JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Characterization of Two Antifungal Chitinases Extracellularly Produced by *Bacillus amyloliquefaciens* V656 in a Shrimp and Crab Shell Powder Medium

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A Gram-positive bacterium with antagonistic activity was isolated from the soil. It has been identified as *Bacillus amyloliquefaciens* strain V656 on the basis of 16S ribosomal DNA analysis and standard bacteriological tests. *B. amyloliquefaciens* V656 produced antifungal enzymes when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine waste. The antifungal enzymes displayed chitinase activities. Two extracellular antifungal chitinases (FI and FII) were purified and characterized, and their molecular weights, isoelectric points, pH and thermal stabilities, and antifungal activities were determined. The characteristics of V656 chitinases are similar to those of the known bacterial chitinases in terms of their isoelectric points, thermal instabilities, and lack of lysozyme activity. In contrast to other known bacterial chitinases, the unique characteristics of V656 chitinases include extremely low molecular weights and nearly neutral optimum pH. Furthermore, this is the first report of the isolation of chitinases from *B. amyloliquefaciens* that are active against fungi.

KEYWORDS: Chitinase; *Bacillus amyloliquefaciens*; antifungal; shrimp and crab shell; chitin

INTRODUCTION

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (Glc-NAc) residues linked by β-1−4 bonds, is a common constituent of insect exoskeletons, shells of crustaceans, and fungal cell walls (*1*, *2*). All organisms that contain chitin also contain chitinases (EC 3.2.1.14), which are presumably required for morphogenesis of cell walls and exoskeletons (*3*, *4*). Some organisms that do not contain chitin also produce chitinases to degrade the polymer for nutritional or defensive purpose (5, *6*); for example, a wide variety of bacteria (*7*−*10*), actinomycetes (*11*), and plants (*12*−*14*) produce chitinolytic enzymes in response to chitin-containing elicitors in their environments. In plants, the chitinases have been considered to be pathogenesis-related proteins, involved in defense mechanisms against pathogenic parasites (fungi, insects) (*15*−*17*). Moreover, reports on chitinolytic microorganisms antagonistic to fungal pathogens are well documented (*7*−*10*, *18*−*24*). In addition to degrading fungal and insect chitin, some animal and higher plant chitinases also have lysozyme activities that can hydrolyze the peptidoglycan of bacterial cell walls, which contains alternating β-1−4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid in its polysaccharide backbone, resembling the structure of chitin. It is suggested that chitinolytic microorganisms or chitinolytic enzymes have potential applications in the biocontrol of plant pathogenic fungi and insects (*23*, *25*), as a target for biopesticides (*26*), and in many other biotechnological areas (*27*, *28*). Biological control of plant pathogen provides an attractive alternative means for management of plant disease without the negative impact of chemical fungicides that are usually costly, can cause environmental pollution, and may induce pathogen resistance.

Recently, we have investigated the bioconversion of shrimp and crab shell powder (SCSP) of marine waste for biofungicide production. We have shown that *Pseudomonas aeruginosa* K-18 is a chitinase-producing strain in an SCSP medium (*29*−*31*). The purification and characterization of two bifunctional chitinase/lysozymes extracellularly produced by this microorganism in SCSP medium have also been described (*31*). We have further demonstrated that *P. aeruginosa* K-187 is an antifungal strain in the SCSP medium exhibiting a broad range of antagonism toward fungal phytopathogens. The antifungal characteristics of this fungicide were also evaluated as seed treatment to prevent damping off in alfalfa caused by *Fusarium oxysporum* (*32*, *33*). In the present work, we further found that a soil-borne strain of *Bacillus amyloliquefaciens*, which displayed antifungal activities when cultured in an SCSP medium, possesses strong chitinolytic activity. The purification and characterization of the antifungal chitinases thus produced were also investigated.

MATERIALS AND METHODS

Materials. The SCSP used in these experiments was purchased from Chya-Pau Co., I-Lan, Taiwan. In the preparation of the SCSP, the

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10.1021/jf010885d CCC: $22.00
Published on Web 03/06/2002

shrimp and crab shells collected from a marine food processing industry were washed thoroughly with tap water and then steamed. The solid material obtained was dried, milled, and sieved to powder with diameters <0.053 mm. DEAE-Sepharose CL-6B was from Pharmacia. *Serratia marcescens* chitinase, *Streptomyces griseus* chitinase, ethylene glycol chitin (EGC), glycol chitin (GC), lyophilized cells of *Micrococcus lysodeikticus*, and powdered chitin were purchased from Sigma Chemical Co., St. Louis, MO. Colloidal chitin was prepared from powdered chitin (Wako Chemicals, Osaka, Japan) according to the method of Jeniaux (*34*). Cell suspensions of *M. lysodeikticus* were prepared as described previously (*31*). All other reagents used were of the highest grade available.

Isolation and Screening of Antifungal Compound Producing Strains. Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 0.2% colloidal chitin, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $NaNO_3$, and 2% agar (pH 7.0). The plates were incubated at 30 °C for 2 days. Colonies that grew well or showed a clear zone around the colonies under such conditions were isolated and retained for subsequent screening. Those organisms obtained from the first screening were subcultured in liquid media (containing 3% SCSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) in shaken flasks at 30 °C and 180 rpm. After incubation for 2 days, the culture broth was centrifuged (4 °C and 820*g* for 20 min) and the supernatants were collected for measurement of antifungal activity using the procedure described below. The strain V656 that showed the highest antifungal activity was isolated, maintained on nutrient agar, and used throughout the study.

Identification of Strain V656. The bacterial strain V656 (Gram-positive) was characterized by sequence analysis (sequencing by Food Industry Research and Development Institute) of the small ribosomal subunit (16S ribosomal DNA [rDNA]) after PCR amplification with primers and cloning. Nucleotide bases of the DNA sequence obtained were analyzed by comparative sequence analysis with MicroSeq database (Applied Biosystem). From the morphological observation and physiological characteristics, the microorganisms were further identified according to the description in *Bergey's Manual of Systematic Bacteriology* (*35*) (identified by Food Industry Research and Development Institute).

In Vitro Antifungal Activity Tests. The antifungal activities for the supernatant obtained above and the purified enzymes described below were estimated using a growth inhibition assay described earlier (*4*, *36*−*39*). Fungal spores of pathogenic *F. oxysporum* were grown on Petri plates filled with potato dextrose agar (PDA). After 10 days of incubation at 25 °C, the fungal spores were removed with sterile water containing 0.1% (v/v) Tween 80. The resulting suspension was filtered aseptically through a sterilized gauze. The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 1×10^6 spores/mL. The spore suspensions were stored at 4 °C before use. To test the antifungal inhibitory effect of the crude and pure enzymes obtained, Petri plates were filled with 5 mL of molten PDA precooled to 45 °C and divided into two groups (triplicate for each). To each plate in the experimental group (E) was added the supernatant or a properly diluted enzyme solution (5 mL). The ratio (v/v) of the tested solution and PDA added in the Petri plates was 1:1. To those of the control group (C) was added an equal amount of sterile water instead of tested solution. After the plates had been cooled, the fungal spores (20 μL) were then placed onto an agar surface. Both groups were incubated for 72 h at 25 °C. The diameters of the largest and smallest fungal colonies were recorded and the averages calculated. The inhibition ratios were calculated with the formula

$$\text{inhibition ratio (\%)} = (C - E)/C \times 100$$

where C is the average diameter of the largest and smallest colonies of the control groups and E is the average diameter of the largest and smallest colonies of the experimental groups.

Generally if the inhibitory ratio was >20%, the tested fungus would be considered to be inhibited. To express the inhibitory activity of the enzyme, one unit of antifungal activity was defined as the amount of enzyme required to obtain a 50% inhibition under the above assay conditions.

Effect of Culture Condition. In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.0) and gradually supplemented with the various ingredients to be investigated. One hundred milliliters of the resultant medium in a 250 mL Erlenmeyer flask was aerobically cultured at 37 °C for 48 h on a rotary shaker (180 rpm). After centrifugation (8000*g*, 4 °C, 20 min, Beckman J2-21 M/E), the supernatant was used for bioassay. The major ingredients being investigated included chitin, chitosan, SCSP, and cellulose as carbon sources and yeast extract, polypeptone, beef extract, sodium L-glutamate, sodium nitrate, or ammonium sulfate as nitrogen sources. They were added and investigated one factor at a time until the optimal culture composition was obtained. With the use of the optimal culture composition, the effects of the initial pH, temperature, cultivation volume, and cultivation time on the production of antifungal enzymes were investigated in the same fashion until the optimal condition was found. Maximum antifungal activity was obtained when *B. amyloliquefaciens* V656 was grown aerobically in 100 mL of the optimum medium (2.0% SCSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) in a 250 mL Erlenmeyer flask at 30 °C and pH 6.0. To reflect the growth of the culture in this medium by the OD 660 nm measurement, the residual chitin was allowed to settle out.

Purification of Antifungal Enzymes FI and FII. *Production of Antifungal Enzymes.* For the production of antifungal enzymes, *B. amyloliquefaciens* V656 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 2.0% SCSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. Two milliliters of the seed culture was transferred into 100 mL of the same medium and grown in an orbital shaking incubator for 24 h at 30 °C and pH 6.0. The culture broth was centrifuged (4 °C and 12000*g* for 20 min), and the supernatant was used for further purification by chromatography.

DEAE-Sepharose CL-6B Chromatography. To the cell-free culture broth (1635 mL) was added ammonium sulfate (608 g/L). The resultant mixture was kept at 4 °C overnight, and the precipitate formed was collected by centrifugation at 4 °C for 20 min at 12600*g*. The precipitate was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7.0) and dialyzed against the buffer. The resultant dialysate (100 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 × 30 cm) pre-equilibrated with 50 mM sodium phosphate eluting buffer (pH 7.0). The unadsorbed materials were washed from the column with the same eluting buffer, and the enzymes were fractionated with a linear gradient of 0−1.0 M NaCl in 50 mM phosphate buffer. The flow rate was 75 mL/h. The eluted fractions were dialyzed against 50 mM sodium phosphate buffer (pH 7.0, 4 °C) for 24 h to remove NaCl and assayed for the antifungal activity. The antifungal fractions were combined separately and concentrated with ammonium sulfate precipitation. The resultant precipitates were collected by centrifugation and dissolved in 9 mL of 50 mM sodium phosphate buffer (pH 7.0), followed by dialysis against the same buffer.

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 (50 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 according to the method of Imoto and Yagishita (*40*) with *N*-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar/min.

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 according to 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 30 °C for 30 min (*41*).

For measuring protease activity, a diluted enzyme solution (0.2 mL) was mixed with 2.5 mL of 1% casein in phosphate buffer (pH 7) and

Table 1. Discrepancies between Sequences of Strain V656 and *Bacillus* Strain Bases

strain	positions										total different bases
	175	197	280	460	467	478	1246	1456	1457	1528	
V656	G	G	G	G	A	C	G	A	T	T	
B. amyloliquefaciens	C	R	G	G	A	C	G	T	T	C	3
B. popilliae	G	A	R	A	G	T	A	T	A	C	9
B. subtilis	G	A	A	A	G	T	A	T	A	C	9

incubated for 10 min at 37 °C. The reaction was terminated by adding 5 mL of 0.19 M trichloroacetic acid (TCA). The reaction mixture was centrifuged, and the soluble peptide in the supernatant fraction was measured according to the method of Todd with tyrosine as the reference compound (*42*).

Xylanase or cellulase activity was measured with larch wood xylan or carboxymethylcellulose (CMC) as substrate. Enzyme solution (0.1 mL) was added to 0.4 mL of substrate solution, which contained 0.5% xylan or 1.25% CMC in an acetate buffer solution (0.125 M, pH 5.0). After the mixture had been incubated at 37 °C for 10 min, it was then centrifuged, and the amount of reducing sugar produced in the supernatant was determined by using the dinitrosalicylic acid (DNS) method (*43*). One unit of xylanase or celluase activity was defined as the release of 1 μmol of reducing sugar/min at 37 °C and a pH of 5.

Determination of Molecular Weight and Isoelectric Point. The molecular weights of the purified enzymes were determined by sodium dodecyl sulfate−polyacryamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (*44*). The standard proteins used for calibration were phosphorylase B (molecular weight = 97400), bovine serum albumin (66200), catalase (57500), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), trypsinogen (24000), soybean trypsin inhibitor (19700), and lysozyme (14400). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7.0) containing 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:5, v/v) and decolorized in 7% acetic acid. The molecular weights of the purified enzymes in the native form were determined by using a gel filtration method. The sample and standard proteins were applied to a PTLC 260138 column (4.6 mm × 25 cm; ISCO), which was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and chromatographed with an elution rate of 0.5 mL/min. The molecular weight markers used were bovine serum albumin (molecular weight = 67000), ovalbumin (43000), lactoglobulin (36800), carbonic anhydrase (29000), chymotrypsinogen A (25000), and ribonuclease A (13700) (Sigma Chemical Co.). The isoelectric points of chitinases FI and FII were estimated by chromatofocusing. The chitinase soultion (1 mL) was loaded onto a chromatofocusing PBE 94 column (0.9 × 27 cm) equilibrated with 50 mM Tris-HCl buffer (pH 6.0), and the elution was done with Polybufer 74/Tris-HCl (pH 6.0) as described in the manufacturer's manual (Pharmicia).

Protein Determination. Protein content was determined according to the method of Lowry (*45*) with crystalline egg albumin as the standard.

RESULTS

Identification of Strain V656. From the morphological observation and physiological characteristics (not shown), strain V656 was identified as a strain of *Bacillus subtilis* according to the description in *Bergey's Manual of Systematic Bacteriology* (*35*). To reaffirm the strain, ribosomal sequence (16S rRNA sequences) analysis was performed. The 1546 nucleotide bases of the DNA sequence obtained were aligned with those of the most closely related isolates, and the discrepancies between sequences of strain V656 and *Bacillus* strains are shown in **Table 1**. The DNA sequence suggests that strain V656 is closer to *B. amyloliquefaciens* than to *B. subtilis*.

Time Course of Cultivation. *B. amyloliquefaciens* V656 was grown aerobically in 100 mL of the optimum medium in a 250 mL Erlenmeyer flask at 30 °C. During the process of incubation,

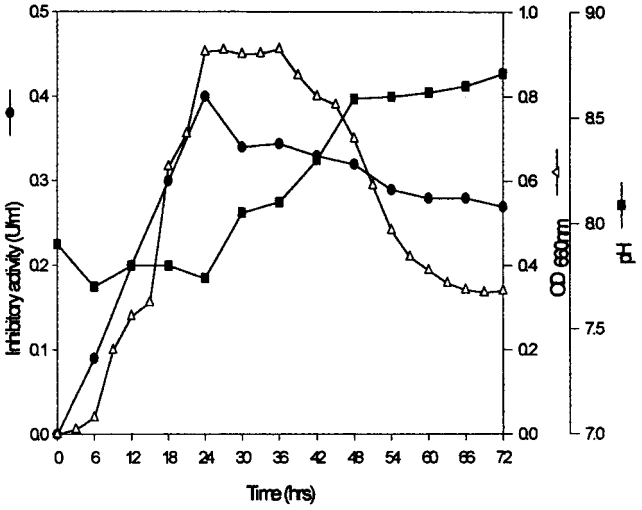

Figure 1. Time courses of growth (△), antifungal activity (●), and pH (■) in a culture of *B. amyloliquefaciens* V656. OD, optical density.

antifungal activities, cell growth, and pH in the broth were measured. The time courses of cell growth and antifungal activity are shown in **Figure 1**. The antifungal activities increased along with the cell growth (as judged by OD_{660}) and reached a maximum (0.4 unit/mL) when the cell growth reached a peak at 24 h of incubation.

Purification of Antifungal Chitinases FI and FII. In the presence of SCSP as a major carbon source, *B. amyloliquefaciens* V656 released antifungal enzymes into the culture fluid. These antifungal enzymes displayed chitinolytic activities as shown in **Table 2**. The purification of these antifungal chitinases from the culture supernatant (1635 mL) was described under Materials and Methods. Upon ion exchange chromatography on a DEAE-Sepharose CL-6B column, two protein peaks (FI and FII) exhibiting antifungal activities were resolved in the fractions eluted by buffer containing 0.2−0.6 M NaCl (**Figure 2**), allowing each to be pooled separately. The purification procedures are summarized in **Table 2**. The purification steps were very effective and combined to give overall purifications of 34-fold for FI and 10-fold for FII. The overall activity yields of the purified chitinases were 13 and 25% for FI and FII, with specific chitinase activities of 0.34 and 0.11 unit/mg of protein, respectively. The final amounts of chitinases FI and FII obtained were 11 and 62 mg, respectively. The purified enzymes FI and FII were also confirmed to be homogeneous by SDS-PAGE (**Figure 3**) and high-pressure liquid chromatography (HPLC) and chromatofocusing (data not shown).

Molecular Weight and p*I*. The molecular weight of each enzyme was calculated on the mobilities of the bands on SDS-PAGE using a standard curve established with proteins of known molecular weight. The molecular weights of chitinase FI and FII were estimated to be 14.4 and 16.9 kDa, respectively (**Figure 3**). Gel filtration on an HPLC column gave molecular weights of 14 kDa for chitinase FI and 17 kDa for chitinase FII. These results indicate that both enzymes were monomeric. The

Table 2. Purification of Antifungal Chitinases FI and FII from *B. amyloliquefaciens* V656

step	volume (mL)	total protein (mg)	total activity (units)		specific activity (units/mg)		activity yield (%)	
			chitinase[a]	antifungal[b]	chitinase[a]	antifungal[b]	chitinase	antifungal
culture supernatant	1635	2325	27.9	654	0.01	0.28	100	100
$(NH_4)_2SO_4$ precipitate	50	312	9.4	380	0.03	1.22	34	58
DEAE-Sepharose CL-6B								
FI	315	19	3.8	63	0.2	3.32	14	10
FII	510	127	7.6	204	0.06	1.61	27	31
$(NH_4)_2SO_4$ precipitate								
FI	30	11	3.7	51.6	0.34	4.69	13	8
FII	70	62	7	196	0.11	3.16	25	30

[a] One unit of chitinase activity was defined as the amount of the enzyme that produced 1 μmol of reducing sugar/min under standard assay conditions. [b] One unit of antifungal activity was defined as the amount of fungicide required to obtain a 50% inhibition under the assay conditions described under Materials and Methods.

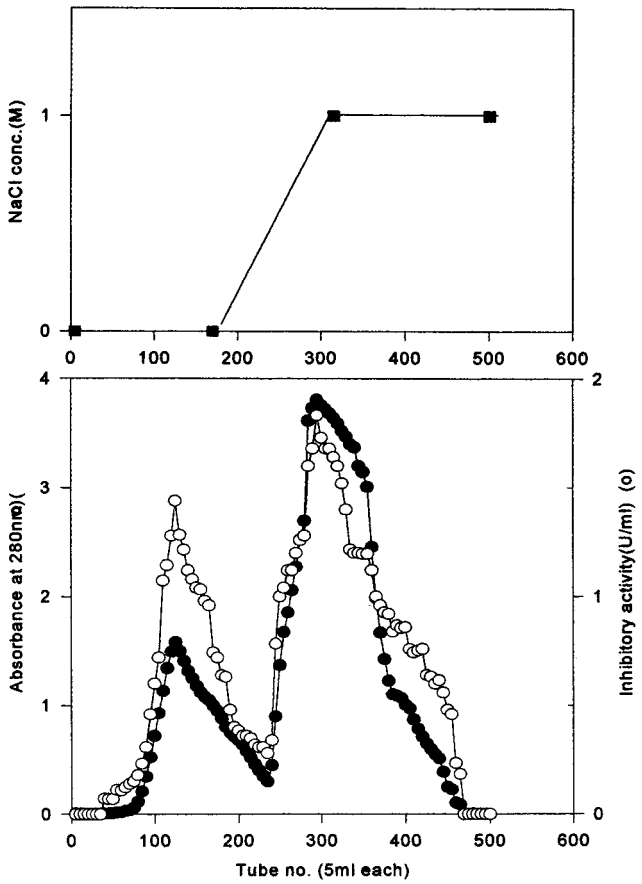

Figure 2. Elution profile of antifungal compounds (chitinases) on DEAE-Sepharose CL-6B. The DEAE-Sepharose CL-6B column (5 × 30 cm) pre-equilibrated with 50 mM sodium phosphate eluting buffer (pH 7.0) was eluted with a linear gradient of 0–1.0 M NaCl in 50 mM phosphate buffer at a flow rate of 75 mL/h.

isoelectric points of chitinases FI and FII were found to be pH 5.8 and 5.3, respectively, by chromatofocusing.

Enzymatic Activity. Chitinases FI and FII were assayed with various substrates, that is, colloidal chitin (chitinases activity), EGC (EGCase activity), *M. lysodeikticus* cells (lysozyme activity), CMC (cellulase), xylane (xylanase), and casein (protease). Hen egg white lysozyme (HEWL) and *S. marcescens* chitinase were used as reference enzymes. The enzyme activities are shown in **Table 3**. Under the assay conditions with 50 mM phosphate buffer (pH 6), V656 chitinase FI showed higher chitinase-specific activities against colloidal chitin than did the other enzymes, and chitinase FI was ~3 times as potent as chitinase FII. When EGC and *M. lysodeikticus* cells were used as substrates, both chitinases FI and FII showed much lower

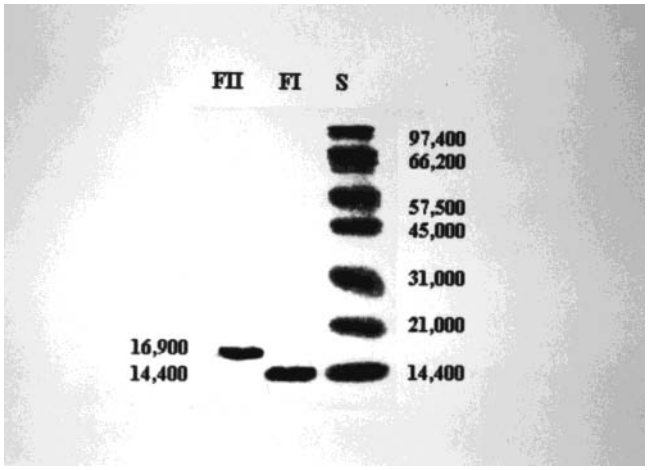

Figure 3. SDS-PAGE of the purified chitinases.

lysozyme activities than those of HEWL. As shown in **Table 3**, chitinases FI and FII possessed no cellulase or xylanase activity and little protease activity. Therefore, it was concluded that FI and FII are chitinase specific.

Effects of Various Chemicals on the Chitinase Activities of FI and FII. The effects of various chemicals on the enzymatic 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 by using colloidal chitin as a substrate. The results are presented in **Table 4**. The enzymatic activity of FI was enhanced by Zn^{2+} but strongly inhibited by Cu^{2+}, whereas the activity of FII was slightly enhanced by Fe^{2+}. Both enzymes were completely inactivated by acetone and Hg^{2+}.

Effect of pH and Temperature on the Chitinase Activities of FI and FII. 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 chitinase FI and FII are shown in **Figure 4**. The optimal pH values for FI and FII were at pH 7 and 6, respectively. The effect of temperature on the activities of chitinases was also studied using colloidal chitin as a substrate. The optimum temperature for both chitinases FI and FII was at 40 °C (**Figure 5**).

Temperature and pH Effect on the Antifungal Activities of FI and FII. The pH stability of the chitinolytic enzymes (FI and FII) before and after purification was determined by the measurement of the residual inhibitory activity at pH 6 after incubation in a 50 mM buffer solution of various pH values (pH 3.0−11.0) at 37 °C for 30 min. The inhibitory effects of the crude and purified enzymes on *F. oxysporum* have been studied, and the results are shown in **Figure 6**. The pH stability of the crude enzyme was at pH 6−8; FI was at pH 6−9 and

Table 3. Enzyme Activities with Various Substrates

enzyme	activity[a] [mean ± SEM (n = 3)] on colloidal chitin	EGC	*M. lysodeikticus* cells	casein	CMC	xylan
chitinase FI	0.32 ± 0.02	0.22 ± 0.02	0.03 ± 0.01	0.05 ± 0.01	0	0
chitinase FII	0.11 ± 0.03	0.11 ± 0.02	0[b]	0.04 ± 0.01	0	0
HEWL (chitinase/lysozyme)	0.018 ± 0.002	2.4 ± 0.1	24.1± 3.8	–[c]	–	–
S. marcescens chitinase	0.276 ± 0.026	0	0	–	–	–

[a] Enzyme activities are expressed as units per milligram except that the lysozyme activity against *M. lysodeikticus* cells is expressed as units per micrograms. [b] No activities were detected. [c] Activities were not measured.

Table 4. Effects of Various Substances on Chitinase Activities of FI and FII[a]

substance	concn	relative activity[b] (%) FI	FII
none	0 mM	100	100
$ZnSO_4 \cdot 7H_2O$	1 mM	127	90
$CuSO_4 \cdot 5H_2O$	1 mM	68	82
$MgSO_4 \cdot 7H_2O$	1 mM	74	70
$FeSO_4 \cdot 7H_2O$	1 mM	75	102
$HgCl_2 \cdot 2H_2O$	1 mM	18	22
$CaCl_2$	1 mM	80	85
K_2SO_4	1 mM	76	86
NH_4NO_3	1 mM	72	87
EDTA	1 mM	90	81
methanol	50%	71	90
ethanol	50%	79	86
acetone	50%	14	20

[a] The reaction mixture of enzyme solution and colloidal chitin suspension was incubated with each of the tested substances in 50 mL of phosphate buffer (pH 6) for 10 min at 37 °C. [b] Activities were assayed under the standard conditions and expressed as a percentage of the activity in the absence of the tested substance.

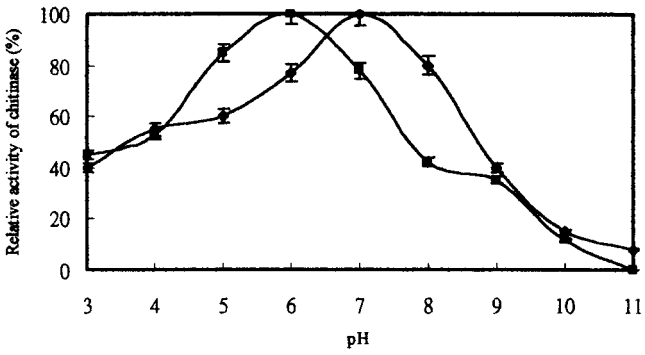

Figure 4. Effects of pH on the enzyme activity of chitinases FI (◆) and FII (■). Chitinase activities were measured at various pH values at 37 °C for 10 min.

FII was at pH 5−9. At such pH, the inhibitory activities were highly retained (>80%). The thermal stabilities of the crude enzymes FI and FII were studied by heating the samples at 100 °C for various time periods, and the residual inhibitory activities on *F. oxysporum* were measured. As shown in **Figure 7**, neither the crude nor the purified enzymes (FI and FII) were thermally stable. They lost >50% of their initial activities only 3 min after heating at 100 °C and were inactivated completely after 9 min of heating. These results are consistent with those shown in **Figure 5**, where it is shown that the chitinase activities of FI and FII were nearly inactivated at high temperatures.

DISCUSSION

In this study we demonstrated that the culture supernatant of *B. amyloliquefaciens* V656, a newly isolated and identified

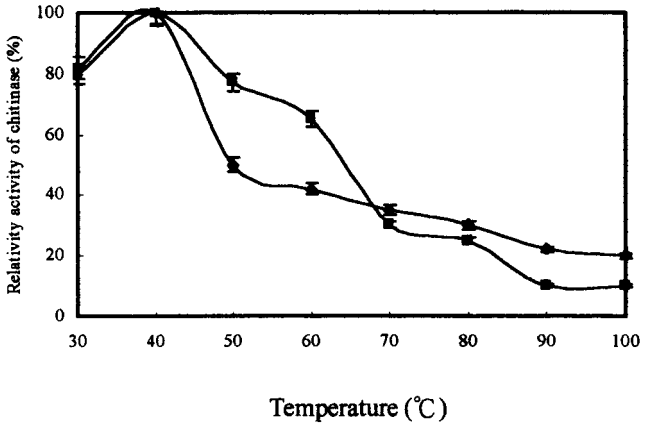

Figure 5. Effects of temperature on the enzyme activity of chitinases FI (◆) and FII (■). Chitinase activities were measured at various temperatures at pH 6.

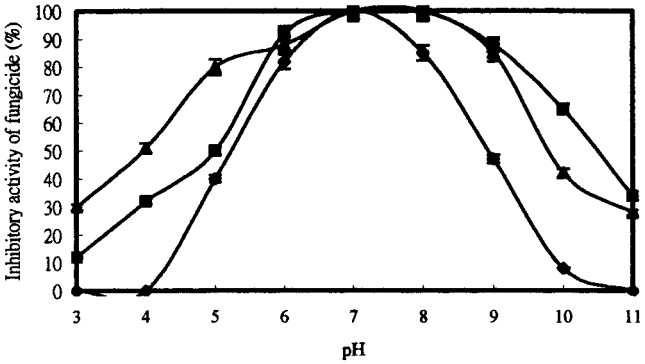

Figure 6. pH effect on the antifungal activity of crude and purified chitinases.

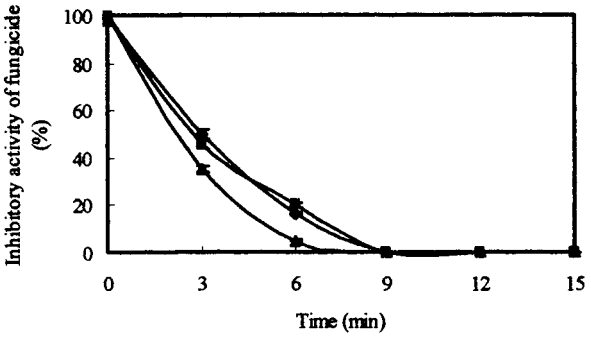

Figure 7. Temperature effect on the antifungal activity of crude and purified chitinases.

bacterium, grown in an SCSP medium displayed inhibitory activity on fungal growth (**Figure 1**). The inhibitory activities of *B. amyloliquefaciens* V656 increased along with the cell growth (as judged by OD_{660}) and reached a maximum (0.4 unit/mL) when the cell growth reached a peak at 24 h of incubation.

Although the inhibitory activities decreased afterward, they were significantly retained (0.27 unit/mL) even at 72 h of incubation. The crude culture supernatant was tested for pH and thermal stabilities, and the results (**Figures 6** and **7**) showed that the inhibitory activity was stable at neutral pH but was reduced significantly below pH 5. The results also showed that the inhibitory activity of crude culture supernatant was unstable at high temperature; it lost >70% of its initial activity after heating at 100 °C for only 3 min and was inactivated completely after heating at 100 °C for 9 min. These phenomena suggested that the inhibitory activities might result from the presence of proteinous enzymes in the culture broth. In fact, two enzymes (FI and FII) were isolated and purified from the culture broth, and both displayed antifungal activity on pathogenic *F. oxysporum*. In addition, both enzymes were shown to be chitinolytic. Comparisons of pH and thermal profiles of purified FI and FII with those of crude supernatant showed that the extents of impairment of inhibitory activities during pH and thermal stability testing were practically the same for the tested materials (**Figure 6** and **7**). The rapid inactivation of the inhibitory activities of FI and FII at high temperature was consistent with the fact that the chitinase activities of FI and FII were nearly inactivated at high temperatures as shown in **Figure 5**. Furthermore, the chitinase activities of FI and FII were coexistent with antifungal activities throughout the whole purification procedure (**Table 2**). These facts reaffirmed that the antifungal activities shown in the culture broth of *B. amyloliquefaciens* V656 were due to the presence of chitinolytic enzymes FI and FII. Therefore, we conclude that *B. amyloliquefaciens* V656, isolated from the soil of northern Taiwan, is capable of releasing antifungal chitinases into the culture broth when it is grown aerobically in a medium containing SCSP. 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 that both enzymes were monomeric.

Numerous microorganisms with antifungal activities have been identified, and many have been effective in field experiments (*46*, *47*). So far, Gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated as biological control agents (*48*−*51*). In contrast, the Gram-positive bacteria such as *Bacillus* spp. were not studied as often for such purposes, although *B. subtilis* is considered to be a safe biological agent (*52*−*55*). *B. subtilis* is an organism known for protease production (*56*); it is, however, rarely used for fungicide production. Previous studies showed that *B. subtilis* NB22 and *B. subtilis* RB14 produced antifungal peptide antibiotic iturin A and surfactin in solid-state fermentation using soybean curd residue (okara) (*57*, *58*). Iturin A is a cyclolipopeptide containing seven residues of α-amino acids and one residue of a β-amino acid; it is a small molecule, yet displays strong antifungal activity. The other lipopeptide, surfactin, in contrast, has weak antibiotic activity. The characteristics of these two compounds clearly distinguished them from the antifungal enzymes found in this study.

The molecular weights of microbial chitinases range from 20000 to 120000 with little consistency. The molecular weights of bacterial chitinases are mostly ∼60000−110000 (*9*, *10*), whereas those of actinomycetes are mostly 30000 or lower (*24*, *59*) and those of fungi are >30000 (*20*, *23*). All plant chitinases are small proteins with molecular weights varying between 25000 and 40000 (*60*). The molecular weights of V656 chitinases are approximately 14000 (FI) and 17000 (FII) by SDS-PAGE and HPLC, which are apparently much lower than those of known chitinases. The molecular weights of chitinases for many other *Bacillus* spp. are known, for example, 36000 for *B. cereus* 6E1 (*61*); 35000, 47000, 58000, and 64000 for *B. cereus* CH (*62*); 71000, 62000, and 53000 for *Bacillus* sp. MH-1 (*63*); 36000 for *B. cereus* 65 (*64*); 68000, 38000, and 52000 for *B. cereus* VKPM B-6838 (*65*); and 74000, 69000, 38000, 38000, 39000, and 52000 for *B. circulans* WL-12 (*66*, *67*). They are apparently much higher than those of FI and FII. The molecular weight of HEWL, which possesses chitinase activity, has a molecular weight of 14400. It was speculated that V656 might have lysozyme activity. However, the study performed to compare the lysozyme and chitinase activities of the V656 chitinases with those of HEWL and *S. marcescens* chitinase revealed that the V656 chitinases lacked lysozyme activity. It is known that chitinases from various sources have bifunctional chitinase/lysozyme activity. Plant and animal sources of chitinase/lysozyme have been frequently reported (*17*, *68*−*72*), whereas bifunctionality of microbial chitinases is rare. Therefore, the lack of lysozyme activity of V656 chitinases is not surprising.

Most of the bacterial chitinases have acidic p*I* values (*41*, *67*, *73*, *74*), and actinomyces chitinases have neutral or alkaline p*I* values (*75*, *76*). Plant chitinases generally have very basic or very acidic isoelectric points (*60*, *71*, *77*, *78*). Chitinases produced by *B. amyloliquefaciens* V656 have acidic p*I* values, which are similar to almost all of the other bacterial chitinases. When colloidal chitin was used as a substrate for measuring chitinase activity, the optimum pH values for FI and FII were nearly neutral or slightly acidic (7 and 6, respectively). These optimum pH values are unusual because other *Bacillus* chitinases usually work better at an acidic or alkaline pH; for example, the optimum pH of *B. cereus* 6E1 is at 5.8 (*61*), that of *Bacillus* sp. BG-11 is at 8.5 (*79*), that of *B. cereus* CH is at 5.0−7.5 (*62*), that of *Bacillus* sp. MH-1 is at 6.5, 5.5, or 5.5 (*63*), and that of *B. thuringiensis* var. *caucasicus* INMI Arm.837 is at 8.0 (*80*). Only the chitinase of *Aermonas hydrophila* subsp. *Anaerogenes* A-52 has a similar optimum pH (pH 7) (*74*). The low thermal stabilities of chitinases produced by *B. amyloliquefaciens* V656 are similar to those observed for chitinases of other origins (*41*, *67*, *81*−*83*).

In summary, the characteristics of V656 chitinases are similar to the known bacterial chitinases in terms of their isoelectric points, thermal instabilities, and lack of lysozyme activity. In contrast to other known bacterial chitinases, the unique characteristics of V656 chitinases include extremely low molecular weights and nearly neutral optimum pH values. Furthermore, this is the first report of the isolation of chitinases from *B. amyloliquefaciens* that are active against fungi.

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Received for review July 5, 2001. Revised manuscript received December 31, 2001. Accepted January 14, 2002. This work was supported in part by a grant from the National Science Council, the Republic of China (NSC89-2313-B-212-003).

JF010885D