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Purification and Characterization of Two Bifunctional Chitinases/Lysozymes Extracellularly Produced by *Pseudomonas aeruginosa* K-187 in a Shrimp and Crab Shell Powder Medium

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Two extracellular chitinases (FI and FII) were purified from the culture supernatant of *Pseudomonas aeruginosa* K-187. The molecular weights of FI and FII were 30,000 and 32,000, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 60,000 and 30,000, respectively, by gel filtration. The pIs for FI and FII were 5.2 and 4.8, respectively. The optimum pH, optimum temperature, pH stability, and thermal stability of FI were pH 8, 50°C, pH 6 to 9, and 50°C; those of FII were pH 7, 40°C, pH 5 to 10, and 60°C. The activities of both enzymes were activated by Cu^{2+}; strongly inhibited by Mn^{2+}, Mg^{2+}, and Zn^{2+}; and completely inhibited by glutathione, dithiothreitol, and 2-mercaptoethanol. Both chitinases showed lysozyme activity. The purified enzymes had antibacterial and cell lysis activities with many kinds of bacteria. This is the first report of a bifunctional chitinase/lysozyme from a prokaryote.

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) residues linked by β1-4 bonds, is the most abundant renewable natural resource after cellulose (4). It is widely distributed in nature as the integuments of insects and crustaceans and as a component of fungi and algae (23). It is estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates, for example, is 37,300 metric tons (32). Chitin and its derivatives are of interest because they have various biological activities, such as those of an immunoadjuvant and a flocculant of wastewater sludge, and agrochemical uses (7). Chitooligosaccharides are prepared by partial hydrolysis of chitin with hydrochloric acid or enzymatically by degradation and transglycosylation. This pretreated chitin is then mixed with a chitinolytic enzyme to hydrolyze it to the monomer or oligomer of *N*-acetylglucosamine.

Chitinases, a group of enzymes capable of degrading chitin directly to low-molecular-weight products, have been shown to be produced by a number of microorganisms. Almost all of the reported chitinase-producing strains will use chitin or colloidal chitin as a carbon source (42). Commercial interest in the utilization of chitin and its derivatives has led to the need for inexpensive, reliable sources of active and stable chitinase preparations. The production of inexpensive chitinolytic enzymes is an important element in the utilization of shellfish wastes that not only solves environmental problems but also promotes the economic value of the marine products (42).

Some animal and higher plant chitinases have lysozyme activity (chitinase/lysozyme), while bifunctional chitinases have not been isolated from microorganisms. We reported the medium composition and the character of crude chitinase from *Pseudomonas aeruginosa* K-187 in a previous paper (42). In the present study, we isolated two kinds of bifunctional chitinases/lysozymes from K-187 cell-free culture broth. This paper describes the purification and characterization of these enzymes.

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MATERIALS AND METHODS

Materials. The shrimp and crab shell powder (SCSP) used in these experiments was purchased from Chya-Pau Co., I-Lan, Taiwan. Hen egg white lysozyme (HEWL), turkey egg white lysozyme (TEWL), human milk lysozyme (HML), *Serratia marcescens* chitinase, *Streptomyces griseus* chitinase, ethylene glycol chitin (EGC), lyophilized cells of *Micrococcus lysodeikticus*, and powdered chitin were purchased from Sigma Chemical Co., St. Louis, Mo. DEAE-Sepharose CL-6B was from Pharmacia, and Econo Pac q was from Bio-Rad. Cell suspensions of *M. lysodeikticus* were prepared as described previously (43, 44). Colloidal chitin was prepared from powdered chitin by the method of Jeniaux (13). All other reagents used were of the highest grade available.

Microorganism and enzyme production. *P. aeruginosa* K-187 was isolated from the soil in Taiwan (42) and maintained on nutrient agar plates at 37°C. For maximum production of the enzyme, we checked the activities of chitinase and lysozyme (using *M. lysodeikticus* cells as a substrate) in the culture supernatant at different stages of growth of *P. aeruginosa* K-187. As shown in Fig. 1, the enzyme activities were highest at 3 days.

For the production of chitinase, *P. aeruginosa* K-187 was grown in 175 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 3.0% (wt/vol) SCSP, 0.1% (wt/vol) carboxymethyl cellulose, 0.1% (wt/vol) $(NH_4)_2SO_4$, 0.1% (wt/vol) K_2HPO_4, 0.1% (wt/vol) $MgSO_4 \cdot 7H_2O$, and 0.1% (wt/vol) $ZnSO_4 \cdot 7H_2O$, pH 9.0. Two milliliters of the seed culture was transferred into 175 ml of the same medium and grown in an orbital shaking incubator for 72 h at 45°C. The culture broth was centrifuged for 15 min at 8,000 × *g*, and the supernatant was used for the purification of the enzyme.

Purification of chitinases I and II. (i) DEAE-Sepharose CL-6B chromatography. To the cell-free culture broth (850 ml), 515 g of ammonium sulfate was added. The resultant precipitate was collected by centrifugation, dissolved in a small amount of 50 mM sodium phosphate buffer (pH 6.0), and dialyzed against the buffer. The resultant dialysate (40 ml), equilibrated with the same buffer containing 0.2 M NaCl, was loaded onto a DEAE-Sepharose CL-6B column (5 by 17 cm) equilibrated with the dialysis buffer. The unadsorbed proteins were washed from the column with the same buffer, and the enzyme was eluted with a linear gradient of 0.2 to 1.0 M NaCl in 50 mM phosphate buffer. In the fractions eluted by buffer containing 0.2 to 0.4 M NaCl, two protein peaks exhibiting chitinase/lysozyme activity were obtained (peaks A and B). The enzyme fractions of peaks A and B were combined and concentrated with ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 9 ml of 50 mM phosphate buffer (pH 6.0), followed by dialysis against the same buffer.

(ii) Econo-Pac q chromatography. The dialysate (11 ml) was chromatographed on a column of Econo-Pac q (Bio-Rad) which had been equilibrated with 50 mM phosphate buffer. After application of the enzyme and washing of the column with 50 mM phosphate buffer (pH 6.0), the column was eluted with a linear gradient between 0 and 1.0 M NaCl in the same buffer. Two protein peaks (FI and FII) exhibiting chitinase/lysozyme activity were obtained, combined, and used as a purified enzyme preparation.

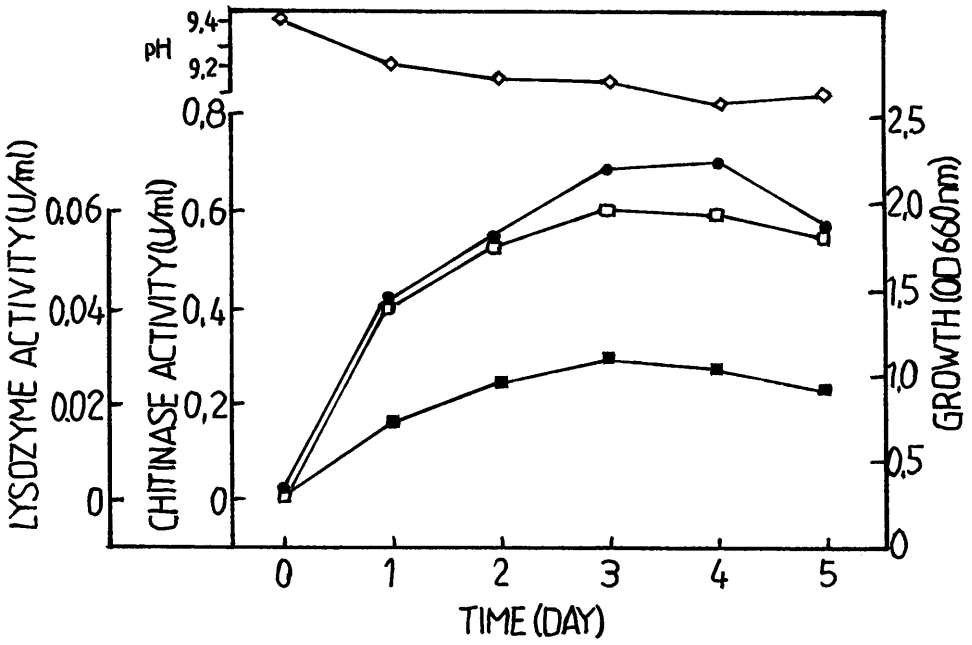

FIG. 1. Time courses of growth (●) and chitinase (□) and lysozyme (■) production in a culture of *P. aeruginosa* K-187. ◇, pH. OD, optical density.

Measurement of enzyme activity. Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained a 1.3% suspension of colloidal chitin in a phosphate buffer (75 mM, pH 6), and the mixture was incubated at 37°C for 10 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita (11) with *N*-acetylglucosamine as a reference compound.

Lysozyme activity was determined spectrophotometrically by measuring the decrease in optical density at 660 nm. The reaction mixture contained 1.5 ml of an *M. lysodeikticus* cell suspension (optical density of 1.7) in 50 mM phosphate buffer (pH 7) and 1.5 ml of the enzyme solution. The mixture was incubated at 37°C for 30 min, and the optical density at 660 nm was measured. The control sample contained 1.5 ml of the buffer instead of the enzyme. The turbidimetric assay for bacterial cell-lytic enzyme was performed by the same method described above. Lysozyme activity was also measured as an increase in reducing power resulting from hydrolysis of EGC (in 50 mM phosphate buffer, pH 7) at 37°C for 30 min (21).

When colloidal chitin or EGC was used, one unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing sugars per min. When *M. lysodeikticus* cells were used as a substrate, one unit of lysozyme activity was defined as the amount of enzyme required to decrease the optical density at 660 nm by 0.01 per min.

Determination of molecular weight and isoelectric point. The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (45a) with bovine serum albumin (molecular weight, 67,000), ovalbumin (43,000), soybean trypsin inhibitor (20,500), and HEWL (14,500) as standard proteins. Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7.0) containing SDS and 2-mercaptoethanol. The molecular weights of the purified enzymes in the native form were determined by a gel filtration method. The sample and standard proteins were applied to a PTLC 260138 column (4.6 mm by 25 cm; ISCO) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and chromatographed with an elution rate of 0.5 ml/min. Bovine serum albumin (molecular weight, 67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400) (Sigma Chemical Co.) were used as molecular weight markers. The isoelectric points of chitinases I and II were estimated by chromatofocusing. The chitinase solution (about 1 ml) was loaded onto a chromatofocusing PBE 94 column (0.9 by 27 cm) equilibrated with 50 mM Tris-HCl buffer (pH 6.0). Elution was done with Polybuffer 74–Tris-HCl (pH 6.0) as described in the manufacturer's manual (Pharmacia).

Protein determination. Protein was determined by the method of Lowry et al. (16) with crystalline egg albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

Amino acid analysis. The purified enzymes were hydrolyzed with 6 N HCl at 110°C for 24 h in a sealed and evacuated tube, and the amino acid compositions were determined with a Beckman system 6300E.

Antimicrobial action of chitinases I and II. The action of chitinases I and II against both gram-positive and gram-negative bacteria was examined. The enzyme solution (2 mg/ml; 50 mM phosphate buffer) was used as the lytic enzyme for the measurement of growth inhibition, and buffer without enzyme was used as a blank for the control experiment. The test bacteria used were *Bacillus cereus* CCRC 14689, *Bacillus subtilis* CCRC 10029, *Bacillus thuringiensis* subsp. *israelensis* CCRC 11501, *B. thuringiensis* subsp. *kurstaki* CCRC 11498, *Bacillus bassiana* CCRC 31767, *Enterobacter faecalis* CCRC 10789, *Escherichia coli* CCRC 51445, *Lactobacillus bavaricus* CCRC 12933, *Lactobacillus lactis* subsp. *lactis* CCRC 10791, *M. lysodeikticus* CCRC 11034, *Staphylococcus aureus* CCRC 10451, *S. aureus* CCRC 10777, and *P. aeruginosa* M-1001. *P. aeruginosa* M-1001, an HEWL inhibitor-producing strain, was isolated from the soil in Taiwan (43). The other

TABLE 1. Purification of chitinases I and II from *P. aeruginosa* K-187

Step	Total protein (mg)	Chitinase		Yield (%)	Lysozyme		Ratio (*A*/*B*)
		Total U (*A*)	Sp act (U/mg)		Total U (*B*)	Sp act (U/mg)	
Culture supernatant	3,410	510	0.15	100	39.2	0.011	13.01
$(NH_4)_2SO_4$ ppt[a] (80%)	1,494	380	0.25	75	28.9	0.019	13.15
DEAE-Sepharose CL-6B	310	300	0.97	59	24.5	0.079	12.24
$(NH_4)_2SO_4$ ppt (80%)	203	210	1.03	41	16.7	0.082	12.57
Econo-Pac q							
Chitinase I	92	140	1.52	27	9.5	0.103	14.74
Chitinase II	40	35	0.88	7	3.4	0.085	10.29

[a] ppt, precipitate.

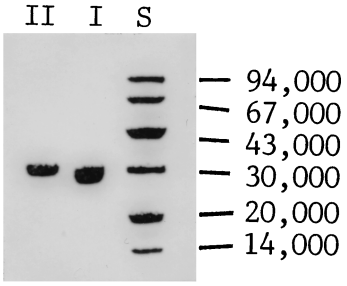

FIG. 2. SDS-PAGE of the purified chitinases. Lane S, low-molecular-weight standards (Pharmacia); lane I, chitinase I; lane II, chitinase II.

strains used for antibacterial activity tests were purchased from the Culture Collection and Research Center, Hsin-Tsu, Taiwan.

RESULTS

Purification of chitinases I and II. In the presence of SCSP as a major carbon source, *P. aeruginosa* K-187 released chitinases into the culture fluid. These were purified from the culture supernatant (850 ml) of *P. aeruginosa* K-187 as described in Materials and Methods. Econo-Pac q chromatography yielded two protein peaks, corresponding to chitinases I and II (peaks F1 and F2, respectively). The purification procedures are summarized in Table 1. The purifications of chitinase I and II were 10- and 6-fold, with overall yields of 27 and 7%, respectively. The final amounts of chitinases I and II obtained were 92 and 40 mg, respectively. The specific activities of these chitinases were 1.52 and 0.88 U/mg of protein, respectively. The chitinase and lysozyme activities could not be separated by using standard methods for protein separation and purification, and their ratio remained almost constant throughout all of the purification steps. The purified enzymes I and II were also confirmed to be homogeneous by SDS-PAGE (Fig. 2) and high-pressure liquid chromatography (HPLC) and chromatofocusing (data not shown).

Molecular weight, pI, and amino acid composition. The molecular weight of each enzyme was calculated on the basis of semilogarithmic plots of the mobilities of the bands on SDS-PAGE, using a standard curve established with proteins of known molecular weight. The type I and II chitinases were almost indistinguishable; the molecular weights of the two forms were estimated to be 30,000 and 32,000, respectively (Fig. 2). Gel filtration on an HPLC column gave molecular weights of 60,000 for chitinase I and 30,000 for chitinase II. These results indicate that chitinase I has a dimeric structure and chitinase II has a monomeric structure. The isoelectric points of chitinases I and II were found to be pH 5.2 and 4.8, respectively, by chromatofocusing. Table 2 summarizes the data obtained from amino acid analyses of the purified chitinases.

Enzymatic activity. The type I and II chitinases were assayed with three different substrates, i.e., colloidal chitin (chitinase activity), EGC (lysozyme activity), and *M. lysodeikticus* cells (lysozyme activity) (Table 3). HEWL, TEWL, HML, *S. marcescens* chitinase, and *S. griseus* chitinase were used as reference enzymes.

The chitinase and lysozyme activities are shown in Table 3. Under the assay conditions with 50 mM phosphate buffer (pH 6), K-187 chitinases I and II showed higher chitinase specific activities against colloidal chitin than did the other enzymes, and chitinase I was approximately two times as potent as type II. The K-187 chitinases I and II also showed a higher lysozyme specific activities against EGC than the other enzymes. Chitinase I was approximately two times as potent as type II. When *M. lysodeikticus* cells were used as a substrate, the specific activity of chitinase I was also higher than that of chitinase II but lower than those of HEWL, TEWL and HML. The commercial bacterial chitinases, *S. marcescens* and *S. griseus* chitinases, have no lysozyme activity when EGC or *M. lysodeikticus* cells are used as a substrate.

TABLE 2. Amino acid compositions of chitinases I and II

Amino acid[a]	mol % in:	
	Chitinase I	Chitinase II
Asx	0.3	0.4
Thr	6.7	0.2
Ser	5.7	19.7
Glx	19.1	4.2
Pro	4.1	0.2
Gly	11.0	0.3
Ala	14.0	3.1
Cys	2.3	9.7
Val	0.2	9.9
Met	11.2	6.2
Ile	0.2	9.7
Leu	7.8	18.4
Tyr	0.6	2.8
Phe	4.8	6.9
His	5.9	6.5
Lys	5.9	1.9
Arg	0.3	0.3

[a] Tryptophan was not measured.

pH-activity and pH-stability profiles. The effect of pH on the catalytic activity was studied by using colloidal chitin as a substrate under the standard assay conditions. The pH-activity profiles of chitinases I and II were bell shaped, with maximum values at pH 7 and 8, respectively (Fig. 3A). The pH-stability profiles of the two enzymes were determined by the measurement of the residual activity at pH 6 after incubation at various pHs at 37°C for 30 min. As shown in Fig. 3B, chitinases I and II were stable at pH 6 to 9 and 5 to 10, respectively. Lysozyme activities showed the same extents of impairment at various pHs.

To further compare the chitinase/lysozyme activities of chiti-

TABLE 3. Enzyme activities with various substrates[a]

Enzyme	Activity (mean ± SEM [n = 3][b] on:		
	Colloidal chitin	EGC	*M. lysodeikticus* cells
Chitinase I	1.5 ± 0.3	16.9 ± 0.2	0.10 ± 0.03
Chitinase II	0.80 ± 0.3	9.1 ± 0.2	0.08 ± 0.03
HEWL (chitinase/lysozyme)	0.018 ± 0.002	2.4 ± 0.1	24.1 ± 3.8
TEWL (chitinase/lysozyme)	0.005 ± 0.001	0.08 ± 0.01	22.7 ± 4.5
HML (chitinase/lysozyme)	0.003 ± 0.001	0.02 ± 0.01	24.7 ± 4.1
S. marcescens chitinase	0.276 ± 0.026	0[c]	0
S. griseus chitinase	0.678 ± 0.023	0	0

[a] Seven different enzymes were assayed on three different substrates at a constant ionic strength (50 mM phosphate buffer) and temperature (37°C) at pH 6 (10 min) for colloidal chitin and pH 7 (30 min) for EGC or *M. lysodeikticus* cells. The amount of enzyme used was less than 100 μg, and it was controlled to a concentration that showed a optical density range of 0.05 to 0.20. The assay conditions are described in Materials and Methods.

[b] Expressed as units per milligram for colloidal chitin and EGC and as units per microgram for *M. lysodeikticus* cells.

[c] Where no activity was detected, 100 μg of protein was assayed for 30 min.

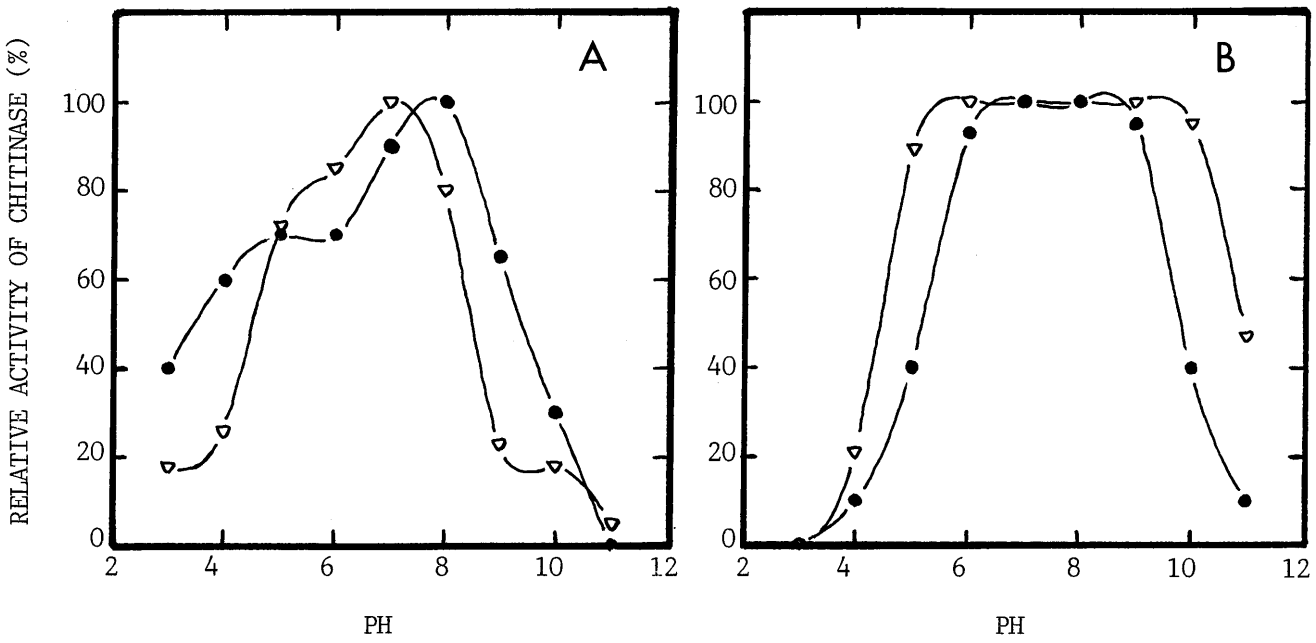

FIG. 3. Effects of pH on the activity and stability of chitinases I (●) and II (▽). (A) Chitinase activities were measured at various pHs at 37°C for 10 min. (B) Enzyme solutions were incubated at various pHs at 37°C for 30 min, and residual activities were assayed at pH 6.

nases I and II to that of HEWL at their respective optimum pHs, colloidal chitin, EGC, and *M. lysodeikticus* cells were used as substrates. The results indicated that chitinases I and II have an optimum pH range of 7 to 8 on all three substrates. The optimum pH for HEWL on *M. lysodeikticus* cells is also 7 to 8, but its optimum pH with colloidal chitin and EGC as substrates is around 5.

Effect of temperature on activity and stability. The effect of temperature on the activities of chitinases was studied with colloidal chitin as a substrate. The optimum temperatures for chitinases I and II were 50 and 40°C, respectively (Fig. 4A). To examine the heat stabilities of the chitinases, the enzyme solution in 50 mM phosphate buffer (pH 6.0) was allowed to stand for 10 min at various temperatures, and then the residual activity was measured. As shown in Fig. 4B, chitinase I maintained its initial activity from 25 to 50°C and had 15% of its activity at 80°C but was completely inactivated at 90°C. On the other hand, chitinase II showed markedly high thermostability

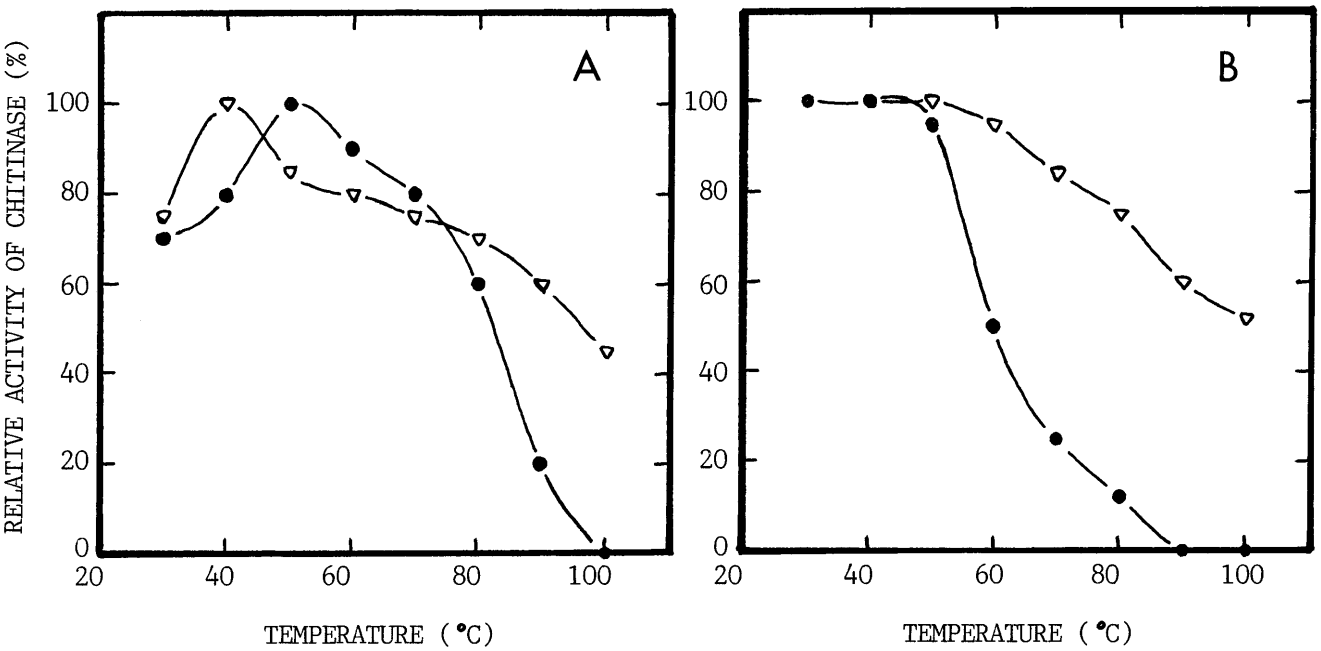

FIG. 4. Effects of temperature on the activity and stability of chitinases I (●) and II (▽). (A) Chitinase activities were measured at various temperatures at pH 6. (B) Enzyme solutions were incubated at pH 6 for 10 min, and remaining activities were measured at 37°C.

TABLE 4. Effects of various substrates on enzyme activity

Substrate	Concn	Relative activity (%)[a]			
		FI		FII	
		Chitinase[b]	Lysozyme[c]	Chitinase	Lysozyme
None	0	100	100	100	100
$CaCl_2$	1.0 mM	94	95	88	91
$CoCl_2$	1.0 mM	54	61	54	59
$CdCl_2$	1.0 mM	94	92	88	90
$MgSO_4$	1.0 mM	64	65	9	13
$MnSO_4$	1.0 mM	26	25	3	5
$ZnSO_4$	1.0 mM	78	81	27	30
$CuSO_4$	1.0 mM	150	146	105	107
$CaSO_4$	1.0 mM	100	100	90	100
$FeSO_4$	1.0 mM	85	91	48	52
K_2SO_4	1.0 mM	61	63	18	17
NaCl	1.0 mM	50	54	24	27
NH_4NO_3	1.0 mM	90	96	48	51
$HgCl_2$	0.05 mM	0	0	16	12
$C_2H_2COH_2(COOK)COONa$	1.0 mM	73	75	15	18
EDTA	1.0 mM	60	64	56	58
Dithiothreitol	1.0 mM	0	0	0	0
2-Mercaptoethanol	1.0 mM	0	0	0	0
Glutathione	1.0 mM	0	0	0	0
KIO_3	1.0 mM	100	98	84	88
p-Chloromercuribenzoate	0.1 mM	100	100	105	104
n-Ethylmaleimide	0.1 mM	105	106	106	104
Iodoacetate	0.1 mM	100	98	92	94
GlcNAc	10 mM	0	5	32	35
GalNAc	10 mM	3	6	24	23
Galactose	10 mM	8	10	20	18
Methanol	50%	45	48	52	54
Ethanol	50%	68	67	76	73
Acetone	50%	36	40	57	56
Acetonitrile	50%	69	67	68	65

[a] The activities were assayed under the standard conditions and expressed as a percentage of the activity in the absence of the compound.
[b] The reaction mixture of enzyme solution and colloidal chitin suspension was incubated with each of the compounds in 50 mM phosphate buffer (pH 6) for 10 min at 37°C.
[c] The reaction mixture of enzyme solution and EGC was incubated with each of the compounds in 50 mM phosphate buffer (pH 6) for 30 min at 37°C.

(Fig. 4B). Chitinase II maintained 55% of its activity at 100°C. The chitinase and lysozyme activities of both enzymes showed the same dependence on temperature.

Effects of various chemicals. The effects of various chemicals on the enzyme activity were investigated by preincubating the enzyme with chemicals in 50 mM phosphate buffer (pH 6) for 10 min at 37°C and then measuring the residual activities of chitinase and lysozyme by using colloidal chitin and EGC, respectively, as substrates. The results are presented in Table 4. Only in the case of copper addition was there a slight increase in the activity. In some cases, e.g., with dithiothreitol, 2-mercaptoethanol, and glutathione, complete inhibition was observed.

The effect of copper ion on chitinase activity was further examined. A slight increase in the chitinase activities of chitinase I, chitinase II, and crude chitinase was observed. The optimum concentrations of copper ion added were 5, 4, and 4 mM for chitinase I, chitinase II, and the crude chitinase, respectively. A further increase of the concentration of copper ion above the optimum value, however, resulted in a decrease in chitinase activity.

Bacterial cell-lytic activities of chitinases I and II. The bacterial cell-lytic activities of the type I and II chitinases against both gram-negative and gram-positive bacteria were examined. Comparisons of the lytic spectra of the two chitinases with those of HEWL and the lysozyme of the other *P. aeruginosa* strain, M-1001 were also made. The results are given in Table 5. The two chitinases showed potent lytic activities toward *S. aureus*, *P. aeruginosa* M-1001, and *P. aeruginosa* K-187. The *P. aeruginosa* K-187 chitinases have lytic spectra similar to that of M-1001 lysozyme but different from that of HEWL.

Antimicrobial effects of chitinases I and II. The actions of lytic enzymes against gram-positive and gram-negative bacteria

TABLE 5. Comparison of the lytic spectra of the lytic enzymes from strain K-187 and other organisms[a]

Substrate[a]	Mean specific act (U/mg) ± SEM (n = 3)		
	HEWL	FI	FII
M. lysodeikticus CCRC 11034	24.1 ± 3.8	0.100 ± 0.031	0.080 ± 0.033
B. cereus CCRC 14689	0	0.056 ± 0.022	0.040 ± 0.018
B. subtilis CCRC 10029	1.2 ± 0.4	0.029 ± 0.011	0.026 ± 0.014
E. coli CCRC 51445	2.4 ± 0.3	0.057 ± 0.019	0.024 ± 0.017
P. aeruginosa K-187	1.2 ± 0.5	0.100 ± 0.047	0.086 ± 0.036
P. aeruginosa M-1001	40.5 ± 4.7	0.195 ± 0.038	0.160 ± 0.043
E. cloacae M-1002	12.0 ± 2.4	0.013 ± 0.042	0.008 ± 0.003
S. aureus CCRC 10451	1.2 ± 0.3	0.094 ± 0.029	0.073 ± 0.035

[a] Cell suspensions of the bacterial cells were prepared as described previously (43, 44). The reaction mixtures (50 mM phosphate buffer, pH 7), containing 1.5 ml of a cell suspension (optical density of 1.7) of the substrate and 1.5 ml of the enzyme solution, was incubated at 37°C for 30 min, and the enzyme activity was determined spectrophotometrically by measuring the decrease in optical density at 660 nm.

were examined. Cells of each organism were suspended on molten nutrient agar medium and then poured into petri plates. Paper discs were placed on the surface of the medium, and the enzyme solution (10 μl) to be assayed was pipetted into each disc. After 3 days of incubation at 37°C, the susceptible cells grew uniformly in the medium except for the area where antibiotic had diffused into the medium. This was indicated by the formation of clear zones of inhibition; as chitinases I and II inhibited growth, zones of microbial inhibition were visible. Chitinases I and II inhibited the growth of all of the tested bacteria except *P. aeruginosa* M-1001 and *P. aeruginosa* K-187.

DISCUSSION

The molecular weights of microbial chitinases range from 20,000 to 120,000, with little consistency. The molecular weights of bacterial chitinases are mostly around 60,000 to 110,000, while those of actinomycetes are mostly 30,000 or lower and those of fungi are higher than 30,000. The molecular weights of plant chitinases are mostly around 30,000. The molecular weight of K-187 chitinase (types I and II) is approximately 30,000 (by SDS-PAGE) which is similar to those of actinomycetes and plants.

Most of the bacterial chitinases have acidic pIs (22, 25, 45, 46), and actinomycete chitinases have neutral or alkaline pIs (13, 18, 26, 35, 40). Like almost all of the other bacterial chitinases, strain K-187 chitinase has an acidic pI. Plant chitinases generally have very basic or very acidic isoelectric points (7, 19, 24, 30, 37, 47).

When colloidal chitin was used as a substrate for measuring chitinase activity, the optimum pHs for FI and FII were found to be 7 and 8, respectively. These optimum values are different from the pH 5 reported for HEWL. The optimum pH for the chitinase produced by K-187 is nearly neutral or slightly alkaline. This is unusual for bacterial chitinases; only the optimum pH for the chitinase of *Aeromonas hydrophila* subsp. *anaerogenes* A (pH 7) is similar. Most bacterial chitinases work better at an acidic or alkaline pH (20, 22, 25, 33, 38). The optimum pHs for the chitinases of actinomycetes (36, 40) or fungi (41) are acidic, unlike that for K-187. In addition, chitinases produced by plants such as ivy (1) have an optimal pH of 5, while that produced by kidney beans (2) has an optimal pH of 6.5. The thermostability of chitinase II is quite remarkable; it is stable up to around 60°C and maintains half of its activity even at the high temperature of 100°C. This stability is similar to that of the thermostable chitinases of *Bacillus licheniformis* X-7u (34) and markedly higher than the thermal stabilities observed for chitinases of other origins (8, 22, 27, 33, 38, 39, 45).

The two extracellular chitinases from strain K-187 are also unique because of the activation by Cu^{2+}. This is different from the case for chitinases from other organisms, for instance, *Streptomyces antibioticus* (13), *Streptomyces cinereoruber* (26), and a *Vibrio* sp. (25). Chitinases I and II are inhibited by Hg^{2+}. This is similar to the case for chitinases from other organisms, for example, *Nocardiopsis albus* subsp. *prasina* OPC-131 (36), *Nocardia orientalis* IFO 12806 (40), *Streptomyces cinereoruber* (26), *Streptomyces orientalis* (35), and *Streptomyces viridificans* (10), etc. The activities of chitinase I and II are not inhibited by the presence of *p*-chloromercuribenzoate or iodoacetate, which strongly inhibited the activities of chitinases of *A. hydrophila* A52 (46), *Aeromonas* sp. strain 10S-24 (38), and *Streptomyces cinereoruber* (26), etc. Reducing substances (such as glutathione, cysteine, or bisulfite) are activators of enzymes that contain sulfhydryl groups as an essential part of the active center of the enzyme. Papain, ficin, and bromelain are such enzymes. Both activities of chitinases I and II were completely inhibited by 2-mercaptoethanol, glutathione, and dithiothreitol, which is different from the case for papain, ficin, and bromelain. It is postulated that chitinases I and II have a disulfide bond in their molecular structures, which contributes to the inhibition of enzyme activity. Cysteine may not be present in the active sites of the two chitinase molecules. The amino acid composition profiles of chitinases I and II were dissimilar; chitinase I had Glx, Ala, Met, and Gly as the major amino acids (55.3%), and chitinase II had Ser, Leu, Val, and Cys as the major amino acids (57.7%). The presence of four amino acids as the major components of the chitinase were also found for bacterial (*Aeromonas* sp.) chitinases (38) and plant (pokeweed leaf) chitinases (24).

The purity of the enzymes after purification was supported by the results of SDS-PAGE, HPLC, and chromatofocusing performed for the measurement of the molecular weights and isoelectric points. Furthermore, it was confirmed by the results that the chitinase and lysozyme activities could not be separated, the ratio of the two activities remained almost constant throughout the whole purification procedure, and the extents of impairment during pH and thermal stability testing were the same. Chitinases from various sources have the bifunctional chitinase/lysozyme activity. Plant and animal sources of chitinase/lysozyme have been frequently reported (1, 2, 9, 14, 17, 19, 47), whereas, bifunctionality of microbial chitinases is rare. It is interesting that the lytic activity (against *M. lysodeikticus* cells) of chitinases from plant sources is low in comparison to chitinase activity and that the chitinolytic activity (against colloidal chitin) of chitinases from animal sources is low in comparison to lytic activity (21). The K-187 chitinases seem to be similar to the enzymes from plant sources. A study was performed to compare the lysozyme activity of the K-187 chitinases with those of *S. marcescens* chitinase and *S. griseus* chitinase, and the lack of lysozyme activity from the *S. marcescens* and *S. griseus* chitinases was confirmed (Table 3). As far as we are aware, K-187 chitinases I and II are the first bacterial chitinases reported to possess the bifunctional chitinase/lysozyme activity. Reports on the antimicrobial effect of chitinase are scant, with most reports addressing the lysozyme activity provided by plant chitinases (29, 31, 47). Although there are reports on chitinolytic microorganisms antagonistic to fungal plant pathogens (3, 5, 6, 12, 15, 28), the chitinases produced by these microorganisms are not known to have antibacterial effects similar to those of the K-187 chitinases.

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