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Purification and Characterization of an Antimicrobial Chitinase Extracellularly Produced by *Monascus purpureus* CCRC31499 in a Shrimp and Crab Shell Powder Medium

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Monascus purpureus CCRC31499 produced an antimicrobial chitinase when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine wastes. An extracellular antimicrobial chitinase was purified from the culture supernatant to homology. The chitinase had a molecular weight of ~81000 and a p*I* of 5.4. The optimal pH, optimum temperature, and pH stability of the chitinase were pH 7, 40 °C, and pH 6−8, respectively. The activity of the chitinase was activated by Fe^{2+} and strongly inhibited by Hg^{2+}. The unique characteristics of the purified chitinase include high molecular weight, nearly neutral optimum pH, protease activity, and antimicrobial activity with bacteria and fungal phytopathogens. This is also the first report of isolation of a chitinase from a *Monascus* species.

KEYWORDS: Chitinase; chitin; shrimp and crab shell; *Monascus*; antimicrobial

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*). 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. All organisms that contain chitin also contain chitinases, which are presumably required for morphogenesis of cell walls and exoskeletons (*3*, *4*). Some other organisms that do not contain chitin also produce chitinases to degrade the polymer for nutritional or defensive purpose (*5*, *6*). It is suggested that chitinolytic microorganisms or chitinolytic enzymes have potential applications in the biocontrol of plant pathogenic fungi and insects, as a target for biopesticides, and in many other biotechnological areas (*7*−*10*).

Due to undesirable side effects on the environment and human health, the use of chemical pesticides and food preservatives has been widely criticized in recent years (*11*, *12*). As a consequence, microorganisms have been studied to develop safer alternatives to chemical treatments (*5*). Recently, we have investigated the bioconversion of shrimp and crab shell powder (SCSP) of marine waste for biofungicide production. We have shown that *Pseudomaonas aeruginosa* K-18 is a chitinase-producing strain in an SCSP medium (*13*−*17*). The purification and characterization of two bifunctional chitinase/lysozymes extracellularly produced by this microorganism in SCSP medium have also been described (*15*). We have further demonstrated that *P. aeruginosa* K-187 is an antifungal strain in the SCSP medium, and it exhibits a broad range of antagonism toward fungal phytopathogens. Among fungi, only the chitinases from *Trichoderma harzianum* (*5*, *18*), *Trichoderma* (*Gliocladium*) *virens* (*19*), *Penicillium oxalicum* (*20*), *Piromyces communis* (*21*), *Rhizopus oligosporus* (*22*), and *Verticillium lecanii* (*5*) have been characterized. The fermentation of shellfish chitin wastes by *Monascus* for the production of antifungal chitinases was investigated because *Monascus* is a safe and widely used traditional food microorganisms. In the present work, we further found that *Monascus purpureus* CCRC31499 displayed antibacterial and antifungal activities when cultured in an SCSP medium, possessing strong chitinase activity. The purification and characterization of the antimicrobial chitinase 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 shrimp and crab shells collected from the 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 of <0.053 mm. Ethylene glycol chitin (EGC), lyophilized cells of *Micrococcus lysodeikticus*, powdered chitin, *p*-nitrophenyl *N*-acetylglucosamine, xylan, carboxymethyl cellulose (CMC), and casein were purchased from Sigma Chemical Co., St. Louis, MO. DEAE-Sepharose CL-6B and Sephacryl S-200 were from Pharmacia. Flaked chitin and powdered chitosan from crab shell were purchased from Biotech Co. (Kau-shyuon, Taiwan). Yeast extract, polypeptone, nutrient agar, malt extract agar, and potato dextrose agar were purchased from DIFCO Laboratories, Detroit, MI. Cell suspensions of *M. lysodeikticus* were prepared as described previously (*13*−*15*). Colloidal chitin was prepared from powdered chitin according to the method of Jeniaux (*23*). All other reagents used were of the highest grade available.

The microorganisms used in this study were *Monascus purpureus* CCRC31499, *M. purpureus* CCRC32966, *M. purpureus* CCRC31530, *Monascus ruber* CCRC31535, and *Monascus pilosus* CCRC31527.

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These strains were purchased from the Culture Collection and Research Center (CCRC), Taiwan.

Effect of Culture Conditions. The above five strains of *Monascus* species were maintained on potato dextrose agar plates at 25 °C. In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% yeast extract, 0.1% polypeptone, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $FeSO_4 \cdot 7H_2O$, 0.3% $NaNO_3$, and 0.05% KCl (pH 7) and gradually supplemented with the various carbon sources to be investigated. The major ingredients being investigated included sucrose, SCSP, and chitin. They were added and investigated in one kind at a time fashion. One hundred milliliters of the resultant medium in a 250-mL Erlenmeyer flask was aerobically cultured at 25 °C for 48 h on a rotary shaker (180 rpm). After centrifugation (12000*g*, 4 °C, for 20 min, Beckman J2-21 M/E), the supernatant was collected for measurement of chitinase activity and antifungal activity against *Fusarium oxysporum*. Usually an effective experimental prior condition was used as the basis for the later experiment until the optimal culture composition was obtained. With the use of the optimal culture composition, the effects of the initial pH, temperature, culture volume, and cultivation time on the production of antimicrobial chitinase were investigated in the same fashion until the optimal condition was found. *M. purpureus* CCRC 31499 showed the highest capability in producing antifungal chitinase.

Microorganism and Enzyme Production. *M. purpureus* CCRC31499 was maintained on potato dextrose agar plates at 25 °C. For maximum production of the enzyme, we checked chitinase activity and antifungal activity (using fungal phytopathogen of *F. oxysporum* as target) in the culture supernatant at different stages of growth of *M. purpureus* CCRC31499.

For the production of chitinase, *M. purpureus* CCRC31499 was grown aerobically in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SCSP, 0.1% yeast extract, 0.1% polypeptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.3% $NaNO_3$, and 0.05% KCl (pH 7). Two milliliters of the seed culture (10^5 spores/mL) was transferred into 100 mL of the same medium and grown in an orbital shaking incubator for 4 days at 25 °C. The culture broth was centrifuged for 15 min at 12000*g*, and the supernatant was used for the purification of the enzyme.

pH and Thermal Stability of the Antifungal Compounds. The pH stability of the chitinase was determined by measuring the residual inhibitory activity at pH 7 as described above after the samples had been dialyzed against a 50 mM buffer solution of various pH values (pH 3−11) in seamless cellulose tubing (Sankyo). The buffer systems used were glycine−HCl (50 mM, pH 3), acetate (50 mM, pH 4.5), phosphate (50 mM, pH 6−8), and Na_2CO_3−$NaHCO_3$ (50 mM, pH 9−11). The thermal stability of the chitinase was studied by heating the samples at 100 °C for various time periods. The residual activity was measured as described above.

Purification of the Chitinase. *(i) Production of the Chitinase.* For the production of the chitinase, *M. purpureus* CCRC31499 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SCSP, 0.1% yeast extract, 0.1% polypeptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.3% $NaNO_3$, and 0.05% KCl (pH 7). Two milliliters of the seed culture was transferred into 100 mL of the same medium and grown in a shaking incubator for 4 days at 25 °C and pH 7. The culture broth was centrifuged (4 °C and 12000*g* for 20 min), and the supernatant was used for further purification by chromatography.

(ii) DEAE-Sepharose CL-6B Chromatography. To the cell-free culture broth (1600 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 12000*g*. The precipitate was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7) and dialyzed against the buffer. The resultant dialysate (90 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 × 30 cm) pre-equilibrated with 50 mM sodium phosphate eluting buffer (pH 7). 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 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, 4 °C) for 24 h to remove NaCl and assayed for antifungal and chitinase activities.

(iii) Sephacryl S-200 Chromatography. The resultant dialysate (25 mL) was loaded onto a Sephacryl S-200 gel filtration column (2.5 × 120 cm), which had been equilibrated with 50 mM phosphate buffer (pH 7), and then eluted with the same buffer at a flow rate of 20 mL/h. Fractions (5 mL each) were automatically collected and assayed for antifungal and chitinase activities.

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 7), 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 (*24*) with *N*-acetylglucosamine as a reference compound. The activity of ethylene glycol chitinase was 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 (*15*). One unit of chitinase activity was defined as the amount of the enzyme that produced 1 μmol of reducing sugar per minute.

N-Acetylglucosaminidase activity was assayed using *p*-nitrophenyl *N*-acetylglucosamine (*p*NAG) as a substrate. The reaction mixture contained 0.1 mL of 5 mM *p*NAG in 100 mM sodium phosphate buffer (pH 7). The reaction was stopped by the addition of 2 mL of 1 M Na_2CO_3 after incubation for 10 min at 37 °C, and the absorbance of the *p*-nitrophenyl released was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenyl or reducing sugars per minute.

Lysozyme activity was determined spectrophotometrically by measuring the decrease in optical density at 660 nm. The reaction mixture contained 1.5 mL of a *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 using the same method described above.

For measuring protease activity, a diluted solution of purified enzyme (0.2 mL) was mixed with 2.5 mL of 1% casein in phosphate buffer (pH 7) and 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 (*25*).

Xylanase or cellulase activity was measured with larch wood xylan or CMC as substrate. Enzyme solution (0.1 mL) was added to 0.4 mL of substrate solutions, which contained 0.5% xylan or 1.25% CMC in an acetate buffer solution (125 mM, pH 5). After the mixture had been incubated at 37 °C for 10 min, it was then centrifuged, and the amount of reducing sugars produced in the supernatant was determined by using the dinitrosalicylic acid (DNS) method (*26*). One unit of xylanase or celluase activity was defined as the release of 1 μmol of reducing sugar per minute 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 (*27*). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing 2-mercaptoethanol. The gels (12.5%) 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 isoelectric point of the antifungal enzyme was estimated by chromatofocusing. The enzyme soultion (1 mL) was loaded onto a chromatofocusing PBE 94 column (0.9 × 27 cm) equilibrated with 50 mM Tris-HCl buffer (pH 6), and the elution was done with Polybuffer 74−Tris-HCl (pH 6) as described in the manufacturer's manual (Pharmicia).

Protein Determination. Protein content was determined according to the method of Bradford (*28*) using Bio-Rad protein dye reagent concentrate and bovine serum albumin as the standard.

Antimicrobial Action of the Chitinase. The antifungal activity for the purified chitinase was estimated using a growth inhibition assay

Table 1. Purification of the Chitinase from *M. purpureus* CCRC31499[a]

		chitinase			antifungal		
step	total protein (mg)	total units (A)	specific activity (units/mg)	yield (%)	total units (B)	specific activity (units/mg)	ratio (A/B)
$(NH_4)_2SO_4$ precipitate[a] (80%)	2122	191	0.09	100	546	0.25	0.35
DEAE-Sepharose CL-6B	243	110	0.45	58	92	0.37	1.20
$(NH_4)_2SO_4$ precipitate[a] (80%)	135	80	0.59	42	66	0.48	1.21
Sephacryl S-200	40	41	1.02	21	34	0.83	1.20

[a] *M. purpureus* CCRC31499 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% shrimp and crab shell powder, 0.1% yeast extract, 0.1% polypeptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.3% $NaNO_3$, and 0.05% KCl (pH 7) in an shaking incubator for 4 days at 25 °C.

described earlier (*16*, *17*). Fungal spores were grown on Petri plates filled with potato dextrose agar. After 10 days of incubation at 25 °C, the fungal colonies were removed with sterile water containing 0.1% (v/v) Tween 80. The resulting suspension was filtered aseptically through 0.45-μm pore size membrane filters. 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 antagonistic effect of the chitinase purified from *M. purpureus* CCRC31499, Petri plates were filled with molten PDA precooled to 45 °C and divided into two groups (triplicate for each). To each plate in the experimental group (E) was added an appropriate amount of the chitinase (80 μg). To those of the control group (C) was added an equal amount of sterile buffer. After the plates (10 mL) had cooled, the fungal inoculum was 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 following formula. If the inhibitory ratio was >20%, the test strain would be considered inhibited and the minimal inhibitory concentration (MIC) for that strain was then determined.

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

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.

The test fungi used were *M. purpureus* CCRC31499, *M. purpureus* CCRC32966, *M. purpureus* CCRC31530, *M. ruber* CCRC31535, *M. pilosus* CCRC31527, *F. oxysporum* CCRC35100, and *Fusarium solani* (kindly supplied by Dr. Chaur-Tseuen Lo, Department of Plant Pathology, Taiwan Agricultural Research Institute, Taichung, Taiwan).

The action of the purified chitinase against both Gram-positive and Gram-negative bacteria was examined as described previously (*15*). The chitinase would be used for measurement of the growth of inhibition, and buffer without enzyme was used as a blank for the control experiment. The test bacteria used were *Bacillus cereus* CCRC10603, *Bacillus subtilis* CCRC10255, *Escherichia coli* CCRC 10239, *Pseudomonas aeruginosa* K-187, *Staphylococcus aureus* CCRC10780, and *Streptomyces actuosus* A-151.

RESULTS

Purification of the Antifungal Enzyme. In the presence of SCSP as a major carbon source, *M. purpureus* CCRC31499 released antimicrobial chitinase into the culture fluid. As shown in **Figure 1**, the activities were highest at 4 days. The purification of the antifungal chitinase from the culture supernatant (1600 mL) was described under Materials and Methods. The purification procedures are summarized in **Table 1**. The purification steps were very effective and combined to give an overall purification of 11-fold. The overall activity yield of the purified chitinase was 21%, with specific chitinase activities of 1.02 units/mg. The final amount of this chitinase obtained was 40 mg. The purified enzyme was also confirmed to be homogeneous by SDS-PAGE (**Figure 2**) and chromatofocusing (data not shown).

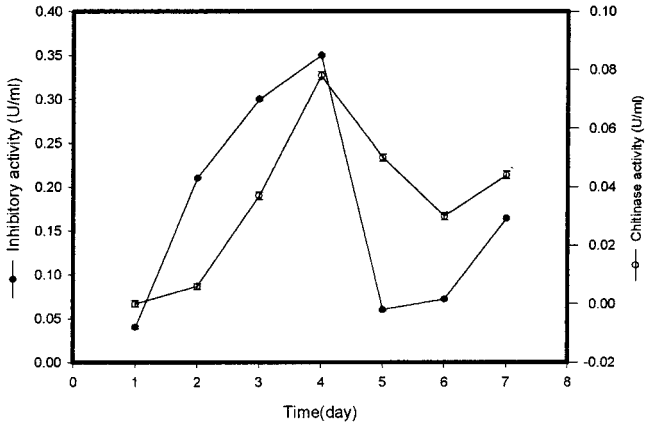

Figure 1. Time courses of chitinase activity (○) and antifungal activity (●) in a culture of *M. purpureus* CCRC31499.

Figure 2. SDS-PAGE of the purified chitinase: (lane S) low molecular weight standards (Pharmacia); (lane I) chitinase.

Molecular Weight and p*I*. The molecular weight of the 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 weight of the chitinase was estimated to be 81 kDa (**Figure 2**). The isoelectric point of the enzyme was found to be pH 5.4 by chromatofocusing.

Enzymatic Activity. The antimicrobial chitinase was assayed with various substrates, that is, colloidal chitin (chitinase activity), *p*-nitrophenyl *N*-acetylglucosamine (*N*-acetylglucosaminidase activity), EGC (ethylene glycol chitinase activity), *M. lysodeikticus* cells (lysozyme activity), CMC (cellulase activity), xylan (xylanase activity), and casein (protease activity). Under the assay conditions with 50 mM phosphate buffer (pH 7), the antifungal chitinase showed higher chitinase specific activity against colloidal chitin (1.02 units/mg) than EGC (0.85

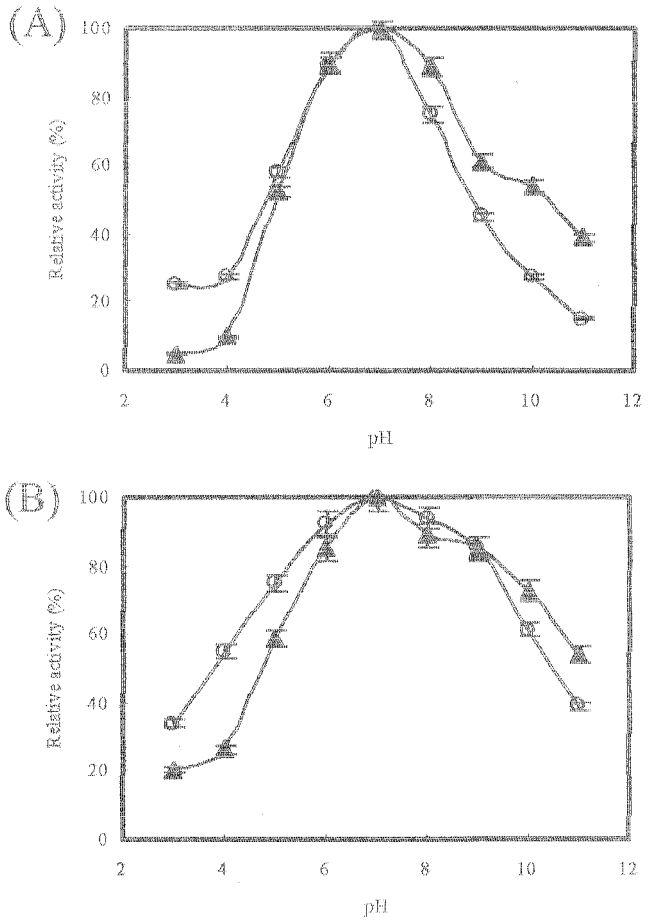

Figure 3. Effects of pH on the activity and stability of chitinase (○) and protease (▲): (A) enzyme activities were measured at various pH values at 37 °C for 10 min; (B) enzyme activities were measured at various pH values at 37 °C for 30 min, and residual activities were assayed at pH 7.

unit/mg). The antifungal enzyme possessed no lysozyme, cellulase, or xylanase activity and little protease activity (0.07 unit/mg).

pH Activity and pH Stability Profile. The effects of pH on the catalytic activities of chitinase and protease were studied under the standard assay conditions. The pH activity profiles of chitinase and protease were bell shaped, with maximum values at pH 7 (**Figure 3A**). The pH stability profiles of the two activities were determined by the measurement of the residual activity at pH 7 after incubation at various pH values at 37 °C for 30 min. As shown in **Figure 3B**, activities of chitinase and protease were stable at pH 6−8. Protease activities showed the same extent of impairment at various pHvalues.

Effect of Temperature on Activity and Stability. The optimum temperature for the chitinase and protease activities was 40 °C (**Figure 4A**). To examine the heat stability of the chitinase and protease activities, the enzyme solution in 50 mM phosphate buffer (pH 7) was heated at 100 °C for various time periods, and then the residual activity was measured. As shown in **Figure 4B**, the enzymes lost >40% of their initial activities after only 2 min of heating at 100 °C and were inactivated completely after 8 min of heating.

Effect of Various Chemicals. The effects of various chemicals on chitinase and protease activities were investigated by preincubating the enzyme with chemicals in 50 mM phosphate buffer (pH 7) for 10 min at 37 °C and then measuring the residual activities of chitinase and protease by using colloidal

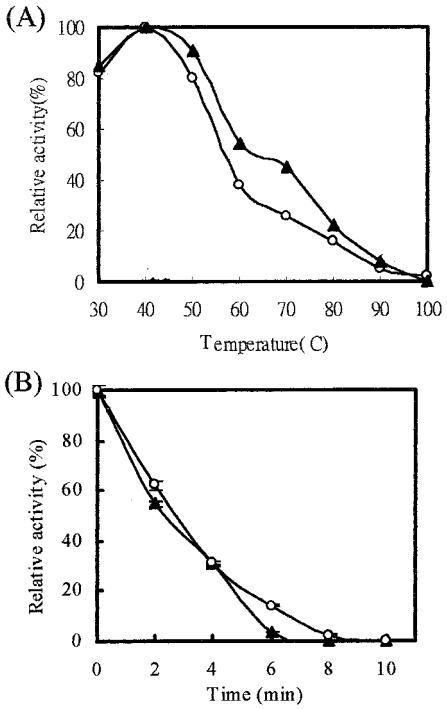

Figure 4. Effects of temperature on the activities of chitinase (○) and protease (▲): (A) enzyme activities were measured at various temperatures at pH 6: (B) enzyme solutions were incubated at pH 7 for various times, and remaining activities were measured at 37 °C.

Table 2. Effects of Various Chemicals on Enzyme Activity

substrate	concn	relative activity (%)[a] chitinase[b]	protease[c]
none	0	100	100
$ZnSO_4 \cdot 7H_2O$	1.0 mM	67	55
$CuSO_4 \cdot 5H_2O$	1.0 mM	80	90
$MgSO_4 \cdot 7H_2O$	1.0 mM	78	51
$FeSO_4 \cdot 7H_2O$	1.0 mM	141	123
$HgCl_2$	1.0 mM	19	17
$CaCl_2 \cdot 2H_2O$	1.0 mM	91	95
NaCl	1.0 mM	98	96
K_2SO_4	1.0 mM	74	67
NH_4NO_3	1.0 mM	96	77
EDTA	1.0 mM	65	51
methanol	50%	57	46
ethanol	50%	51	47
acetone	50%	6	12

[a] Activities were assayed under standard conditions and expressed as a percentage of the activity in the absence of the compound. [b] The reaction mixture of the enzyme solution and colloidal chitin suspension was incubated with each of the compounds in 50 mM phosphate buffer (pH 7) for 10 min at 37 °C. [c] The reaction mixture of enzyme solution and casein was incubated with each of the compounds in 50 mM phosphate buffer (pH 7) for 10 min at 37 °C.

chitin and casein as substrate, respectively. The results are presented in **Table 2**. Only in the case of Fe^{2+} addition was there a slight increase in the activity. The activities of chitinase and protease were significantly inactivated by acetone and Hg^{2+}.

Antimicrobial Action of the Enzyme. The enzyme was evaluated for its antifungal activity by incubating the test fungi with 0.08% enzyme (w/v). Among seven tested strains, the enzyme effectively inhibited two strains. Meanwhile, it was found that the enzyme was the most effective on *F. oxysporum* and *F. solani* with inhibitory ratios of >70% (85 and 70%, respectively) and least effective on *M. pilosus* CCRC31527 and *M. ruber* CCRC31535 (<10%). In contrast, it was not effective

on *M. purpureus* CCRC32966, *M. purpureus* CCRC31530, or the enzyme-producing strain *M. purpureus* CCRC31499 itself.

dThe action of the enzyme against numerous bacteria was also examined. Cells of each test bacteria were suspended on molten nutrient agar medium and then poured into Petri plates. Paper disks were placed on the surface of the medium, and the enzyme solution (80 μg) to be assayed was pipetted into each disk. After 3 days of incubation at 37 °C, the susceptible cells grew uniformly in the medium except for the area where enzyme had diffused into the medium. This was indicated by the formation of clear zones of inhibition; as the enzyme inhibited growth, zones of microbial inhibition were visible. It is considered positive inhibition when the diameter of the inhibited zone is >1 cm. The enzyme inihibited the growth of all of the tested bacteria.

DISCUSSION

M. purpureus CCRC31499 released antimicrobial enzymes into the culture broth when it was grown aerobically in a medium containing SCSP. An antimicrobial enzyme isolated and purified from the culture broth displayed antifungal activity on pathogenic *F. oxysporum* and a broad range antibacterial effects. This antimicrobial enzyme was shown to be chitinolytic and proteolytic. The purity of the enzyme after purification was supported by the results of SDS-PAGE and chromatofocusing performed for the measurement of the molecular weight and isoelectric point. Furthermore, it was confirmed by the results that the chitinase and antifungal activities could not be separated; the ratio of the two activities remained almost constant throughout the whole purification procedure. Numerous microorganisms with antifungal activities have been identified, and many have been effective in field experiments (*29*−*32*). So far, antifungal chitinases of fungi, only *Trichoderma harzianum* (*18*, *30*), *T.* (*Gliocladium*) *virens* (*19*), *Beauveria bassiana* (*33*), and *Verticillium lecanii* (*5*), have been reported; *T. harzianum* and *G. virens* have been intensively investigated as biological control agents.

Beni-koji, a rice koji prepared by fermentation with *Monascus* spp., is a traditional food in Asian countries with beneficial health properties. *Monascus* species are also organisms known for pigment production (*34*−*36*); it is, however, never used for chitinase production. Kono and Himeno (*36*) reported that the beni-koji extract (extracted with ethanol and followed by ethyl acetate extraction) prepared with *M. pilosus* IFO4520 at 30 mg/mL inhibited growth of *Aspergillus sojae* and *B. subtilis*; however, the yeasts tested were fairly resistant. The antimicrobial activity of these compounds was heat stable at 100 °C for 10 min (*36*). Martinkova et al. (*37*) reported that the significant antibiotic activities against *B. subtilis* and *Candida pseudotropicalis* were found with two pigments of rubropunctatin and monascorubrin purified from the mycelium of *M. purpureus*. The characteristics, such as thermal instability and ethanol instability, of the antimicrobial chitinase of *M. purpureus* CCRC31499 clearly distinguished it from the antimicrobial compounds reported in previous studies.

Almost all of the reported chitinase-producing microorganisms used chitin or colloidal chitin as a substrate or an elicitor for chitinase production, and the chitinases thus produced were presumably responsible for their antifungal activities (*10*, *38*−*40*). The use of SCSP as substrate or elicitor for chitinase production is rarely seen, although we have shown that chitinases could be produced by *P. aeruginosa* K-187 in the presence of SCSP in the medium. However, such chitinases displayed no antifungal activity (*15*). Therefore, the finding that *M. purpureus* CCRC31499 was able to produce chitinase with antibacterial and antifungal activities in the presence of SCSP is notable. Why these strains showed higher enzyme production with SCSP than with chitin is not clear. The shell of shrimp and crab is composed mainly of chitin impregnated with proteins, which has been variously modified with lipid and mineral salts. The impregnated mineral salts are primarily calcium carbonate with minor amounts of magnesium, phosphate, silica, and sulfur. SCSP is more effective than chitin (data not shown) in antimicrobial chitinase production by *M. purpureus* CCRC31499 because of the difference in composition of SCSP and chitin.

The molecular weights of microbial chitinases range from 20000 to 120000 with little consistency. The molecular weights of fungal chitinases are mostly ∼41000−45000 (*5*, *18*, *19*), whereas those of bacterial chitinases are mostly ∼60000−110000 (*41*, *42*) and those of actinomycetes are mostly 30000 or lower (*43*, *44*). All plant chitinases are small proteins with molecular weights varying between 25000 and 40000 (*45*). There are only a few extracellularly fungal chitinases that have been purified and analyzed for their molecular weights. For example, 41000 for *T. harzianum* ATCC74058 (*18*), 41000 for *G. virens* ATCC20906 (*19*), 45000 for *V. lecanii* A3 (*5*), 42000 for *P. communis* (*21*), 54000 for *P. oxalicum* (*20*), and 25000 *Aspergillus carneus* (*46*). The molecular weight of *M. purpureus* CCRC31499 chitinase is ∼81000 by SDS-PAGE, which is apparently much higher than those of known fungi chitinases. The p*I* values of reported fungal chitinases were 3.95 for *T. harzianum* ATCC74058 (*18*), 7.8 for *G. virens* ATCC20906 (*19*), 4.9 for *V. lecanii* A3 (*5*), and 4.9 for *P. communis* (*21*). Most of the bacterial chitinases have acidic p*I* values, and *Actinomyces* chitinases have neutral or alkaline p*I* values. Plant chitinases generally have very basic or very acidic isoelectric points (*15*). Chitinase produced by *M. purpureus* CCRC31499 has a p*I* value of 5.4, which is different from those of the other fungal chitinases and similar to those of bacterial chitinases.

The antibacterial chitinases of *P. aeruginosa* K-187, which also use SCSP as a major carbon source, possess lysozyme activity (*15*). It was speculated that *M. purpureus* CCRC31499 might have lysozyme activity. However, the results revealed that *M. purpureus* CCRC31499 chitinase lacked lysozyme activity. Plant and animal sources of chitinase/lysozyme have also been frequently reported (*47*−*52*), whereas bifunctionality of microbial chitinases is rare. Therefore, the lack of lysozyme activity of *M. purpurus* CCRC31499 chitinase is of no surprise. The antimicrobial chitinase of *M. purpurus* CCRC31499 possesses protease activity as has been confirmed by the same extents of impairment of activities during chemical addition, optimum pH, optimum temperature, pH stability, and thermal stability testing.

The neutral optimum pH (pH 7) of the antimicrobial chitinase is unusual for fungal chitinases. All reported fungal chitinases work better at an acidic pH, for example, pH 4 for *T. harzianum* ATCC74058 (*18*), pH 4−6 for *G. virens* ATCC20906 (*19*), pH 4 for *V. lecanii* A3 (*5*), pH 6.2 for *P. communis* (*21*), 5.0 for *P. oxalicum* (*20*), and pH 5.2 for *A. carneus* (*46*).

Roberts and Selitrennikoff (*53*) studied plant and bacterial chitinases for antifungal activity and enzyme specificity. According to their results, plant chitinases isolated from the grains of wheat, barley, and maize functioned as endo-chitinases and inhibited hyphal elogation of test fungi. In contrast, bacterial chitinases from *Serratia marcescens*, *S. griseus*, and *Pseudomonas stutzeri* act as exo-enzymese and had no effect on hyphal extension of test fungi such as *T. reesei*, *T. harzianum*, *G. virens*,

and *Phycomyces blackesleeanus*. We have observed that the antimicrobial chitinase of *M. purpureus* CCRC31499 inhibited spore germination and germ tube elongation of *F. solani* (data not shown). Similar phenomena were seen when *Trichoderma viride* was treated with chitinase purified from pea tissue (*54*) or when pathogenic *Botrytis cinerea* was treated with endochitinase produced by *T. harzianum* (*30*). From these results, it is presumed that CCRC31499 chitinase may act as endochitinase, and the inhibition of hyphal growth of test fungus is presumably attributed to the action of endochitinase. In this aspect, the antifungal activity and enzyme specificity of CCRC31499 chitinase seem to be similar to those of plant chitinases.

Studies on the antibacterial and antifungal effects of chitinase are scant, with most papers addressing lysozyme activities provided by plant chitinases (*52*, *53*). Although there are reports of chitinolytic microorganisms antagonistic to fungal phytopathogens (*19*, *41*, *42*, *53*, *55*−*57*), the chitinases produced by these microorganisms are not known to have antibacterial effects as broad as those of the CCRC31499 chitinase. Considering the fact that CCRC31499 chitinase has no lysozyme activity, it is still not clear why this enzyme displays such a broad range of antibacterial activities. The protease activity of this enzyme is presumed to play an important role in inhibiting bacterial growth. Answers to this question are important in understanding the role of these activities in biocontrol and in determining how best to use this enzyme or the encoding gene in pest management strategies.

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