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# Purification and Properties of the Elastase from *Aspergillus fumigatus*†

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**Elastase, a potential virulence factor from the opportunistic pathogen *Aspergillus fumigatus*, was purified 220-fold from culture broth by fast-performance liquid chromatography employing anion exchange (Q Sepharose fast flow), cation exchange (S Sepharose fast flow), and gel filtration (Superose 12). Purified to near homogeneity, the elastase had an apparent molecular mass of 32 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (silver stain) but a mass of about 19.1 kDa as determined by gel filtration on Superdex 75. The elastase is not glycosylated and is positively charged at neutral pH, having a pI of 8.75. Inhibition by 0.2 mM phenylmethylsulfonyl fluoride (100%) and 0.21 mM leupeptin (60%) implies that the elastase is a serine protease. However, the enzyme is also inhibited by 5 mM EDTA (100%) and 10 mM 1,10-orthophenanthroline (30%), suggesting a requirement for divalent cations. The enzyme acts optimally at pH 7.4 and 45°C in 50 mM sodium borate buffer, but in Tris HCl, the pH optimum shifts to 8.8.**

While strains of *Aspergillus* species can cause allergic reactions upon inhalation of high spore concentrations, proliferation of the fungus is generally not a problem in individuals with normal immune status. Invasive aspergillosis occurs in individuals predisposed to infection through diseases such as acute leukemia and diabetes mellitus and in patients undergoing cancer chemotherapy or immunosuppressive therapy following organ transplants. Several *Aspergillus* species, including *Aspergillus flavus*, *A. niger*, *A. glaucus*, and *A. terreus*, can cause invasive aspergillosis, but *A. fumigatus* is the predominant causative organism (2).

Elastases have been implicated in the pathogenesis of bacteria, fungi, and schistosomes (9, 13, 14, 19). In 1984, we showed that environmental strains of *A. fumigatus* were capable of producing an extracellular elastase (8). Production of this enzyme was correlated with the ability to cause invasive aspergillosis in immunocompromised mice. In 1988, Rhodes et al. (21) examined 38 clinical specimens and reported that all isolates of *Aspergillus* causing invasive aspergillosis produced elastase. The elastase from an isolate of *A. flavus* was purified and characterized (20). However, there is no direct evidence in these reports that the fungal elastase is a virulence factor actually required for invasion of *Aspergillus* spp. into lung tissue. By determining whether murine antibodies to this enzyme can ameliorate invasive aspergillosis in immunocompromised mice, our research aims to determine if the elastase plays a direct role in pathogenesis. The present report deals with the purification and characterization of the *A. fumigatus* elastase used in preparation of monoclonal and polyclonal antibodies (6) for this study.

## MATERIALS AND METHODS

**Screening method for elastase production.** The strains of *A. fumigatus* described in 1984 (8), which were isolated from air samples collected near a sewage sludge-composting facility in Camden, N.J., were rescreened for elastase production on agar medium. These cultures had been maintained on malt extract agar (Difco Laboratories, Detroit, Mich.) and stored under mineral oil at 4°C. Fresh slants (malt extract agar) of the cultures were prepared (4 days, 37°C), and spores were plated on agar medium containing 0.2% elastin (no. E-60; Elastin Products Co., Owensville, Mo.) and 0.01% yeast extract suspended in 0.5% Triton X-100–1.5% agar. After 5 to 7 days at 37°C, the agar surrounding fungal colonies was observed for clearing zones, which indicate elastin degradation. The diameters of the clearing zones surrounding isolated colonies were used to estimate elastase production by the different strains.

**Production of elastase.** Spores of *A. fumigatus* 18 were collected aseptically from a malt extract agar slant by washing the surface with 0.1% Tween 80. Mycelia and other debris were removed by filtration of the spore suspension through sterile glass wool, and the concentration of the filtrate was adjusted to $3 \times 10^7$ spores per ml. For elastase production, 250 ml of liquid medium (1.17% yeast carbon base [Difco], 0.2% elastin, and 0.3% $CaCO_3$) (7) in 2.8-liter Fernbach flasks was inoculated with 3.3 ml of the spore suspension. The cultures were incubated at 37°C with shaking (150 rpm) until the elastase concentration was ~0.3 U/ml (72 to 96 h). The culture broth was filtered through cheese cloth, double Whatman 114 filters, a 1.2-μm-pore-size membrane filter, and finally through a 0.45-μm-pore-size membrane filter. This cell-free broth was used as the source of crude enzyme in purification experiments.

**Measurement of elastase activity.** A modified colorimetric assay (22) employing elastin-Congo red (Elastin Products) as the substrate was used to quantify the elastase. In brief, 250 μl each of 50 mM sodium borate (pH 8.5), elastin-Congo red (20 mg/ml) in 50 mM sodium borate (pH 8.5), and test sample were mixed (by Vortex mixer) in a 1.7-ml microcentrifuge tube. After 30 min at 37°C, insoluble material in the assay mixture was removed by centrifugation, and the $A_{495}$ of released dye in the supernatant was measured spectrophotometrically. Standard curves were constructed by measuring the absorbance of dilutions of elastin-Congo red which had been completely solubilized with porcine pancreatic

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elastase. One unit of elastase is that amount of enzyme catalyzing the release of soluble dye equivalent to 1 mg of elastin-Congo red in 30 min at 37°C. Purified *A. fumigatus* elastase, tested at pHs 7.5 and 8.5, released 40 times more dye in 30 min than did an equimolar amount of purified (thrice crystallized) trypsin (Worthington Biochemical Corp., Freehold, N.J.). In some experiments, elastin-Remazol (Elastin Products) was used as the substrate, and the $A_{595}$ was measured.

The increased concentration of elastin-Congo red (6.67 versus 1.0 mg/ml in the earlier method [8]) increased the sensitivity of the assay, allowing measurement of activity in 30 min rather than 3 h. Absorbance values in the linear range (0.02 to 0.12) were used in calculating elastase activity.

Activity of elastase on casein was determined by using the method of Kunitz as described by Laskowski (11). Casein was suspended in 50 mM sodium borate buffer, pH 7.5, and heated for 20 min in a boiling water bath to dissolve the casein. The temperature of the casein was adjusted to 37°C for use in the assay. Prior to determining whether phenylmethylsulfonyl fluoride (PMSF) or EDTA inhibited caseinolytic activity of the purified elastase, the enzyme was mixed with the inhibitor and held for 45 min at 37°C.

**Elastase purification.** Crude elastase (1,200 ml) was concentrated and equilibrated against 50 mM diethanolamine HCl, pH 8.8, by ultrafiltration (YM10 filter; Amicon, Danvers, Mass.). The concentrated elastase (20 ml) was subjected to anion- and cation-exchange and gel filtration chromatographies at room temperature on a fast-performance liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Piscataway, N.J.) with procedures performed as described by the manufacturer. Columns and matrices, also from Pharmacia, are described in the figure legends. Between chromatography steps, ultrafiltration with a YM10 membrane was used to concentrate pooled samples and equilibrate the concentrates against [2-(*N*-morpholino) ethane-sulfonic acid]-NaOH, (MES-NaOH; Fisher Scientific, Springfield, N.J.) at either 50 mM (pH 5.5) or 75 mM (pH 5.8). The sample used for gel filtration was further concentrated with Centriprep 10 and Centricon 10 concentrators (both from Amicon).

**Protein determination.** Protein was measured as described by Lowry et al. (12), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard, or by the protein dye binding assay, with the Bio-Rad reagent (Bio-Rad, Rockville Centre, N.Y.) and procedures performed as outlined by the manufacturer. Protein concentration of fractions from column chromatography was estimated by measuring the $A_{280}$ of the sample.

**Monoclonal antibody production.** The purified elastase was used as the immunogen for preparation of murine monoclonal antibodies as described in the accompanying paper (6). Monoclonal antibody KD5 (approximately 100 μg/ml) from cell-free culture supernatant was diluted 1:3 with Tris-buffered saline (TBS; pH 8.0) for use as a reagent in Western blots (immunoblots).

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (10). Briefly, protein samples were boiled in sample buffer (3% SDS, 5% 2-mercaptoethanol, and 10% glycerol in 25 mM Tris HCl; pH 6.8). Proteins were electrophoresed at 4°C (30 mA through stacking gel and 75 mA through resolving gel) in a 1.5-mm, 12.5% polyacrylamide slab gel (Hoefer SE 600 apparatus) with a reservoir buffer consisting of 25 mM Tris HCl (pH 8.3), 192 mM glycine, and 0.1% SDS. Resolved proteins were fixed in the gel with a 10% methanol–10% acetic acid solution and subsequently silver stained (17) for visualization. Estimation of molecular mass was based on masses of commercially prepared standards (Bio-Rad). Proteins in duplicate gels were stained for carbohydrate or used for Western blot analysis.

**Carbohydrate detection.** Proteins in SDS-PAGE gels were stained with periodic acid-Schiff reagent to detect glycosylation by following the procedure described by Fairbanks et al. (4).

**Nondenaturing PAGE.** Nondenaturing PAGE was performed as described by Thomas and Hodes (27) except that 0.045% (wt/vol) ammonium persulfate was used to polymerize the stacking gel and 0.0005% (wt/vol) riboflavin was used to polymerize the resolving gel. In brief, proteins were dissolved in 65 mM MOPS-KOH [3-(*N*-morpholino)propanesulfonic acid; Fisher Scientific]-buffered glycerol solution, pH 8.0, and electrophoresed at 30 mA for 5 h at 4°C in a 7.5% polyacrylamide slab gel (Hoefer SE 600 apparatus), with 22 mM MOPS, pH 6.8, containing 100 mM histidine as reservoir buffer. Resolved proteins were fixed in gels and silver stained (17). Duplicate gels were used for Western blot analysis.

**Western blot analysis.** Proteins resolved by SDS-PAGE were transferred electrophoretically (Hoefer TE 42 apparatus) (28) onto nitrocellulose paper (BA 83; Schleicher & Schuell, Keene, N.H.) in cooled 25 mM Tris buffer, pH 8.3, containing 192 mM glycine, 0.05% SDS, and 10% methanol. Proteins resolved by nondenaturing PAGE were transferred in the same manner except that the transfer buffer consisted of 4 mM MOPS, pH 6.8, containing 20 mM histidine (diluted electrode buffer), and the polarities of the electrodes were reversed. Subsequent Western blot steps were performed at 37°C. Between steps, the nitrocellulose sheets were washed (three times, 10 min each time, with shaking) in TBS (50 mM Tris, 200 mM NaCl; pH 8.0) containing 0.05% Tween 20 at room temperature. To block residual binding sites, the nitrocellulose was soaked for 1 h with shaking in 3% bovine serum albumin in TBS. The sheets were immersed in 18 to 24 ml of the monoclonal antibody reagent and maintained for 3 h at 37°C or overnight at 4°C on a rocking plate. To visualize the monoclonal antibody bound to elastase, sheets were soaked in 60 to 90 ml of alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (0.25 μg/ml) for 1 h, washed, and then immersed in 60 to 90 ml of phosphatase substrate (4-bromo-3-chloro indolyl phosphate [16.7 μg/ml] and Nitro Tetrazolium Blue [33.3 μg/ml] in 100 mM Tris [pH 9.0]) until color developed (all three reagents were from Boehringer Mannheim, Indianapolis, Ind.). The reaction was stopped by flooding the nitrocellulose sheets with distilled water.

**Isoelectric focusing.** Isoelectric focusing was performed in gels (backed with GelBond film) which contained 1% agarose-IEF (Fisher), 11% sorbitol, and premixed ampholytes, pH 3 to 10 (Sigma), 0.07% (vol/vol), in Milli-Q (Millipore, Bedford, Mass.) grade water. Wicks soaked in anolyte (50 mM $H_2SO_4$) or catholyte (1.0 M NaOH) were placed on appropriate ends of the gel. Standards (Sigma) and samples (20 to 30 μl) were applied to the gel near the anode, and proteins were focused in the gel on a flat-bed electrophoresis apparatus (FBE 3000; Pharmacia). Several tubes, each containing 1.0 M NaOH, were placed inside the apparatus, the unit was sealed shut, and the chamber of the sealed unit was flushed with nitrogen to prevent $CO_2$ absorption by the gel. The initial voltage was set at 100 V and increased every 30 min (200, 400, 600, 800, and 1,000 V) (total run, 3 h). Proteins were fixed by soaking the gel in 5% sulfosalicylic acid in 10% trichloroacetic acid (30 min) and then in methanol (30 min).

Gels were dried and visualized by staining with Coomassie brilliant blue R250. Duplicate gels were used for zymograms.

**Zymograms for protease and elastase detection.** Zymogram gels containing 1% agar and 0.5% soluble elastin (Elastin Products) in 50 mM Tris HCl, pH 7, or 1% casein in 37.5 mM Tris HCl, pH 7.5, were cast on GelBond film (Pharmacia) as described by Westergard et al. (29). The backed gels were laid onto the surface of PAGE or isoelectric focusing gels and marked to facilitate comparison with stained protein bands. Gels were kept at 37°C for 2 to 6 h, and residual substrate protein in the zymogram gel was fixed with 5% sulfosalicylic acid in 10% trichloroacetic acid. The dried gel was stained with Coomassie brilliant blue R250. The elastin or casein in the zymogram stained blue, while the zone of proteolytic activity destained completely (clear zone against a dark blue background), indicating areas of casein or elastin hydrolysis.

**Analytical reagents.** PMSF, *N*-ethylmaleimide, 1,10-orthophenanthroline, iodoacetic acid, tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK), tosylsulfonyl lysyl chloromethyl ketone (TLCK), leupeptin, and pepstatin were purchased from Sigma. Dithiothreitol and EDTA were purchased from Fisher Scientific.

## RESULTS

**Screening isolates for elastase production.** Since the isolates of *A. fumigatus* described earlier (8) had been stored for some years on slants under oil, we retested them for elastase production to choose a strain for enzyme production. Inclusion of 0.05% Triton X-100 in an elastin-yeast extract agar medium yielded well-separated colonies surrounded by obvious zones of clearing of elastin in the medium (data not shown). Inclusion of either 0.5% glucose or 50 mM Tris HCl, pH 7.6, inhibited production of clearing zones; therefore, these constituents were not included in plates for observing elastase production. However, glucose was found to be necessary for elastase production in broth. Strains 2, 8, 10, and 31, which did not grow on the elastin-Rose Bengal agar employed earlier, grew and produced clearing zones within 7 days on elastin–Triton X-100 agar, as did 23 other strains earlier reported as not producing elastase in elastin broth.

Table 1 shows elastase production on solid and liquid media by strains 2, 8, 10, and 31 and by four strains (18, 35, 44, and 58) earlier reported as high elastase producers. While elastase production in liquid medium does not correlate well with diameter of the clearing zone in solid medium, it does correlate well with previous results in liquid medium (8), although the newer medium was slightly different (buffered with $CaCO_3$ rather than sodium borate and containing 1.17% rather than 0.2% yeast carbon base); evidently, the level of elastase production in liquid medium is a stable characteristic of strains of *A. fumigatus*. Isolate 18 was chosen for production of elastase for further study.

**Purification of elastase.** Concentration of the crude elastase by ultifiltration removed approximately 90% of the protein present in the broth, presumably low-molecular-mass degradation products of elastin in the medium. Removal of this competing substrate presumably also explains the apparent doubling of activity recovered.

The concentrated elastase was chromatographed on Q Sepharose Fast Flow (Fig. 1), doubling the specific activity but with only a 45% overall recovery. However, when the eluate was concentrated to 20 ml and equilibrated with 50 mM MES-NaOH, pH 5.5, the apparent activity increased by one-third.

TABLE 1. Elastase production by selected isolates of *A. fumigatus*

| Isolate no. | Elastase activity: Previous results[a]: Solid medium[b] | Previous results[a]: Liquid medium[c] (U/ml) | Present results: Diam (cm) on solid medium[d]: Colony | Diam (cm) on solid medium[d]: Clearing zone | Present results: Liquid medium[e] (U/ml) |
|---|---|---|---|---|---|
| 2 | – | 0.023 | 1.65 | 2.08 | 0.20 |
| 8 | – | 0.010 | 1.55 | 1.55 | 0.02 |
| 10 | – | 0.003 | 1.75 | 1.75 | 0.07 |
| 31 | – | 0.013 | 1.40 | 1.65 | 0.00 |
| 18 | + | 0.48 | 1.35 | 1.40 | 0.35 |
| 35 | + | 0.40 | 1.35 | 1.40 | 0.30 |
| 44 | + | 0.38 | 1.35 | 1.40 | 0.26 |
| 58 | + | 0.45 | 1.25 | 1.35 | 0.40 |

[a] Data are from reference 8.

[b] +, growth and clearing of elastin after 7 days at 37°C on 1.5% agar containing 0.05% elastin, 0.05% yeast carbon base, 0.01% Rose Bengal, and 1.5% agar in 50 mM sodium borate, pH 7.6; –, no growth under these conditions.

[c] Elastase production after 72 h at 37°C in liquid medium containing 0.2% elastin, 0.2% yeast carbon base, and 50 mM sodium borate, pH 7.6. Elastase activity was measured by mixing 1 ml of culture broth with 7 ml of elastin-Congo red (1 mg/ml in 50 mM sodium borate, pH 8.8) and holding the mixture for 3 h at 37°C. The results have been converted to the unit used in this paper: milligrams of elastin solubilized in 30 min per milliliter of culture broth (though determined at a lower elastin concentration than that used in the present study).

[d] Measured after 6 days at 37°C on elastin–Triton X-100 agar medium (see Materials and Methods).

[e] Elastase production after 96 h at 37°C in liquid medium containing 0.2% elastin, 0.3% $CaCO_3$, and 1.17% yeast carbon base. Elastase was assayed as described in Materials and Methods, and results are shown as milligrams of elastin solubilized in 30 min per milliliter of culture broth.

The elastase was further purified by cation exchange on S Sepharose Fast Flow (Fig. 2), resulting in fivefold further purification with practically no loss of activity. The eluate was concentrated to 15 ml by ultrafiltration, with equilibration with 75 mM MES-NaOH, pH 5.8, and further concentrated to 0.5 ml by a Centriprep 10 concentrator and then a Centricon 10. This sample was subjected to gel filtration on

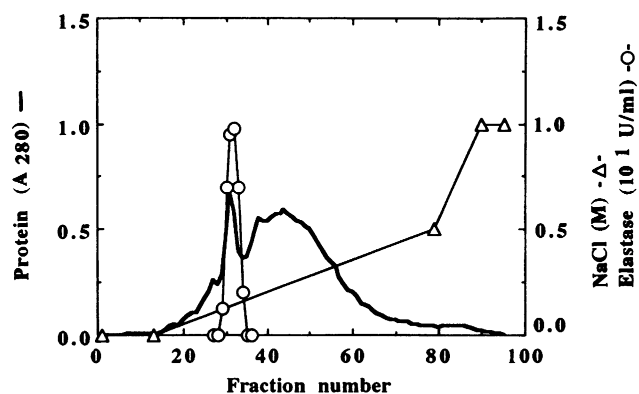


FIG. 1. Anion-exchange chromatography of elastase on Q Sepharose Fast Flow. Concentrated crude elastase (20 ml, 91 mg of protein) in 50 mM diethanolamine HCl, pH 8.8, was applied to an XK 16/20 column containing 30 ml of the matrix, which had been previously equilibrated with the same buffer. Proteins were eluted with 330 ml of gradient (0 to 500 mM) NaCl in the same buffer with a flow rate of 3.5 ml/min, and 5-ml fractions were collected.

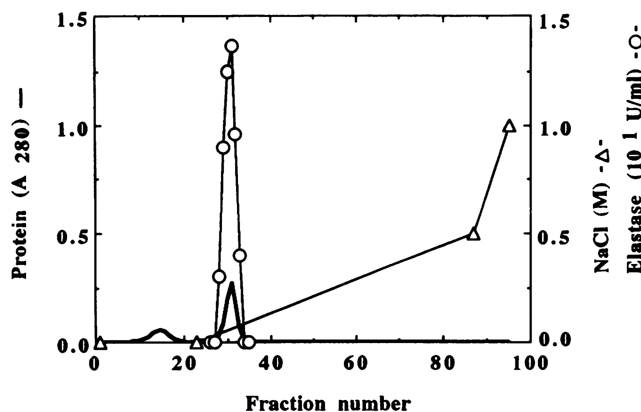


FIG. 2. Cation-exchange chromatography of elastase on S Sepharose Fast Flow. Fractions 29 to 34 of Fig. 1 were pooled, concentrated to 20 ml (10.5 mg of protein), equilibrated with 50 mM MES-NaOH (pH 5.5), and applied to an XK 16/20 column containing 60 ml of the matrix, which had been previously equilibrated with the same buffer. Proteins were eluted with 330 ml of gradient (0 to 500 mM) NaCl in the same buffer with a flow rate of 3.0 ml/min, and 5-ml fractions were collected.

Superose 12 (Fig. 3), resulting in an overall 220-fold purification with 34% recovery. Calibration of the Superose 12 column with several low-molecular-mass proteins suggested a molecular mass of 12 kDa for the elastase. A summary of the purification process is shown in Table 2.

SDS-PAGE of the final preparation showed a number of protein bands (Fig. 4) of 32, 28, and 23 kDa and less. These same bands were visualized by monoclonal antibody KD5 throughout the purification (Fig. 4B), indicating that they represent products of proteolytic degradation of the same protein. The same bands were observed when purified elastase was prepared for SDS-PAGE by boiling without 2-mercaptoethanol (Fig. 4C and D).

SDS-PAGE of samples of culture broth taken during growth showed an increasing amount of a form slightly smaller than the original 32-kDa species followed by the appearance of the 23-kDa form by 72 h (data not shown).

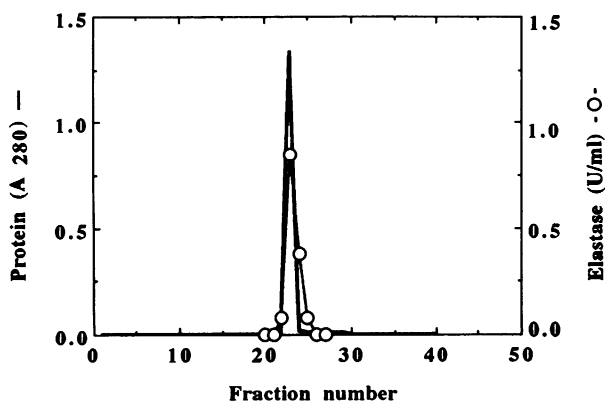


FIG. 3. Gel filtration chromatography of elastase on Superose 12. Fractions 28 to 33 of Fig. 2 were pooled, concentrated to 0.5 ml (2.1 mg of protein), equilibrated with 75 mM MES-NaOH (pH 5.8), and applied to an HR 10/30 column containing 25 ml of the matrix, which had been previously equilibrated with the same buffer. The proteins were eluted from the column at 0.5 ml/min, and 0.75-ml fractions were collected.

TABLE 2. Purification of elastase from *A. fumigatus*

| Purification step | Vol (ml) | Total U | Total protein (mg) | Sp act (U/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|
| Clarified culture broth | 1,100 | 400 | 907.5 | 0.4 | 100 | 1.0 |
| Ultrafiltration (YM10 filter) | 20 | 800 | 91.2 | 8.8 | 200 | 19.9 |
| Anion exchange (Q Sepharose FF[a]) | 32 | 181 | 10.4 | 17.4 | 45 | 39.6 |
| Ultrafiltration (YM10 filter) | 20 | 240 | | | 60 | |
| Cation exchange (S Sepharose FF) | 30 | 235 | 2.6 | 90.7 | 59 | 206.2 |
| Gel filtration (Superose 12) | 7.5 | 135 | 1.3 | 106.8 | 34 | 220.0 |

[a] FF, fast flow.

Elastase degradation apparently occurs during fungal growth as well as during the purification procedure.

**Inhibition of elastase.** Divalent metals inhibited the elastase, but reagents reactive with SS and SH groups were without effect (Table 3). The elastase was inhibited totally by EDTA, 60% by leupeptin, and 30% by 1,10-orthophenanthroline (Table 3). Initially, we found that 20 min of incubation of the enzyme in 2 mM PMSF inhibited elastase activity only 92% at pH 8.5. However, 0.2 mM PMSF totally inhibited the enzyme after 45 min at pH 7.5, probably because this inhibitor is more stable at the lower pH. In a separate experiment, we found that 0.2 mM PMSF at pH 7.5 totally inhibited activity of the purified elastase on 0.5% casein as substrate.

To minimize proteolytic degradation during purification, PMSF (5 mM) and EDTA (10 mM) were added to the culture broth before the broth was concentrated. The inhibited enzyme was then purified by the procedure described above except that the purification was followed by use of SDS-PAGE and Western blots rather than activity assays. SDS-PAGE of 3 μg of purified enzyme showed only the 32-kDa

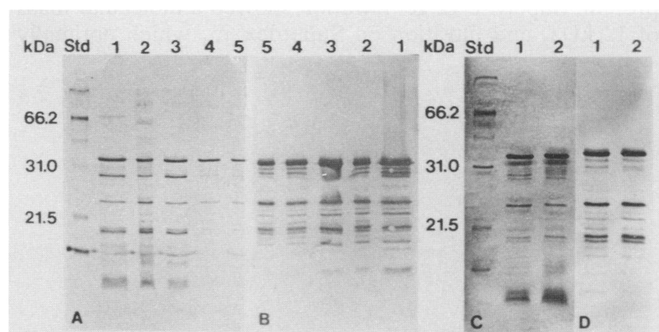


FIG. 4. (A and B) SDS-PAGE of elastase (0.1 U per lane) at various steps during purification: culture broth (lanes 1), crude enzyme preparation (lanes 2), after anion exchange (lanes 3), after cation exchange (lanes 4), and after gel filtration (lanes 5). Panel A is a silver-stained gel, and panel B is a Western blot of a duplicate gel, with monoclonal antibody KD5 (18 ml of 30-μg/ml KD5 in TBS, pH 8.0) for detection. (C and D) SDS-PAGE of purified elastase (2 μg per lane) prepared for electrophoresis by being boiled with (lanes 1) or without (lanes 2) 2-mercaptoethanol. Panel C is a silver-stained gel, and panel D is a Western blot of a duplicate gel. Also shown in panels A and C are commercial molecular mass standards (lanes Std).

TABLE 3. Effects of inhibitors on elastase from *A. fumigatus*

| Inhibitor | Concn (mM) | Residual activity[a] (%) |
|---|---|---|
| PMSF | 0.2 | 0 |
| *N*-Ethylmaleimide | 10.0 | 90 |
| 1,10 orthophenanthroline | 5.0 | 70 |
| EDTA | 5.0 | 0 |
| Pepstatin | 0.15 | 100 |
| TPCK | 1.0 | 100 |
| TLCK | 1.0 | 100 |
| Leupeptin | 0.21 | 40 |
| Dithiothreitol | 1.0 | 100 |
| Iodoacetamide | 10.0 | 100 |
| $MnSO_4$ | 1.0 | 53 |
| | 10.0 | 38 |
| $MgSO_4$ | 1.0 | 42 |
| | 10.0 | 40 |
| $FeSO_4$ | 1.0 | 74 |
| | 10.0 | 23 |
| $CaCl_2$ | 1.0 | 49 |
| | 10.0 | 39 |
| $ZnSO_4$ | 1.0 | 100 |
| | 10.0 | 6 |
| Sodium phosphate | 1.0 | 92 |
| | 10.0 | 52 |

[a] Elastase (0.2 U, 250 μl) was mixed with the inhibitor (250 μl) and held at 37°C for 20 min. Residual elastase activity was measured by adding elastin-Congo red (250 μl; 20 mg/ml in sodium borate, pH 8.5) to the elastase-inhibitor mixture, holding the mixture for 30 min at 37°C, and measuring the $A_{495}$ of the supernatant. PMSF was mixed with the enzyme for 45 min at pH 7.5 to minimize degradation of the inhibitor, which occurs more rapidly at the higher pH. Residual activity was measured as just described except at pH 7.5. Concentrations of inhibitors were maintained at initial levels during measurement of residual elastase activity. Appropriate solvent and buffer controls were tested simultaneously.

species by silver staining (Fig. 5); bands at 28 and 30 kDa were seen when 16 μg of purified enzyme was electrophoresed. Western blots show a number of bands at lower molecular masses, but as these are not detected by the silver stain, they must be present at very low concentrations.

**Molecular mass and isoelectric point.** While SDS-PAGE indicates a molecular mass of 32 kDa for the undegraded protein, Superose 12 gel filtration showed a molecular mass of 12 kDa, and filtration on Superdex 75, which optimally resolves proteins of 10 to 70 kDa (16), gave a molecular mass of 19.1 kDa. The discrepancy is not due to glycosylation (which can result in migration rates on SDS-PAGE that correspond to molecular masses higher than actual [3, 23]), as periodic acid-Schiff reagent failed to stain the protein band in SDS-PAGE gels. The protein is positively charged at neutral pH, the isoelectric point being 8.75 (Fig. 6).

**Nondenaturing PAGE.** Electrophoresis of partially purified enzyme without SDS yielded three bands ($R_f$s = 0.13, 0.26, and 0.40), with elastolytic activity demonstrated by zymograms with soluble elastin as substrate (Fig. 7). These bands were excised, boiled in SDS sample buffer, and electrophoresed in SDS; they gave identical patterns after silver staining (major bands at 32 and 28 kDa and a minor band at 31 kDa) (Fig. 8).

**pH and temperature optima.** As shown in Fig. 9, the pH optimum for action on elastin-Remazol is 7.4 in sodium borate buffer but 8.8 in Tris HCl. The enzyme was only weakly active in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 6.5 to 8.5) and was inactive in

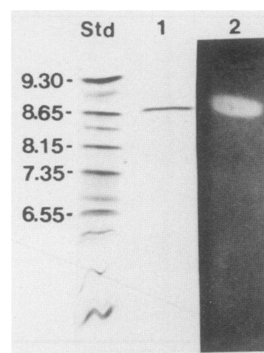

FIG. 6. Isoelectric focusing of purified elastase. Commercial pI standards (lane Std) and elastase (lane 1, 7 μg) were stained with Coomassie brilliant blue R250. Lane 2 is a zymogram of focused elastase. Zone of clearing against dark background (0.5% soluble elastin stained with Coomassie brilliant blue R250 after reaction) indicates elastase activity.

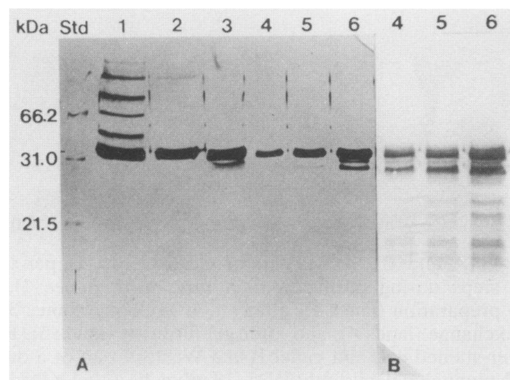

FIG. 5. (A and B) SDS-PAGE of inactivated elastase during purification: crude elastase (lane 1, 15 μg), after anion exchange (lane 2, 10 μg), after cation exchange (lane 3, 15 μg), and after gel filtration (lanes 4, 3.2 μg; lanes 5, 6.4 μg; lanes 6, 16 μg). Panel A is a silver-stained gel, and panel B is a Western blot of a duplicate gel with monoclonal antibody KD5 for detection, as in Fig. 4.

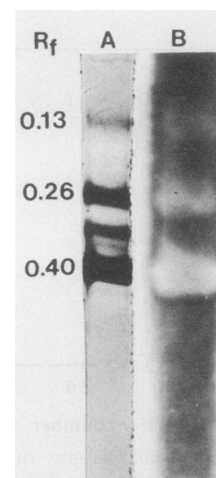

FIG. 7. (A and B) Nondenaturing PAGE of partially purified elastase (~15 μg of protein). Panel A is a silver-stained gel, and panel B is a zymogram of a gel showing elastase activity as clear zones against a dark background of stained (Coomassie brilliant blue R250) 0.5% soluble elastin.

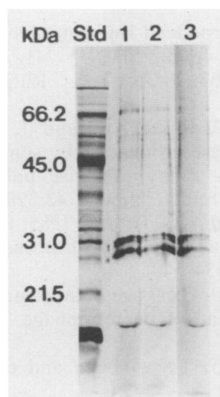


FIG. 8. SDS-PAGE (silver stained) of elastolytic proteins from Fig. 7: $R_f$s = 0.13 (lane 1), 0.26 (lane 2), and 0.40 (lane 3). Commercial molecular mass standards (lane Std) are also shown.

sodium citrate (pH 4.0 to 6.6), MOPS (pH 6.0 to 7.0), Tris-maleate (7.0 to 8.0), and sodium barbital (pH 7.0 to 9.2). The optimal temperature for a 30-min assay in either Tris HCl or sodium borate was 45°C (data not shown).

## DISCUSSION

We found that Triton X-100 incorporated into agar medium was a much better agent for limiting colony size than Rose Bengal, which was used earlier (8). Distinct clearing zones were evident around colonies, and all isolates tested grew and produced elastase, even those strains (2, 8, 10, and 31) which did not grow on the elastin-Rose Bengal medium. Other colony-limiting agents tested, alone or in combination, included Rose Bengal (0.005 to 1.0%), oxgall (0.005 to 1.0%), sodium deoxycholate (0.005 to 1.0%), sorbose (1 to 5%), 2-deoxyglucose (0.2 to 0.5%), saponin (0.05 to 0.5%), and phosphon D (0.000005 to 0.05%). None were as effective as Triton X-100, nor were clearing zones as distinct (data not shown).

With the elastin-Congo red assay (sodium borate buffer, pH 8.5), we found that increasing the substrate concentration from 1.67 to 6.67 mg/ml increased the dye liberation

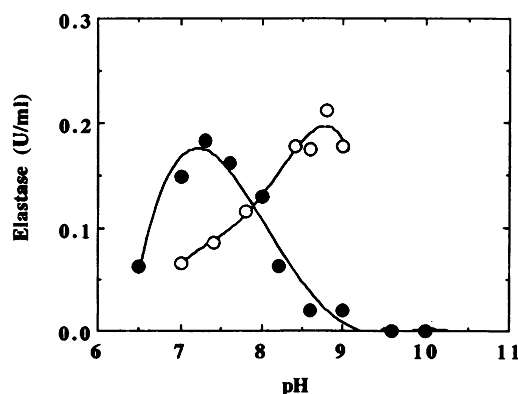


FIG. 9. Elastase activity over pHs 6.5 to 10. Reaction mixtures contained 0.25 ml each of elastin-Remazol (20 mg/ml), elastase (0.2 U), and buffer (150 mM Tris HCl [○] or sodium borate [●]; 50 mM final concentration in reaction mixture). After 30 min at 37°C and then centrifugation, the $A_{595}$ of the supernatant solution was measured to determine elastase activity.

about threefold (data not shown). This suggested that the substrate was limiting at the lower concentration. Elastin has a heterogeneous structure, and it is likely that the enzyme attacks some sites faster than others; we found that the rate of dye liberation was linear with time only up to 10% degradation of the elastin-Congo red present versus 35% for porcine elastase acting on elastin-orcein (22).

The multiple protein bands seen in SDS-PAGE of the purified enzyme (Fig. 4) presumably are due to autoproteolytic cleavage, leaving major fragments containing the immunodominant epitope. The same bands are seen in samples throughout the purification, but the degradation is much diminished by the addition of PMSF and EDTA to the culture broth before purification is begun, indicating that the multiple bands are indeed due to autolysis. In other experiments reported earlier (5), elastolytic and caseinolytic activities from culture broth were inseparable by column chromatography on either Sephadex G-75 or hydroxyapatite. Only one protease, the elastase, could be isolated, so it is presumably responsible for its own degradation. Reichard et al. (18) also encountered autoproteolysis in purification of a serine protease from *A. fumigatus*. Similarly, Shotton (24) reported that porcine pancreatic elastase undergoes rapid autolysis if precautions are not taken. The proteolytic fragments apparently remain associated by noncovalent forces, since a single sharp peak is seen in gel filtration of the purified enzyme (Fig. 3), although reduction with 2-mercaptoethanol is not necessary for separation of the fragments in SDS-PAGE (Fig. 4C and D).

The molecular mass as determined by SDS-PAGE was 32 kDa, and presumably this value is a better estimate than the lower values (12 and 19 kDa) obtained by gel filtration. Rhodes et al. (21) reported that the elastase from *A. flavus* had a molecular mass of 23 kDa, was not glycosylated, and had a pI of 7.6. Reichard et al. (18) described a serine protease from *A. fumigatus* with a molecular mass of 32 kDa and a pI of 7.9. Neither of these reports included data from gel filtration. An alkaline protease isolated from *A. oryzae* had a molecular mass of 34 kDa by SDS-PAGE (25), 12 kDa by gel filtration (26), and 29 kDa by amino acid sequence (25, 26). Although the sequence had a potential site for N glycosylation, the protein did not stain with periodic acid-Schiff reagent, indicating that it was not glycosylated. Our elastase similarly shows no indication of glycosylation.

The elastase is not inhibited by TPCK or TLCK, and thus the specificity does not resemble that of chymotrypsin or trypsin. The inhibition of the enzyme by 0.2 mM PMSF and leupeptin but also by 5 mM EDTA (Table 3) suggests that it is a serine protease stabilized by a metal ion. Another possibility is that the enzyme is contaminated with a copurifying protease. If this were so, one might suspect that enzyme inhibited by PMSF would retain residual activity on casein; however, this inhibitor totally blocked activity on this substrate as well as on elastin.

Inhibition by both PMSF and EDTA is not unique to our elastase. Barthomeuf et al. (1) describe an alkaline protease from *A. niger* that, although quite different in many aspects from our elastase, is blocked by both these inhibitors. Heat stability of this alkaline protease was dependent on calcium ions. However, the elastase of *A. flavus* described by Rhodes et al. (20) appears to be different from that of *A. fumigatus*, as it is a metalloprotease and is inhibited completely by 2 mM 1,10-orthophenanthroline and 26% by EDTA but not by PMSF or leupeptin. The protease isolated by Reichard et al. (18) was completely inhibited by 0.1 M PMSF and was thus described as a serine protease. Although

many properties of the elastase we isolated and the serine protease Reichard et al. isolated are similar, there are still some differences between the two proteases that lead us to believe that, although similar, they are not the same enzyme. Major differences lie in the pI values (7.9 versus 8.75) and the patterns of inhibition (e.g., results with TPCK and EDTA).

The elastase which we purified is probably the same as the one described by Monod et al. (15) in a paper published after submission of this paper. Those authors isolated an extracellular alkaline protease from *A. fumigatus* which had a molecular mass of 33 kDa, a pI of 8.2, and a pH optimum of 9.0 in Tris HCl and was inhibited 42 to 50% by leupeptin and totally by PMSF. The pattern of inhibition by divalent cations was also similar to what we found; however, EDTA did not inhibit their enzyme's activity on azocollagen.

PAGE of partially purified elastase under nondenaturing conditions showed three well-separated bands with elastase activity (Fig. 7). Since the purified enzyme showed only a single band in isoelectric focusing (Fig. 6), it is unlikely that these represent charge isomers. More probably, they represent association of the enzyme to dimers and tetramers. However, the purified enzyme appears homogeneous with respect to size (Fig. 3). It is possible that oligomers in the cruder preparations result from multiple association with single elastin chains or that autolysis during purification abolishes self-association.

The pH optimum of the elastase varied with the buffer employed (pH 7.4 in sodium borate, pH 8.8 in Tris HCl), as previously observed with porcine pancreatic elastase (24). Our earlier work (7) had indicated a pH optimum of 8.8 in sodium borate, and unfortunately, this pH value was used in activity assays reported here, particularly during the purification studies. Although the reported activity of elastase would have been higher if the assays had been carried out at pH 7.4 in sodium borate or at 8.8 in Tris HCl, the results of the purification are still valid, as evidenced by SDS-PAGE (Fig. 4) and zymograms (Fig. 6).

We have prepared monoclonal and polyclonal antibodies to the elastase (6), and studies are in progress to determine the role of this enzyme in the invasion process by seeing whether specific antibodies can arrest invasive infection in immunocompromised mice.

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