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Purification and Characterization of a Novel Chitinase from the Entomopathogenic Fungus, *Metarhizium anisopliae*

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A novel chitinase was detected in extracellular culture fluids of the entomopathogenic fungus *Metarhizium anisopliae* (ATCC 20500) grown in liquid medium containing chitin as a sole carbon source. A chitinase was purified to near homogeneity from culture broth of *M. anisopliae* by DEAE-Sephacel, CM-Sepharose CL-6B ion-exchange chromatography, and gel filtration with Superose 12HR. The molecular mass of the enzyme determined by SDS–polyacrylamide gel electrophoresis was approximately 60 kDa and the optimum pH of the enzyme was 5.0. This molecular mass is different from values of 33, 43.5, and 45 kDa for endochitinases and 110 kDa for an exochitinase (N-acetylglucosaminidase) from *M. anisopliae* ME-1 published previously. In addition, N-terminal sequences of 60-kDa chitinase are different from those of 43.4- and 45-kDa endochitinases. The purified enzyme showed high chitinolytic activity against colloidal, crystalline chitin of crab shells as well as against *p*-nitrophenyl-β-D-*N*-acetylglucosamide, *p*-nitrophenyl-β-D-*N*, *N*′-diacetylchitobiose, and *p*-nitrophenyl-*N*, *N*′-*N*″-triacetylchitotriose, indicating that this enzyme has both endo- and exochitinase activity.

***Key Words: Metarhizium anisopliae;* entomopathogenic fungus; enzyme purification; chitinase; chitinolytic activity; chitobiase; biological control.**

INTRODUCTION

Insect cuticles are composite structural materials with mechanical properties that are optimal for their biological functions. The cuticle consists of a thin outer epicuticle containing lipids and proteins and a thick procuticle consisting mainly of chitin and proteins (Andersen *et al.*, 1995; Samson *et al.*, 1988). Entomopathogenic fungi enter their hosts by direct penetration of the cuticle, which is a barrier against most microbes. Consequently, fungal pathogens have a potential as a biological means of controlling sap-sucking insects that have not been easily controlled with chemical pesticides.

Highly pathogenic strains showed detectable amounts of extracellular chitinase, lipase, and protease activities (Samuels *et al.*, 1989). These enzymes are utilized in cutical penetration.

The complexity of chitinases isolated from *M. anisopliae* ME1 has been investigated. St. Leger *et al.* (1993) reported that the fungus produces multiple extracellular chitinase isozymes. Also, two distinct chitinases of *M. anisopliae* were characterized and have been shown to be regulated and induced by chitin degradation product (St. Leger *et al.*, 1986, 1991). In addition, extracellular acidic chitinases have been localized on the cuticle surfaces of hosts during fungal invasion (St. Leger *et al.*, 1996b).

Although the chitinolytic activity has been considered to be important for pathogenicity and has been demonstrated in various entomopathogenic fungi, the enzymes involved in pathogenicity have been poorly characterized using crude chitinase preparation, except for 33-kDa endochitinase, 43.5-kDa endochitinase, 45-kDa endochitinase, and 110-kDa exochitinase of *M. anisopliae* ME1 (St. Leger *et al.*, 1991, 1996b) and 45-kDa endochitinase of *Beauveria bassiana* (Havukkala, 1993). In this paper, we report the purification and some characteristics of chitinase that appear to be distinct from fungal isozymes purified so far.

MATERIALS AND METHODS

Strain and Culture Conditions

Chitinase was purified from *M. anisopliae* (ATCC 20500) which was claimed by the supplier to have a high entomopathogenic activity. Preliminary testing to confirm production of chitinases was performed on agar medium containing colloidal chitin as the sole carbon source.

The strain was maintained on Saboraud dextrose agar (SDA, Difco) plates. Spore stocks were prepared by harvesting spores grown on plates of SDA containing

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1% (w/v) yeast extract and the number of spores were counted using a hematocytometer. A culture medium for liquid cultures contained basal salts (0.05% $MgSO_4$ and 0.5% NaH_2PO_4) supplemented with 2% (w/v) colloidal chitin. *M. anisopliae* was cultivated with an inoculum of 1.0×10^8 conidia in 1 L of culture medium and grown at 28°C for 4 days to the late-logarithmic growth stage.

Preparation of Colloidal Chitin

The colloidal chitin was obtained by a modification of the published method (Hsu and Lockwood, 1975). Chitin (20 g) from crab shell (Sigma Co., practical grade) was dissolved in 200 ml of concentrated HCl with stirring for 3 min at 40°C. The chitin was precipitated as a colloidal suspension by slowly adding water (2 L) adjusted to 5°C. Colloidal suspensions were collected by filtering through coarse filter paper; then the filtered colloidal suspension was washed with tap water until the pH of the suspension was about 4.0.

Enzyme Assay

Chitinase activity was assayed by the method of Yanai *et al.* (1992). The reaction mixture containing 250 µl of 0.5% colloidal chitin, 250 µl of 0.2 M sodium acetate buffer (pH 4), and 500 µl of enzyme solution was incubated for 2 h at 37°C. After centrifugation, 500 µl from the supernatant fluid was mixed with 100 µl of 0.8 M boric acid, and the pH of this mixed solution was adjusted to 10.2 with KOH. The solution was heated for 3 min in boiling water. After the mixture was cooled, 3 ml of *p*-dimethyl aminobenzaldehyde (DMAB)[2] solution (1 g of DMAB dissolved in 100 ml of glacial acetic acid containing 1% [v/v] hydrochloric acid) was added and the mixture was incubated for 20 min at 37°C. Absorbance at 585 nm was measured against water as a blank.

One unit of chitinase activity was defined as the amount of enzyme which produced sugars equivalent to 1 µmol of *N*-acetylglucosamine per min under the above condition. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

Purification of Chitinase

Purification of chitinases from fungi was generally carried out using an affinity chromatography of prepared colloidal chitins (Kuranda and Robbins, 1991; Yanai *et al.*, 1992). However, we performed ion-exchange chromatography of protein extracts first because chitinases were washed out during the affinity chromatography process.

Step 1. Extraction. Fungal cultures were centrifuged at 3000*g* for 15 min and the supernatant was filtered through Whatman filter paper No. 2, No. 5, and No. 44, and then through a 0.22-µm-pore-size filter (Millipore). Ammonium sulfate was added to the filtrate to achieve 85% saturation (608 g/L). After the mixture was allowed to stand overnight, the precipitate was collected by centrifugation (15,000*g*, 15 min) and dissolved in 40 ml of 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA. The solution was dialyzed against three changes of the same buffer at 4°C for 15 h and then centrifuged at 15,000*g* for 15 min. The precipitates were discarded.

Step 2. DEAE–Sephacel chromatography. The supernatant of step 1 was applied to a DEAE–Sephacel (Pharmacia) column (2.5×15 cm) which had been equilibrated with the Tris–HCl buffer of step 1. After being washed with the same buffer to three volumes of column, the column was eluted with 300 ml of linear salt gradient from 0 to 0.5 M NaCl in the same buffer at the flow rate of 100 ml/h. The enzyme activity appeared in both flow-through fractions and fractions that were eluted with 0.2 M NaCl (Fig. 1). Because of higher chitinase activity, the flow-through fractions were concentrated to 20 ml with an Amicon filtration apparatus (MW cut off, 10,000) and dialyzed against the same buffer.

Step 3. CM–Sepharose CL-6B chromatography. The dialyzed sample of step 2 was applied to a CM–Sepharose CL-6B (Pharmacia) column (1.5×15 cm) preequilibrated with the same buffer. After being washed with the same buffer to three volumes of column, the column was eluted with 150 ml of linear salt gradient from 0 to 0.5 M NaCl in the same buffer at the flow rate of 60 ml/h. Most activity eluted at 80–150 mM NaCl and the corresponding fractions were pooled. The pooled fractions were concentrated to 2 ml in a Centriprep-10 (Amicon).

Step 4. Gel filtration by FPLC. The concentrated samples were applied to Superose 12HR 10/20 (Pharmacia) preequilibrated with the same buffer. No more than 0.5 ml of sample was loaded for each run. Elution was continued at the flow rate of 24 ml/h. The fractions showing chitinase activity were collected for the subsequent analyses.

SDS–Polyacrylamide Gel Electrophoresis

SDS–PAGE was carried out according to the published method (Laemmli, 1970) on a 12% polyacrylamide gel and protein bands were stained with Coomassie brilliant blue dye.

[2] Abbreviations used: YEPD, yeast-extract + peptone + dextrose; PMSF, phenylmethylsulfonyl fluoride; DMAB, *p*-dimethyl aminobenzaldehyde.

TABLE 1

Effects of Carbon and Nitrogen Source on the Production of Chitinase by *M. anisopliae*

Media	Cell dry weight (mg/10 ml)	Chitinase activity[a] (mU/ml)
2% Colloidal chitin + basal salt medium[b]	42	8.66
1% Yeast extract + 1% peptone + 2% dextrose	98	1.25
2% Colloidal chitin + 2% dextrose	20	0.16
2% Colloidal chitin + 1% yeast extract	73	0.55
2% Colloidal chitin + 1% peptone + 2% dextrose	89	2.05
2% Colloidal chitin + 1% yeast extract + 2% dextrose	67	0.42
2% Colloidal chitin + 1% yeast extract + 1% peptone + 2% dextrose	87	3.24

[a] Chitinase activity was measured with the supernatant fraction of cultures.

[b] Basal salt medium contains 0.5% $MgSO_4 \cdot 7H_2O$ and 0.5% Na_2HPO_4.

RESULTS

Culture Medium for the Production of Chitinases

We have grown *M. anisopliae* in several types of media and then quantified chitinase activities (Table 1). Markedly higher chitinolytic activity was detected in the culture fluid when *M. anisopliae* were grown in a medium containing colloidal chitin as a sole carbon source compared to that grown in YEPD medium containing 2% yeast extract, 1% peptone, and 2% dextrose. The highest activities were shown at a late stage of growth (Kang *et al.*, in preparation).

Purification of Chitinases

DEAE–Sephacel column chromatography of the protein extracts showed two peaks of chitinase activity, which were eluted at flow-through and at 0.2 M NaCl (Fig. 1). The minor peak contains 33-kDa chitinase, which was similar in the substrate specificity and molecular weight to the endochitinase reported by St. Leger *et al.* (1991) (data not shown). Therefore, fractions of the main peak were concentrated and subjected to CM–Sepharose CL-6B chromatography. The activity was detected as a single peak (Fig. 2). Fractions of the single peak were pooled and subjected to FPLC gel filtration on a Superose 12HR column. The activity peak was coincided with the protein peak (Fig. 3). The results of each purification step for the chitinase are summarized in Table 2.

The preparation of chitinase was almost homogeneous, judged by 12% SDS-polyacrylamide gel electrophoresis, and the molecular mass was estimated as approximately 60 kDa (Fig. 4). Comparison with the result of gel filtration suggested that this enzyme was monomeric.

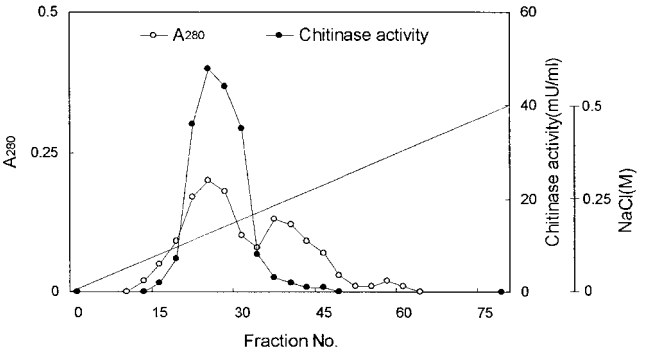

FIG. 2. Elution profile of chitinase activity in a CM-Sepharose CL-6B column. The front activity fraction (concentrated to 1.14 mg) from a DEAE–Sephacel column was dissolved in 20 ml and applied to the column (1.5 × 15 cm). Elution was performed with a 0–0.5 M linear gradient of NaCl for 150 ml. Flow rate was 60 ml/h. Activity fractions corresponding to 80–150 mM NaCl were pooled for the next purification.

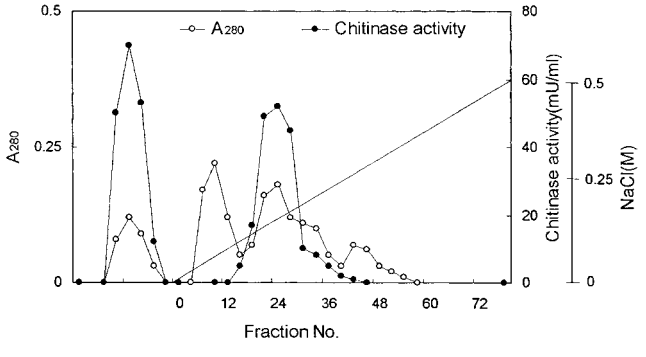

FIG. 1. Elution profile of chitinase activity in a column of DEAE–Sephacel. A sample (5.15 mg) of the concentrated and dialyzed extract from *M. anisopliae* was applied to the column (2.5 × 15 cm). Elution was performed with a 0–0.5 M linear gradient of NaCl for 300 ml. Flow rate was 100 ml/h. Activity fractions of the front peak were pooled for the next purification.

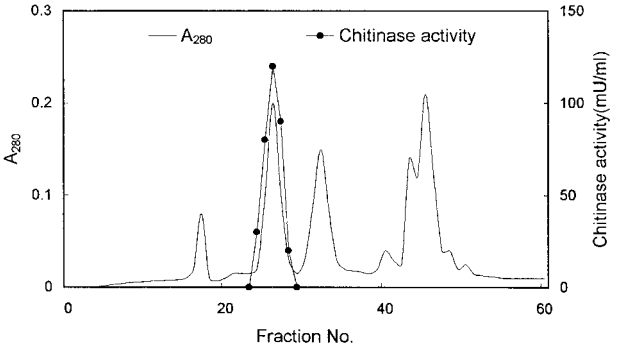

FIG. 3. Separation of chitinase by FPLC gel filtration. The activity fraction from the CM–Sepharose CL-6B column was concentrated to 2 ml. An aliquot (90 μg) of the concentrate was injected into a Superose 12HR column. Flow rate was 24 ml/h. Fraction size was 1 ml. Activity fractions were pooled for the subsequent analyses.

TABLE 2
Purification of Chitinase Isolated from *M. anisopliae*

Purification step	Protein (mg)	Total mU	Specific activity (mU/mg)	Yield (%)	Purification Fold
1. Culture fluid	18.07	8,659	463		
2. Ammonium sulfate precipitation	5.15	5,272	1,024	60.7	2.21
3. DEAE–Sephacel	1.14	2,081	1,825	24.0	3.94
4. CM–Sephacel CL-6B	0.35	807	2,307	9.3	4.98
5. FPLC Superose 12HR gel filtration	0.11	564	5,127	6.5	11.03

Effects of pH and Specificity for Substrates

The effect of pH of the purified enzyme on the chitinolytic activity against the colloidal chitin was examined. The purified chitinase exhibited a pH optimum of 5.0 and remained fully active from 4.0 to 6.0 (Fig. 5).

The endochitinase and exochitinase activities of the purified enzyme were measured by a release of *N*-acetylglucosamine from colloidal chitin and crystalline chitin, and the release of *p*-nitrophenol from several chemicals, *p*-nitrophenyl-β-D-*N*-acetylglucosamide (pNP-NAG1), *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose (pNP-NAG2), and *p*-nitrophenyl-*N*, *N*′,*N*″-triacetylchitotriose (pNP-NAG3), as reported by Yanai *et al.* (1992) and Coudron *et al.* (1984). The purified enzyme degraded colloidal chitin and crystalline chitin as well as pNP-NAG, pNP-NAG2, and pNP-NAG3, indicating that the purified enzyme contains endo- and exochitinase activity (Table 3).

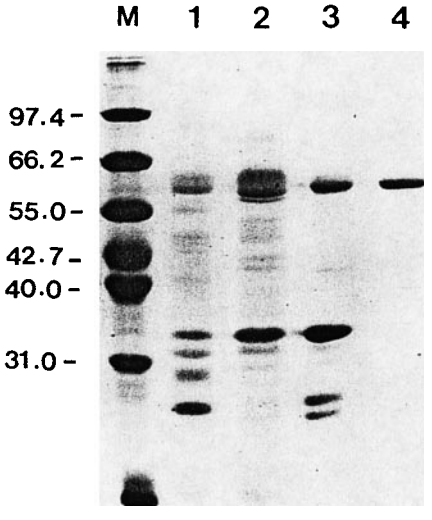

FIG. 4. SDS–PAGE of activity fractions from each purification step. Lane 1, ammonium sulfate precipitation; lane 2, DEAE–Sephacel column chromatography; lane 3, CM–Sepharose CL-6B column chromatography; lane 4, Superose 12HR gel filtration by FPLC; lane M, molecular weight standards from top to bottom, phosphorylase B (97,640), bovine serum albumin (66,200), glutamate dehydrogenase (55,000), ovalbumin (42,700), aldolase (40,000), carbonic anhydrase (31,000).

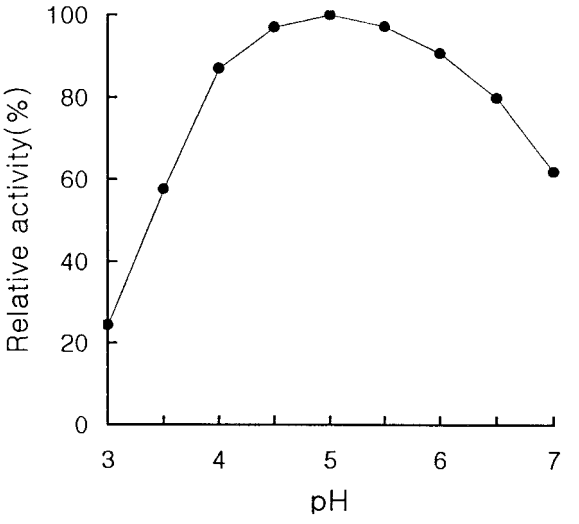

FIG. 5. Effects of pH on chitinase activity. Chitinase activity was measured in citrate–phosphate buffer at pH values ranging from 3.0 to 7.0 as described under Materials and Methods. Each point is the mean of three replicates.

DISCUSSION

The entomopathological activity of fungi against insects seems to be derived from several cuticle-dissolving enzymes, chitinases, and proteases. In the case of *M. anisopliae*, protease plays a major role in the early stage of invasion during penetration (St. Leger *et al.*, 1987, 1991) and chitinases were induced during degradation of encasing cuticle proteins (St. Leger *et al.*, 1986a,b,c). In addition, an overexpression of the protease gene (*pr*1) in *M. anisopliae* increased toxicity against *Manduca sexta* (St. Leger *et al.*, 1996a) and several proteases have been expressed highly during fungal penetration of the host, *Maduca sexta* (St. Leger

TABLE 3
Enzyme Action of the Purified Chitinase from *M. anisopliae* on Chitin and Chitin Oligosaccharides

Substrate	Endochitinase activity[a] (NAG nmol $\mu g^{-1}min^{-1}$)	Exochitinase activity[b] (pNP nmol $\mu g^{-1}min^{-1}$)
Colloidal chitin	5.14	
Crystalline chitin	1.32	
pNP-β-*N*-acetylglucosamide		3.63
pNP-β-*N*,*N*′-diacetylchitobiose		7.80
pNP-β-*N*,*N*′-*N*″-triacetylchitotriose		4.53

[a] Enzyme activity was assayed by the method of Yanai *et al.* (1992).
[b] Enzyme activity was assayed by the method of Coudron *et al.* (1984).

et al., 1998). Therefore chitinases have been regarded as having a minor role in pathogenesis, especially in comparison to protease in *M. anisopliae.* However chitinases have been associated with virulence in several fungi, including *M. anisopliae* (St. Leger *et al.*, 1991), *Beauveria bassiana* (Havukkala *et al.*, 1993), and *Nomuraea rileyi* (El-Sayed *et al.*, 1989). Also, several fungi, including *M. anisopliae, M. flavoviride, B. bassiana,* and *Aspergilus flavus,* have been reported to produce multiple extracellular chitinase isozymes (St. Leger *et al.*, 1993, 1996b).

We purified a chitinase from *M. anisopliae* using chromatography. This purified chitinase is probably new since the molecular mass (60 kDa) is different from the previously published values of 33, 43.5, and 45 kDa for endochitinases and the value of 110 kDa for an exochitinase (*N*-acetylglucosaminidase) from *M. anisopliae* ME-1 (St. Leger *et al.*, 1991, 1996b). In addition, N-terminal sequences of 60-kDa chitinase (NTG-SWNGVNVE, unpublished) are different from those (AGGYVNAVYFYTNGLYLSNYQPA) of 43.4- and 45-kDa endochitinases (St. Leger *et al.*, 1996b). N-terminal sequences of 43.5- and 45-kDa endochitinase of *M. anisopliae* ME-1 are similar to the deduced amino acid sequences of chitinase mRNA (GenBank Accession No: AF027497) of *M. anisopliae* E6 with minor differences (82.6% sequence identity). Chitinase similar in size to that of the 60-kDa chitinase has been detected in the chitinase cDNA clone of *M. anisopliae;* this clone contains 522 deduced amino acids and the calculated molecular mass could be 58 kDa (Kang *et al.*, 1998). In addition, the purified chitinase contains both endo- and exochitinase activity based on the degradation of chitin oligomers and several chemical compounds (Table 3).

In a traditional distinction between endo- and exochitinases, chitin oligomers could be used as a substrate and their degradation products could be examined (Hara *et al.*, 1989). Also a chitin polymer substrate such as colloidal chitin can be used for the identification of endochitinase activity (Havuakkala *et al.*, 1993). The purified enzyme exhibited twofold higher chitinolytic activity against the substrate pNP-NAG2 than the substrates pNP-NAG1 and pNP-NAG3 (Table 2), indicating that this enzyme has a characteristic of chitobiase (St. Leger *et al.*, 1991). Pegg and Young (1982) showed that pure chitinase from *Verticillium albo-utrum* released *N*-acetylglucosamine as either a reaction product or a major end product along with di- and trisaccharides.

Our chitinase was produced at late growth phase in liquid culture medium containing colloidal chitin as the sole carbon source. The chitinolytic activity of the culture fluid in chitin medium was 20-fold higher than in complete media (YEPD) (data not shown). Thus the chitinase seems to be induced and regulated by degradation products of chitin like 33-kDa endochitinase and 110-kDa exochitinase of *M. anisopliae* (St. Leger *et al.*, 1986). In addition, the pH optimum for the chitinase activity was 5.0, which is similar to those of 33-kDa endochitinase (pH 5.3), 43.5-kDa endochitinase (pH 4.8), 45-kDa endochitinase (pH 4.8), and 110-kDa exochitinase (pH 5.0) of *M. anisopliae* (St. Leger *et al.*, 1986, 1996b). Therefore the purified chitinase may be one of 10 chitinase isozymes of *M. anisopliae* (St. Leger *et al.*, 1993, 1997).

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