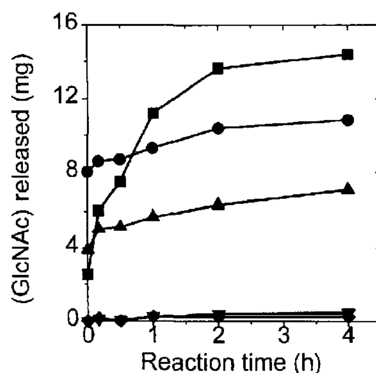

Fig. 9. Time-courses of the chitinase activity for various substrates.
Substrates were used as a concentration of 0.1% (w/v). Colloidal chitin was suspended in buffer A, and chitosan and chitosan derivatives were dissolved in a 100 mM sodium acetate buffer (pH 5.0). Symbols: ■, colloidal chitin; ●, chitosan (D.A., 15-25%); ▲, chitosan (D.A., 7-8%); ▼, N-trimethyl chitosan; ◆, glycol chitosan.

enzymatic reaction products showed that the chitinase produced GlcNAc and $(GlcNAc)_2$ in the early period of reaction. When the reaction was further carried out, more GlcNAc and $(GlcNAc)_2$ were produced, while $(GlcNAc)_3$ was produced in a very small quantity. This was in good agreement with the reports that *Thermoactinomyces vulgaris*

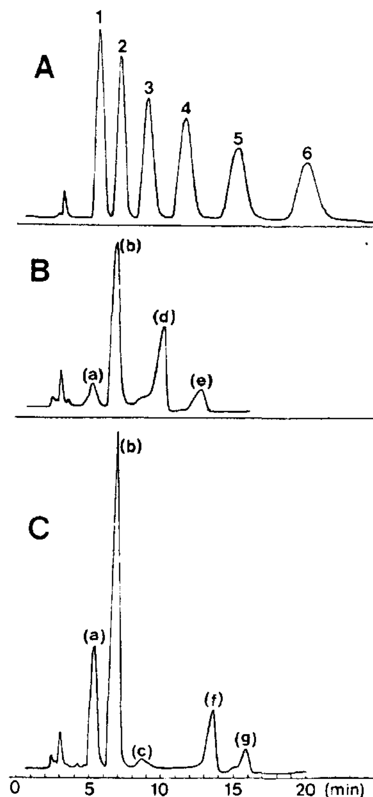

Fig. 10. Hydrolysates of colloidal chitin.
The reaction mixtures containing colloidal chitin (0.09%), buffer A, and the purified chitinase (18 mU) were incubated at 20°C for 30 min or 24 h. They were boiled for 10 min, passed through YM 30 ultrafiltration membrane (Amicon Co.), lyophilized, and analyzed by HPLC with a carbohydrate analysis column (3.9×300 mm, Waters Co.) using a solvent system of 72% acetonitrile and 28% water. A: standards, *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-glucosamine oligomers from dimers $(GlcNAc)_2$ to hexamers $(GlcNAc)_6$. Peak 1: GlcNAc; Peak 2: $(GlcNAc)_2$; Peak 3: $(GlcNAc)_3$; Peak 4: $(GlcNAc)_4$; Peak 5: $(GlcNAc)_5$; Peak 6: $(GlcNAc)_6$. B: Enzymatic hydrolysates after 30 min of incubation. C: Enzymatic hydrolysates after 24 h of incubation. Peak (a) corresponds to GlcNAc, peak (b) corresponds to $(GlcNAc)_2$, Peak (c) corresponds to $(GlcNAc)_3$, and peaks (d), (e), (f), and (g) are unknown.

KFB-C100 [30] produced $(GlcNAc)_2$ and *Pseudomonas* sp. YHS-A2 [11] produced GlcNAc and $(GlcNAc)_2$. The major product in *Cytophaga* sp. HJ and *Thermoactinomyces vulgaris* KFB-C100 [30] was $(GlcNAc)_2$, while GlcNAc dominated in *Pseudomonas* sp. YHS-A2 [11]. Due to the fact that $(GlcNAc)_2$ promotes the growth of bifidobacteria in infants or the elderly suffering with lactose intolerance [1], we can expect that this chitinase may be used in the production of $(GlcNAc)_2$.

Other than peaks representing *N*-acetylchitooligosaccharides, several unknown peaks were detected in the HPLC chromatogram (Fig. 10). Because the extent of acetylation of colloidal chitin used as a substrate in the present study was not determined and that the deacetylated residues could be included in the colloidal chitin, *N*-deacetyloligosaccharides with the same degree of

polymerization could be shown as the various peaks with different retention times in the chromatogram.

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