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# Purification and partial characterization of an elastinolytic proteinase from *Aspergillus flavus* culture filtrates

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**Abstract** A 23-kDa protein with elastinolytic activity was purified from *Aspergillus flavus* (NRRL 18543) culture filtrates by gel-filtration chromatography. Severe inhibition of the elastinolytic activity by 1,10-phenanthrolene (5 mM) and EDTA (0.8 mM) indicated that the protein belongs to the metallo class of proteases. The isoelectric point was 9.0. Natural substrates susceptible to cleavage by this protease, in addition to elastin, included cottonseed storage protein, collagen, ovalbumin and bovine serum albumin. The 23-kDa protein was thermostable to 70°C and retained its elastinolytic activity in concentrated form at 4°C for 6 months. Elastinolytic activity was initially secreted into the culture medium as a 35-kDa protein, which was subsequently converted to a 23-kDa protein, presumably through autolysis. This putative proteolytic degradation product appears to be identical to the 23-kDa protein recovered from the gel-filtration column. The 23-kDa protease may confer selective advantage to the fungus in the extracellular environment because of its temperature and pH stability and wide range of potential natural protein substrates.

## Introduction

The aflatoxin-producing fungus *Aspergillus flavus* is a widely distributed saprophyte that, under special circumstances such as weakened developing seed tissue or immuno-compromised mammalian lung tissue, is capable of opportunistic pathogenesis in plants and animals. The proteinase class of hydrolytic enzymes is but one of several that the fungus can produce to utilize a wide variety of natural substrates (Cotty et al. 1990). One proteinase produced by *A. flavus* degrades the structural protein elastin, found in connective tissues of mammals and insects. The role of elastinolytic proteinases in the utilization of different natural substrates by *A. flavus* has not been well defined.

*A. flavus* has been implicated in invasive aspergillosis in humans (Bodey and Vartivarian 1989), and the production of elastinolytic proteinases (elastase) has been suggested to be a primary determinant of fungal virulence to animals (Kothary et al. 1984). A survey of members of *Aspergillus* section *Flavi* demonstrated elastase activity to be highly conserved within this fungal group (Mellon and Cotty 1995a). Only 2 of 176 *A. flavus* group isolates tested did not possess elastinolytic activity; only one isoform of elastase was observed in filtrates of isolates that secreted activity in liquid culture (Mellon and Cotty 1995a). Conservation of elastinolytic activity within *Aspergillus* section *Flavi* may indicate that elastinolytic proteinases are important components in the *A. flavus* molecular arsenal, allowing the fungus to tap a wide range of biologically derived substrates. Elastinolytic proteinases from *A. flavus* have been purified and characterized, and, in one case, the gene coding for the enzyme has been cloned (Ramesh et al. 1994; Rhodes et al. 1990).

In order to understand better the potential roles and uses of elastinolytic proteinases in *A. flavus*, a purification and characterization of an elastinolytic proteinase activity from a field isolate was undertaken. The results of this investigation are presented in this report. A preliminary report has been given (Mellon and Cotty 1995b).

## Materials and methods

### Biological materials

*Aspergillus flavus* NRRL 18543 was isolated from cottonseed collected in Southern Arizona and maintained on a 5% V-8 medium at

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30°C (Cotty 1989). This strain was selected for these studies because it does not produce aflatoxins and because it has potential commercial use as a biological control agent (Cotty 1994). Culture medium was seeded (200 µl/70 ml) with a conidial suspension containing $10^7$–$10^8$ spores/ml.

### Elastase production and activity assays

Elastase was produced in liquid shake cultures (150 rpm, 31°C) using a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered medium containing elastin as the major carbon source (Mellon and Cotty 1995a). Culture filtrates (5–6 days growth) were used for enzyme purification. Elastase activity was assayed by a previously described radial diffusion technique (Mellon and Cotty 1995a).

The influence of known protease inhibitors on elastase activity was evaluated by incubating appropriate concentrations with test solutions for 1 h prior to assaying. Phenylmethylsulfonyl fluoride (PMSF) was purchased from CalBiochem (San Diego, Calif.); elastin and 1,10-phenanthrolene were obtained from Sigma Chemical Company (St. Louis, Mo.). The following protease inhibitors were obtained in a set from Boehringer Mannheim Biochemica (Mannheim, Germany): antipain dihydrochloride, aprotinin, bestatin, chymostatin, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64), $Na_2EDTA$, leupeptin, pefabloc SC (AEBSF), pepstatin, phosphoramidon. These inhibitors were dissolved as specified by the manufacturer. The inhibitor concentrations tested were selected to fall within the effective concentration range of a given inhibitor for its target protease activity. Appropriate solvent controls were utilized in all inhibitor studies.

To obtain elastase activity from fungal mycelial tissue, freshly grown mycelia were separated from the medium by filtration, washed with deionized water, and ground into fine powder using liquid nitrogen in a mortar and pestle. The powder was resuspended in cold (4°C) 0.05 M HEPES buffer, pH 7.4 and centrifuged 10 000 *g* for 15 min to remove insoluble material. The supernatant was concentrated five-fold with a Centricon unit (10 kDa molecular mass cut-off, Amicon) for use in elastase activity assays and for denaturing polyacrylamide gel electrophoresis (PAGE) analysis.

A radial diffusion system for assaying proteolytic activity against collagen and cottonseed storage protein was developed. Cottonseed storage protein was obtained from defatted cottonseed flour (a gift from E. J. Conkerton, S.R.R.C.) according to the method of Marshall and Conkerton (1991). The protein preparation (in 10% NaCl) was exhaustively dialyzed against deionized water and lyophilized to dryness. Collagen (type I) was purchased from Sigma Chemical Company. The assay medium was constructed in a similar manner to the elastase assay medium, with the substitution of collagen or cottonseed storage protein for elastin and without rose Bengal. Activity zones were visualized after a 24-h incubation at 37°C by flooding the medium with 0.1% (w/v) Coomassie blue R-250 in 10% acetic acid/40% (v/v) methanol for 30–60 min, followed by destaining in the same solvent. Ovalbumin was substituted for elastin in the standard radial diffusion assay (with rose Bengal) to ascertain activity against this glycoprotein.

A filter assay was developed to test proteolysis activity of the 23-kDa protease against bovine serum albumin. A protease sample to be tested was mixed with 0.02 M TRIS-HCl, pH 7.8 to a volume of 2.0 ml and centrifuged at 5000 *g* for 45 min at 25°C in a Centricon unit (Amicon; molecular mass cut-off, 10 kDa). This procedure was repeated following the addition of 2.0 ml TRIS buffer to the retentate. Bovine serum albumin substrate was then added to the enzyme preparation (retentate) in TRIS buffer, and the mixture was incubated at 37°C for 1 h; the reaction mixture was centrifuged again at 5000 *g*. The peptide content of the resultant filtrate was determined by the method of Waddell (Wolf 1983), which was used to estimate quantitatively polypeptide moieties ($< 10$ kDa) that had been hydrolyzed from the parent substrate (66 kDa).

In the temperature-stability study, aliquots of 23-kDa protease (20 µg/ml) were held at various temperatures for 15 min and then evaluated for elastase activity. In the pH optimum study, a series of 0.05 M buffers, including citrate, HEPES, *N*-2-hydroxyethyl piperazine-*N*′-3-propanesulfonic acid (HEPPS), borate, and 3-(cyclohexylamino)propanesulfonic acid (CAPS), was used to cover a pH range of 5.0–11.0. The 23-kDa protease (final concentration, 20 µg/ml) was added to a test buffer at a given pH and incubated at 37°C in an elastin assay plate containing the same test buffer at the same pH (rose-Bengal-derivatized elastin/2% agar in test buffer) before measurement of activity zones by the elastase assay procedure.

### Protein assays

Protein concentrations in culture filtrates were estimated by a Coomassie G-250 binding procedure (Bradford 1976), as well as by the method of Waddell (Wolf 1983). The two procedures were in good agreement (within 5%). Samples subjected to the Waddell procedure had to be placed in a TRIS buffer (0.02 M, pH 7.8), since the HEPES buffer (0.05 M, pH 7.4) interfered with the assay. Major advantages of this second method include its nondestructive characteristics and the nature of the chromophore under analysis. The method specifically measures the level of peptide linkages, not the frequency of chemically distinct amino acid side-groups (e.g. aromatic groups).

### Elastase purification

The BioGel P-60 (BioRad Labs, Richmond, Calif.) gel filtration medium was equilibrated with 0.05 M HEPES buffer, pH 7.4, packed to form a $1.5 \times 95$-cm column (bed volume = 168 ml; void volume = 59 ml), and thoroughly washed with the HEPES buffer. Freshly prepared culture filtrate, 280 ml, was concentrated 110-fold in Centriprep units (10 kDa molecular mass cut-off, Amicon). The entire elastase sample was applied to the column (20°C) and eluted with HEPES buffer. After 50 ml column eluate had passed through, 50 2-ml fractions were collected. The column fractions were monitored for protein (absorbance at 280 nm) and elastase activity (radial diffusion assay). The column fractions were diluted 20-fold with the HEPES buffer before addition to the elastase assay plates or denaturing PAGE analysis.

### Electrophoresis

Denaturing PAGE was performed according to the method of Laemmli (1970), except that 0.05 M dithiothreitol was substituted for 2-mercaptoethanol in the sample buffer. Culture filtrates were used without concentration and were diluted 1:1 (v/v) with sample buffer (sodium dodecyl sulfate, SDS) prior to the heat treatment. The protein bands were visualized using a silver stain procedure (Blum et al. 1987). Native isoelectric focusing gel analysis with elastin overlayer visualization was performed according to a previously published procedure (Mellon and Cotty 1995a).

## Results

Passage of the crude elastase sample (culture filtrate concentrate) through the BioGel P-60 column resulted in the resolution of two peaks of elastase activity (Fig. 1). The first activity peak was considerably smaller than the second. Denaturing (reduced) PAGE analysis of the major peak fractions revealed a homogeneous

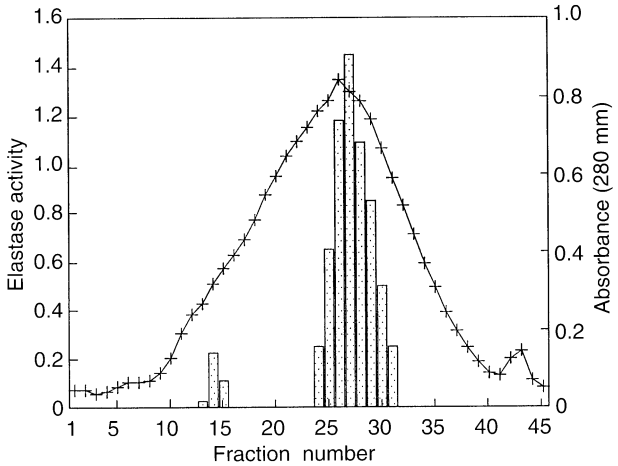


**Fig. 1** Elution profile of *Aspergillus flavus* NRRL 18543 elastase on a BioGel P-60 column. Culture filtrate was concentrated 110-fold, applied to the column and eluted with 0.05 M HEPES buffer, pH 7.4. Fractions were monitored for protein ($A_{280nm}$; +) and elastase activity (radial diffusion assay; bars); 1 unit elastase activity = 1 $cm^2$ digestion zone

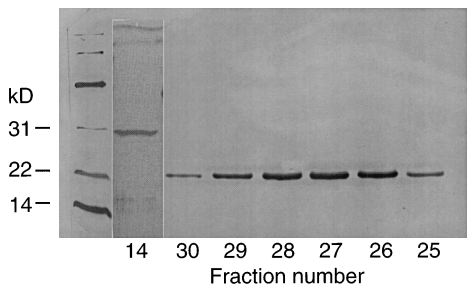


**Fig. 2** Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis of P-60 column fractions. Fraction numbers are shown at the bottom of the figure

preparation of a 23-kDa protein (Fig. 2). The minor activity peak corresponded to a 30-kDa protein (Fig. 2). Molecular mass estimates derived from gel-filtration elution volumes differed greatly from the SDS-PAGE molecular mass estimates. Gel filtration estimates were 23 kDa for the minor peak and 6.3 kDa for the major peak. All of the elastase activity applied to the P-60 column was recovered in the two activity peaks, within the precision limits of the radial diffusion assay. Since there was much less of the material with minor activity available, the investigation focused on the 23-kDa protein. This protein revealed no inhibition by 2 mM PMSF and 60% inhibition by 5 mM 1,10-phenanthrolene (Table 1). Isoelectric focusing analysis coupled with an elastin overlay of the purified protease (23 kDa) demonstrated a single band of activity with a p*I* of 9.0.

Since the protease literature contains examples of activities that readily undergo autolysis, an experiment was designed to detect autolysis in this system. Actively growing cultures were sampled daily; potential autolysis was prevented by immediately adding the sample to SDS sample buffer, followed by the standard heat treatment. When denaturing PAGE analysis was performed with these samples, the presence of 35-kDa and 30-kDa bands in sample aliquots from day 3 through day 8 was evident (Fig. 3). However, the 23-kDa band was missing entirely (Fig. 3). This result suggested that the parent molecule secreted into the culture medium was the 35-kDa protein, which was subsequently proteolytically converted to forms of smaller molecular mass, namely 30-kDa and 23-kDa proteins (by SDS-PAGE estimations).

Indeed, additional correlative evidence suggested that the protease was secreted as a 35-kDa protein which was subsequently converted to a 23-kDa protein. The length of time that culture filtrate preparations were stored (5°C) was correlated to the presence of the 23-kDa protein; the longer the storage, the less 35-kDa

**Table 1** Effect of protease inhibitors on the 23-kDa protease activity. Purified enzyme was diluted 20-fold with HEPES buffer and pre-incubated with test inhibitors for 1 h before addition to standard elastase assay plates. *E-64*, *N*-[*N*-[L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, *EDTA*, ethylenediaminetetraacetic acid, *PMSF*, phenylmethylsulfonylfluoride

| Inhibitor | Concentration | Inhibition (%) |
|---|---|---|
| Antipain | 0.15 mM | 0 |
| Aprotinin | 0.77 μM | 21 |
| Bestatin | 0.13 mM | 27 |
| Chymostatin | 0.17 mM | 27 |
| E-64 | 0.084 mM | 27 |
| $Na_2$ EDTA | 0.75 mM | 80 |
| Leupeptin | 0.01 mM | 27 |
| Pefabloc SC[a] | 0.5 mg/ml | 51 |
| Pepstatin | 7.3 μM | 27 |
| 1,10-Phenanthrolene | 1 mM | 36 |
| | 5 mM | 60 |
| | 8 mM | 100 |
| PMSF | 2 mM | 0 |
| Phosphoramidon | 0.26 mM | 40 |

[a] Molecular mass unknown

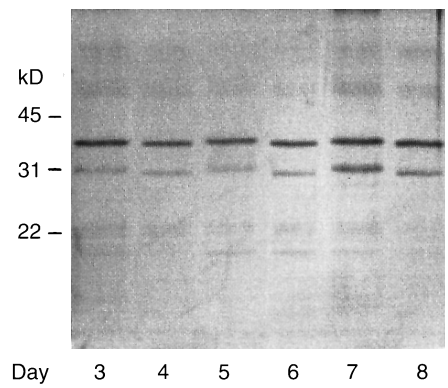


**Fig. 3** Time of production study for *A. flavus* NRRL 18543 elastase activity. Protein profiles were obtained by SDS-PAGE. Culture filtrate samples were not concentrated

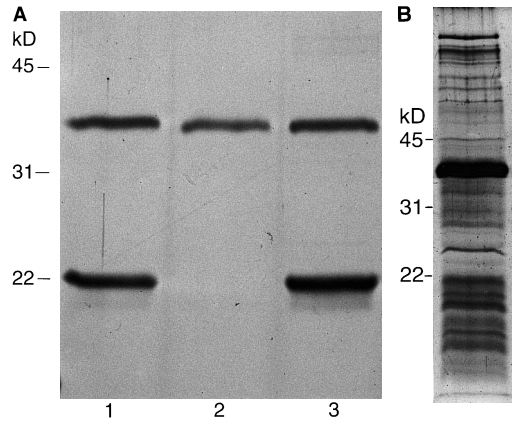


**Fig. 4** **A** Effect of proteinase inhibitors on the protein composition of *A. flavus* NRRL 18543 culture filtrates. Protein profiles were obtained by SDS-PAGE analysis. Culture filtrate samples were incubated at 37°C for 10 days. Lanes: *1* no inhibitor; *2* 1,10-phenanthrolene; *3* phenylmethylsulfonyl fluoride. **B** Protein composition of *A. flavus* NRRL 18543 mycelium-derived extracts. Protein profile obtained by SDS-PAGE analysis. Details of the protein extraction from mycelia are given in Materials and methods

**Table 2** Proteolytic activity of the 23 kDa protease against protein substrates. Radial diffusion wells received 50 µl solution containing 20 µg/ml (0.8 µM) 23-kDa protease in 0.05 M HEPES buffer, pH 7.4; details of radial diffusion assays are given in Materials and methods (Mellon and Cotty 1995a). The 23-kDa protease activity against bavine serumalbumin was measured by means of a filter/centrifugation procedure given in Materials and methods. For each substrate, the range of response linearity to increasing enzyme concentrations was established. The 23-kDa protease molar concentrations were 0.174–2.61 µM. *ND* not determined

| Protein substrate | Activity ($cm^2$) | Linear range (µg/ml) |
|---|---|---|
| Elastin | 1.73 | 4–60 |
| Collagen | 0.85 | 15–60 |
| Cottonseed storage | 4.24 | 7–45 |
| Ovalbumin | 2.86 | 7–60 |
| Bovine serum albumin | 5.0[a] | ND |

[a] Protease activity against bovine serum albumin is expressed as µg polypeptide released (molecular mass < 10 kDa) $h^{-1}$ µg $protease^{-1}$ at 37°C under substrate-saturation conditions. Protein concentrations were estimated by the Waddell method (Wolf 1983)

protein was present. Increasing the temperature to 37°C increased the conversion rate of 35-kDa protease to the 23-kDa form. In addition, concentration of culture filtrate preparations resulted in a partial conversion of the 35-kDa protein to the 23-kDa protein. This latter form retained catalytic activity against elastin for at least 6 months at 4°C.

An inhibitor study was designed to test the autolysis hypothesis. Freshly grown culture filtrate (8-day) samples were incubated with 1,10-phenanthrolene (5 mM) or PMSF (2 mM) at 37°C for 10 days, and then subjected to denaturing PAGE analysis. Samples with PMSF or no inhibitor (control) contained both 35-kDa and 23 kDa proteins, whereas the 1,10-phenanthrolene-containing samples had a single 35-kD band (Fig. 4A). An inhibitor study with 8-day culture filtrate (fresh) demonstrated 90% inhibition in the presence of 5 mM 1,10-phenanthrolene. Further evidence to support the above hypothesis was obtained by extracting freshly prepared fungal mycelia. Grinding this mycelial tissue in liquid nitrogen, followed by re-solubilization in the HEPES buffer resulted in preparations with some elastase activity. Analysis of these preparations with SDS-PAGE revealed a major band at 35 kDa and a very minor band at 23 kDa (Fig. 4B).

An inhibitor profile study that looked at the effects of a wide range of protease inhibitors on the purified 23-kDa protein was conducted to help characterize the activity. At the levels tested, only $Na_2$ EDTA demonstrated an inhibitory capacity similar to that of 1,10-phenanthrolene (Table 1).

The 23-kDa protease was able to utilize other proteins, besides elastin, as substrates. It readily hydrolyzed, in addition to elastin, collagen, cottonseed storage protein, ovalbumin and bovine serum albumin (Table 2).

The 23-kDa protease withstood high temperature conditions, remaining fully active to 70°C (no activity at 80°C). In addition, the 23-kDa elastase displayed a pH optimum of 7.5 (pH range, 6.5–8.0; data not shown). A pH stability study showed that the 23-kDa protease could be maintained in an alkaline pH environment (pH 8.0–11.0) for at least 1 h and still retain elastinolytic activity when returned to a pH 7.5 environment. Although the *A. flavus* protease was not active in this pH range, the inexpression of activity in alkaline conditions was reversible. Thus, the 23-kDa protease appears to be stable to alkaline pH environments, at least on a temporary basis.

## Discussion

*A. flavus* NRRL 18543 secreted a 35-kDa protease in liquid fermentation. This protease is apparently subsequently autolysed to a more stable 23-kDa form. Conversion by autolysis is suggested by (1) observation of the 35-kDa protein, in the absence of the 23-kDa protein, in samples derived from actively growing cultures (Fig. 3), (2) correlation between both temperature-dependent and concentration-dependent loss of the 35-kDa protein and generation of the 23-kDa protein, and (3) generation of the 23-kDa protease being prevented by exposure to 1,10-phenanthrolene (Fig. 4A). Indeed, high-temperature treatment (37°C) of freshly prepared elastase samples in combination with inhibitor exposure delineated a clear relationship between the 35-kDa and 23-kDa proteins. Samples incubated with either no inhibitor or the ineffectual PMSF showed the presence of major protein bands at 23 kDa and 35-kDa, whereas samples with 1,10-phenanthro-

lene revealed only the 35-kDa protein (Fig. 4A). High concentrations of a 35-kDa protein in preparations derived from mycelia further support the hypothesis of an initially secreted 35-kDa protease that is subsequently converted to the 23-kDa protease. The 23-kDa protein is apparently stable to further proteolytic cleavage and sustains no loss of activity after storage for 6 months (4°C).

The straightforward purification of the 23-kDa protein by means of gel filtration chromatography was no doubt aided by the high purity of the initial (crude) culture filtrate preparations. Such high initial purity may indicate that *A. flavus* NRRL 18543 is of potential value in commercial enzyme production. The minor activity peak (30 kDa) obtained from the BioGel P-60 column was presumably a small residual pool of an intermediate form of the degraded 35-kDa protease. Anomalous behavior of fungal proteases with respect to molecular mass estimations obtained by gel filtration has been observed in other systems (Kolattukudy et al. 1993; Frosco et al. 1992).

The purified 23-kDa protease would appear to be a member of the metalloprotease family because of severe inhibition by the chelation agents EDTA and 1,10-phenanthrolene. This protease does not appear to be related to the serine protease of *A. flavus* reported by Ramesh et al. (1994). Instead, it appears to be very similar to (possibly the same as) the protease activity reported by Rhodes et al. (1990). The only real discrepancy between this protease and the enzyme of Rhodes et al. seems to be the p*I* values: 9.0 for the former and 7.6 for the latter. Since Rhodes et al. did not report taking precautions against autolytic catalysis, their purified 23-kDa protein presumably represents a similar degradation product of a parent enzyme.

The high degree of conservation of this protease activity among members of *Aspergillus* section *Flavi* (Mellon and Cotty 1995a) suggests an important role for this enzyme in the fungal arsenal of hydrolytic proteins. Other properties of this protein may impart significant advantages to this group of fungi in their competition for substrate resources. The 23-kDa protease is thermostable, resistant to proteolytic autodegradation, and capable of utilizing a wide range of protein substrates, including both hydrophobic proteins (elastin, cottonseed storage protein, collagen) and soluble proteins (ovalbumin, bovine serum albumin). These enzymatic properties could prove invaluable in the stress-inducing environmental conditions (hot, dry) in which these fungi are competing with other organisms for the available resources. These same characteristics may make the 23-kDa protein useful in certain industrial settings. An alkaline protease derived from another strain of *A. flavus* has been tested as a depilation agent in the tanning industry (Malathi and Chakraborty 1991). It is interesting to note that target seed tissues of *A. flavus* in susceptible crops (cotton, corn and peanuts) all contain hydrophobic storage proteins. Perhaps the 23-kDa protease is an important molecular tool allowing the fungus to tap this carbon and nitrogen resource. As such, this enzyme may play a decisive role in the process through which these crops are contaminated with aflatoxins by toxigenic *A. flavus* strains.

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