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Purification and Characterization of an Elastinolytic Metalloprotease from *Aspergillus fumigatus* and Immunoelectron Microscopic Evidence of Secretion of This Enzyme by the Fungus Invading the Murine Lung

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Extracellular proteases have been suggested to be virulence factors in invasive aspergillosis. Since serine protease gene-disrupted mutants retain virulence, other proteases are suspected to be also involved in the degradation of lung structural material. An elastinolytic neutral metalloprotease was purified 320-fold from the extracellular fluid of *Aspergillus fumigatus* grown on elastin by affinity chromatography on bacitracin-Sepharose 4B and gel filtration on Sephadex G-75. The molecular mass was determined to be 43 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No carbohydrate was attached to this metalloprotease, and its first 22 N-terminal amino acids did not show any homology with the known metalloproteases. The enzyme was completely inhibited by EDTA, 1,10-phenanthroline, and phosphoramidon but not by inhibitors specific for serine, aspartate, and cysteine proteases. Zn^{2+} and, to a lesser extent, Co^{2+} reversed the inhibition caused by 1,10-phenanthroline. The protease hydrolyzed the peptide bonds His-Leu, Ala-Leu, Tyr-Leu, Gly-Phe, and Phe-Phe in the B chain of insulin. Synthetic substrate Abz-Ala-Ala-Phe-Phe-pNA could be used for the fluorimetric assay of the *A. fumigatus* metalloprotease. This enzyme had maximum activity in the pH range 7.5 to 8.0 and at 60°C. It retained 50% of the protease activity when held at 60°C for 1 h. Zn^{2+} and Co^{2+} at 1 mM did not inhibit the protease activity. The metalloprotease was able to hydrolyze elastin, and its elastinolytic activity was comparable to that of the serine protease from this organism. The presence of Zn^{2+} in the culture medium stimulated the metalloprotease production. Rabbit antibodies prepared against the enzyme severely inhibited the enzyme activity. Immunogold electron microscopy revealed that *A. fumigatus* invading neutropenic mouse lungs secretes this metalloprotease.

Aspergillus fumigatus is an important fungal pathogen that causes a respiratory infection, invasive pulmonary aspergillosis, in immunocompromised patients (3). The high mortality caused by this fungal infection is a major threat to the survival of such patients (22). There is a need for an effective means to protect against and treat invasive aspergillosis, and such approaches could be directed against the virulence factors involved in this infection. However, little is known about the virulence determinants of *A. fumigatus*. Since elastin constitutes nearly 30% of the lung (23), the primary site of entry and infection, it has been suggested that the ability of the fungus to degrade elastin may aid its invasion of and growth in lung tissue (8, 9, 19–21). Correlation of the ability of *A. fumigatus* isolates to produce serine protease with their ability to cause invasive aspergillosis suggested that protease is an important virulence factor (9). In support of this conclusion, serine protease-deficient mutants showed decreased virulence (8). Serine protease with elastinolytic activity has been isolated and characterized, and its cDNA and gene were cloned and sequenced (5, 7, 8, 14, 19). Recently, two groups reported that a serine protease gene-disrupted mutant of *A. fumigatus* did not show any decrease in mortality in the murine models for aspergillosis (13, 25). From such results it was concluded that this elastinolytic enzyme was not an important virulence factor. However, it is possible that in the serine protease gene-disrupted mutant other elastinolytic enzymes substituted for this particular serine protease. In fact, other proteases produced by *A. fumigatus* have been reported. For example, an acid protease that hydrolyzed hemoglobin has been described (17). Recently, a metalloprotease (40 kDa) was isolated from a serine protease gene-disrupted mutant of *A. fumigatus* after the fungus was cultivated in collagen-containing medium (12). However, this enzyme was unable to hydrolyze elastin (12). In the present report, we describe the purification and characterization of an elastinolytic neutral metalloprotease (43 kDa) from the wild-type strain of *A. fumigatus*. The properties of this metalloprotease also show other significant differences from those of the recently described nonelastinolytic metalloprotease (12). We also report that the presence of Zn^{2+} in the growth medium promotes the production of the elastinolytic metalloprotease. Immunoelectron microscopic evidence that *A. fumigatus* invading the lungs of neutropenic mice secretes this elastinolytic metalloprotease is presented.

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MATERIALS AND METHODS

Materials. The protease inhibitors antipain, chymostatin, E64, EDTA, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), and phosphoramidon were obtained from Boehringer Mannheim. 1,10-Phenanthroline, bacitracin, and Suc-Ala-Ala-Pro-Leu-pNA were from Sigma. Sepharose 4B was from Pharmacia. Z-Ala-Ala-Leu-pNA, Suc-Ala-Ala-Pro-Leu-pNA, Abz-Ala-Ala-Phe-Phe-pNA, and Z-Phe-Arg-AMC were from Novabiochem.

Fungal culture. The strain of *A. fumigatus* used in this work was isolated from a patient (8) and maintained on YG agar

plates (0.5% yeast extract, 2% glucose, 2 ml of trace elements per liter, and 1.5% agar). For enzyme purification, conidia were harvested from YG plates in water and 0.1 ml of the conidial suspension (5×10^8 conidia) was used to inoculate 100 ml of elastin-containing liquid medium in Roux bottles. This medium consisted of 1.17% yeast carbon base (Difco), 0.3% calcium carbonate, and 0.2% insoluble elastin (Sigma).

Enzyme assays. The activity of the metalloprotease on azocasein (Sigma) was determined as described elsewhere (19). The enzyme samples (5 to 50 μl) were mixed with 150 μl of the substrate (15 mg/ml) and 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) containing 1 mM $CaCl_2$ in a total volume of 500 μl. After incubation at 37°C for 30 to 60 min, 50 μl of 50% trichloroacetic acid was added, and after 5 min at 37°C, the precipitate was collected by centrifugation. The supernatant was mixed with the same volume of 1 M NaOH, and absorption of liberated dye at 436 nm was measured on a 8451A diode array spectrophotometer. The specific activity is expressed in units per milligram of protein. Elastinolytic activity was measured by determining the amount of radioactivity released after incubation of the enzyme with [^{3}H]elastin (0.12 μCi/mg) as described previously (1, 30). The reaction mixture containing the enzyme and 100 μg of [^{3}H]elastin in a total volume of 300 μl was centrifuged after various periods of incubation at 37°C, and aliquots of the supernatant (100 μl) were assayed for radioactivity by liquid scintillation spectrometry. For all inhibition studies, the enzyme samples were preincubated for 10 min with the inhibitor and after addition of the substrate the enzyme activity was measured as described above.

Measurement of protein and radioactivity. Protein was measured with the Bio-Rad protein assay kit, based on the dye-binding assay of Bradford (2). Aliquots of solutions containing tritiated materials were mixed with Scintiverse II BD (Fisher Scientific) and assayed for radioactivity in a Beckman LS 3801 liquid scintillation counter.

Purification of the metalloprotease. *A. fumigatus* was grown in liquid medium in batches of nine Roux bottles. The extracellular fluid (approximately 800 ml), separated from the fungal mat by filtration through Whatman no. 1 filter paper (Fisher Scientific), was applied directly onto a bacitracin-Sepharose 4B column (3.0 by 14.0 cm) which was obtained as described previously (24). The column was washed and equilibrated with buffer A (50 mM Tris-HCl [pH 7.2] containing 1 mM $CaCl_2$). The bound protein was sequentially eluted with 145 ml of buffer B (50 mM Tris-HCl [pH 7.2] containing 1 M NaCl, 1 mM $CaCl_2$, and 7% 2-propanol) and 190 ml of buffer C (50 mM Tris-HCl [pH 7.2] containing 1 M NaCl, 1 mM $CaCl_2$, and 25% 2-propanol). Aliquots of the 5.5-ml fractions collected were assayed for metalloprotease activity, and the fractions containing the enzyme activity were pooled and concentrated by ultrafiltration with PM10 membranes (Amicon). During ultrafiltration, the buffer was changed to buffer D (50 mM Tris-HCl [pH 7.2] containing 100 mM NaCl and 1 mM $CaCl_2$). The concentrated metalloprotease preparation was applied to a Sephadex G-75-50 column (1 by 120 cm) equilibrated with buffer D, proteins were eluted at 0.1 ml/min, 1.2-ml fractions were collected, aliquots were assayed for enzyme activity, and fractions containing the metalloprotease activity were pooled and stored at −20°C.

Hydrolysis of the B chain of insulin. Oxidized B chain of bovine insulin (Sigma) (100 μg) dissolved in 100 μl of 0.1 M HEPES buffer (pH 7.5) was incubated with 1 μl (40 ng) of metalloprotease at 37°C for 5 min. The reaction mixture was immediately applied to a C_{18} high-pressure liquid chromatography column (4.6 by 250 mm), the peptides were eluted with 7.5 to 75% acetonitrile in water with 0.1% trifluoroacetic acid in all solutions, and the effluent was monitored for A_{215}. The peptide fractions were lyophilized and analyzed on a mass spectrometer (Finnigan Mat) and an amino acid analyzer (Applied Biosystems 420A).

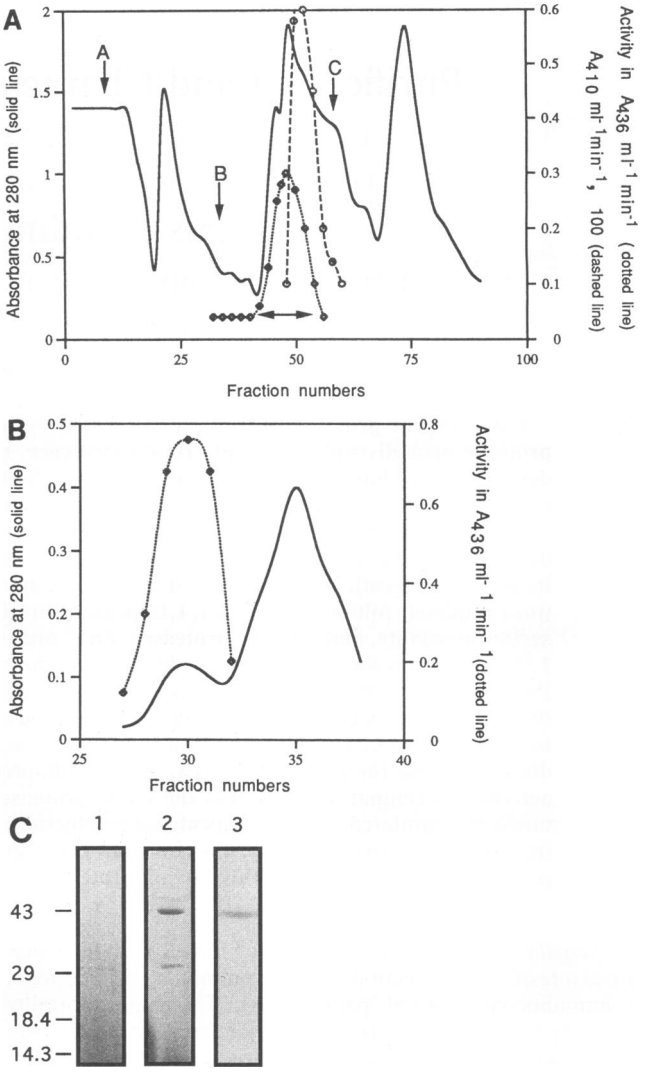

FIG. 1. (A) Chromatography of *A. fumigatus* metalloprotease on bacitracin-Sepharose 4B. The culture supernatant was applied to a bacitracin-Sepharose 4B column, and aliquots of eluant fractions were assayed for protein (A_{280}), for serine protease (dashed line) (using Suc-Ala-Ala-Pro-Leu-pNA [8]), and for metalloprotease activity (closed circle) (using azocasein). Arrows indicate buffers A, B, and C used for elution (see Materials and Methods). The enzyme fractions indicated by the double-headed arrow were pooled for the next step. (B) Gel filtration chromatography of *A. fumigatus* metalloprotease on Sephadex G-75. Fractions from the step shown in panel A were pooled, treated with PMSF (1 mM), concentrated to 2.5 ml (12.5 mg of protein), and applied to Sephadex G-75. The fractions were assayed for protein at an A_{280} and metalloprotease activity (closed circle) with azocasein. (C) SDS-PAGE (13% polyacrylamide) of crude extract (lane 1), bacitracin-Sepharose 4B product (lane 2), and gel filtration chromatography product (lane 3). Molecular mass markers (in kilodaltons) are shown on the left.

Hydrolysis of Abz-Ala-Ala-Phe-Phe-pNa. The hydrolysis of the fluorigenic substrate Abz-Ala-Ala-Phe-Phe-pNA was monitored as described previously (4). The stock solution of the

TABLE 1. Purification of an extracellular elastinolytic metalloprotease from *A. fumigatus*[a]

Purification step	Total protein (mg)	Activity		Yield (%)	Purification (fold)
		Total U (A_{436}/min)	Sp act U (A_{436}/A_{280} · min)		
Culture supernatant	48	12	0.015	100	1
Bacitracin-Sepharose 4B	12	9.35	0.08	78	5.3
Sephadex G-75	0.54	2.6	4.8	22	320

[a] Experimental details are given in Materials and Methods.

substrate (10 mM) was prepared in absolute methanol. The enzymatic reaction was started in 2 ml of 0.1 M HEPES buffer (pH 7.5) containing 1 mM $CaCl_2$ by adding 2 μl of the stock substrate and aliquots of enzyme. The fluorescence of the liberated Abz (2-aminobenzoyl) derivative was measured on an LS-3B fluorescence spectrometer (Perkin-Elmer) with excitation at 340 nm and emission at 420 nm.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (10), with a 13% resolving gel and a 4% stacking gel. For protein sequencing and Western blots (immunoblots), the electrophoretically separated proteins were transblotted onto ProBlott (Applied Biosystems) and Immobilon P polyvinylidene difluoride membranes (Millipore), respectively.

Antibody production and Western blots. The SDS-PAGE gel band corresponding to the metalloprotease was cut out, mixed thoroughly with Freund's adjuvant (complete for the first injection and incomplete for subsequent injections), and injected subcutaneously into two rabbits; two additional booster injections were given at 2-week intervals, after which blood was collected by heart puncture. The serum fraction was isolated and used as a source of antimetalloprotease antibodies in the immunological studies. Western blots were done by the standard protocol (26), with the electrophoretic conditions indicated above. The gel was transblotted onto a membrane for 30 to 60 min at 0.5 A in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid transfer buffer (pH 11)–10% methanol. Blots were visualized by using horseradish peroxidase-protein A and the ECL (Enhanced Chemiluminescence) detection system from Amersham as described by the manufacturer.

Carbohydrate detection. The GlycoTrack kit (Oxford GlycoSystems) was used to detect carbohydrate moieties in protein after SDS-PAGE and transblotting onto Immobilon P membranes as outlined by the manufacturer.

Protein N-terminal sequencing. The purified metalloprotease was subjected to SDS-PAGE and electroblotted to a ProBlott membrane as described elsewhere (11). The stained band of 43 kDa was cut out and subjected to N-terminal sequencing on an Applied Biosystems 475A protein sequencer.

Immunogold localization of metalloprotease produced by *A. fumigatus* in the lungs of neutropenic mice. Neutropenia was induced in mice by irradiation of the mice, and the degree of neutropenia was measured as described previously (8). *A. fumigatus* conidia were introduced intranasally (10^8 conidia), and the actual number of conidia introduced into the lungs was determined to be about 10^7 by a previously described method (8). Lung tissue was sampled after various periods after inoculation. Lung tissue was fixed with paraformaldehyde-glutaraldehyde, dehydrated with ethanol, embedded in L. R. White embedding medium, and polymerized as described previously (8). Blocks were cut with glass knives on a Richert-Ultracut Ultramicrotome. Silver sections were placed on gold single-slot grids (2 by 1 mm) coated with Formvar (6). The coated grids with sections were immersed for 20 min in 1% bovine serum albumin in 0.05 M Tris-buffered saline (pH 7.4) with Tween 20. The grids were placed in 1:400 diluted metalloprotease antiserum solution for 1 h, rinsed in Tris-buffered saline containing 0.5% Tween 20, and then placed in goat anti-rabbit antiserum conjugated with 20-nm-diameter colloidal gold (Polysciences, Warrington, Pa.) for 30 min before being washed in distilled water. Sections were stained with saturated aqueous uranyl acetate before viewing with a Philips

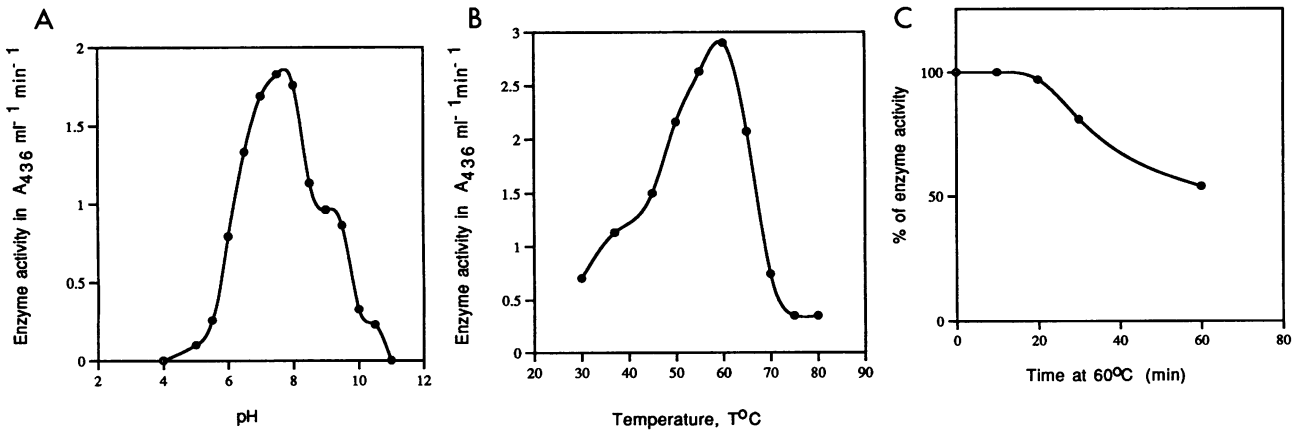

FIG. 2. (A) Effect of pH on activity of *A. fumigatus* metalloprotease. Azocasein was used as the substrate in 0.1 M Tris-citrate buffer at pH 3 to 9 and 0.1 M glycine-sodium hydroxide buffer at pH 9 to 11. (B) Effect of temperature on *A. fumigatus* metalloprotease activity. Assays were done in 0.1 M HEPES buffer (pH 7.5) containing 10 mM $CaCl_2$. Enzymatic activity was measured in 1-h assays with azocasein at the indicated temperatures. (C) Thermostability of *A. fumigatus* metalloprotease. Enzyme in buffer D was held at 60°C for the indicated periods before being assayed for protease activity with azocasein as the substrate.

300 electron microscope at 60 kV. The specificity of labeling was tested with preimmune serum diluted 1:400 in place of the antimetalloproteinase primary antibody and by incubating sections with secondary antibody without prior exposure to primary antibody.

RESULTS

Purification of the *A. fumigatus* metalloprotease. After 48 h of growth of *A. fumigatus* in elastin-containing medium, the extracellular fluid showed proteolytic activity against azocasein in the presence of PMSF, but the protease activity was totally inhibited in the presence of both PMSF and EDTA (data not shown). These results demonstrated that *A. fumigatus* secreted not only the well-characterized serine protease but also a metalloprotease. When the extracellular fluid was passed through a bacitracin-Sepharose 4B column, the metalloprotease activity was retained in the column but could be eluted by 50 mM Tris-HCl buffer (pH 7.2) containing 1 M NaCl, 1 mM $CaCl_2$, and 7% 2-propanol (Fig. 1A). This step did not resolve the metalloprotease from the serine protease secreted by *A. fumigatus*. Obviously, association constants of both proteases with bacitracin are close. However, this step removed part of the pigments and contaminating proteins. After ultrafiltration on an Amicon PM-10 membrane, the metalloprotease showed an increase in specific activity. When the enzyme solution was subjected to gel filtration on Sephadex G-75, the metalloprotease was resolved from the serine protease (Fig. 1B). The purification achieved by the two steps is shown in Fig. 1C. Combination of these procedures resulted in a 320-fold purification of the metalloprotease from the culture medium, with a 22% yield (Table 1). The metalloprotease could also be resolved from the serine protease by an ion-exchange step on a Mono Q column. When the mixture in 20 mM HEPES buffer (pH 7.15) containing 10 mM $CaCl_2$ was applied to a Mono Q column, the serine protease was not bound but all of the metalloprotease was bound. Application of a 0 to 1 M NaCl gradient in the same buffer released the metalloprotease at 0.19 M NaCl. Although the metalloprotease preparation showed only one band upon SDS-PAGE, the preparation contained some pigments. On the other hand, the product of the gel filtration step did not contain any visible pigments, although the yield had to be sacrificed to assure complete resolution from the serine protease.

Molecular and catalytic properties of *A. fumigatus* metalloprotease. SDS-PAGE of the purified metalloprotease showed a single protein band at 43 kDa (Fig. 1C). The elution volume of metalloprotease from the Sephadex G-75 gel filtration column indicated that the enzyme is a monomer. With a highly sensitive carbohydrate detection system, the presence of carbohydrates in the metalloprotease could not be detected, indicating that it is not a glycoprotein (data not shown). The N-terminal amino acid sequence of the metalloprotease with a protein sequencer revealed the sequence of 22-amino acid residues (with at least 75% yield per cycle) A-D-Y-Q-V-Y-A-W-G-I-N-D-P-X-P-E-E-R-(R,T)-X-V-K.

The pH dependence of the metalloprotease activity was measured with azocasein. The optimal pH for the metalloprotease activity was found to be 7.5 to 8.0 (Fig. 2A). The influence of temperature on the metalloprotease activity was tested in the temperature interval between 30 and 85°C. The optimal activity against azocasein was obtained at 60°C (Fig. 2B). Thermostability of the metalloprotease was measured at 60°C. The enzyme lost about 50% of its activity during incubation at 60°C for 1 h (Fig. 2C). The effects of different potential inhibitors on the activity of the metalloprotease are summarized in Table 2. The enzyme was inhibited by the metalloprotease inhibitors, such as EDTA, 1,10-phenanthroline, and phosphoramidon, confirming our preliminary observations with the culture supernatant. In contrast, inhibitors specific for serine, cysteine, and aspartate proteases, such as PMSF, antipain, leupeptin, chymostatin, and pepstatin, had little or no effect on the metalloprotease activity. To determine which metal ion is involved in the active site of the metalloprotease, we inhibited the enzyme with 10 mM 1,10-phenanthroline and tried to restore the enzyme activity by adding 10 mM Zn^{2+} or Co^{2+}. The results showed that Zn^{2+} restored metalloprotease activity to 116% and Co^{2+} restored the protease activity to 87% of the original activity, suggesting that Zn^{2+} may be involved in the active site of this enzyme. In the absence of any chelators, Zn^{2+} and Co^{2+} at 1 mM showed no inhibition of the present metalloprotease (Table 2); even at 10 mM, only 30 to 40% inhibition was observed. The specificity of cleavage of bonds in the insulin B chain by the metalloprotease was examined by analyzing the peptides generated, using mass spectrometry and amino acid analysis. The results showed that the metalloprotease cleaved the bonds shown in Fig. 3. The specificity appears to be for the amino side of hydrophobic residues with bulky side chains. Among various synthetic substrates tested (see Materials and Methods), only Abz-Ala-Ala-Phe-Phe-pNA, which generated a fluorescent product, was used for the detection of the metalloprotease activity. Abz derivatives that do not contain a quenching *p*-nitroanilide group have fluorigenic properties (4). Action of the enzyme on this substrate did not release spectrophotometrically detectable free *p*-nitroaniline (A_{405}) but generated a fluorescent

TABLE 2. Effects of different compounds on the protease activity of metalloprotease purified from *A. fumigatus*

Compound[a]	Concn	% Inhibition
PMSF	10 mM	0
EDTA	10 mM	100
1,10-Phenanthroline	10 mM	100
EGTA	10 mM	0
Phosphoramidon	10 μg/ml	100
Pepstatin	10 μg/ml	0
Chymostatin	100 μg/ml	20
E64	10 μg/ml	15
Antipain	50 μg/ml	25
Leupeptin	10 μg/ml	0
Zn^{2+}	1 mM	0
	10 mM	30
Co^{2+}	1 mM	0
	10 mM	40
DTT	1%	85
2-Mercaptoethanol	1%	40
Diethylpyrocarbonate	10 mM	60
Na citrate	10 mM	0

[a] The purified enzyme was incubated with these chemicals for 10 min before the enzyme activity was measured with azocasein as the substrate. EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol.

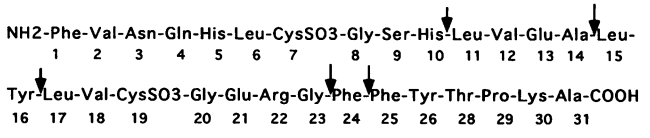

FIG. 3. Hydrolysis of the oxidized B chain of insulin by *A. fumigatus* metalloprotease. Arrows indicate peptide bonds split by the enzyme.

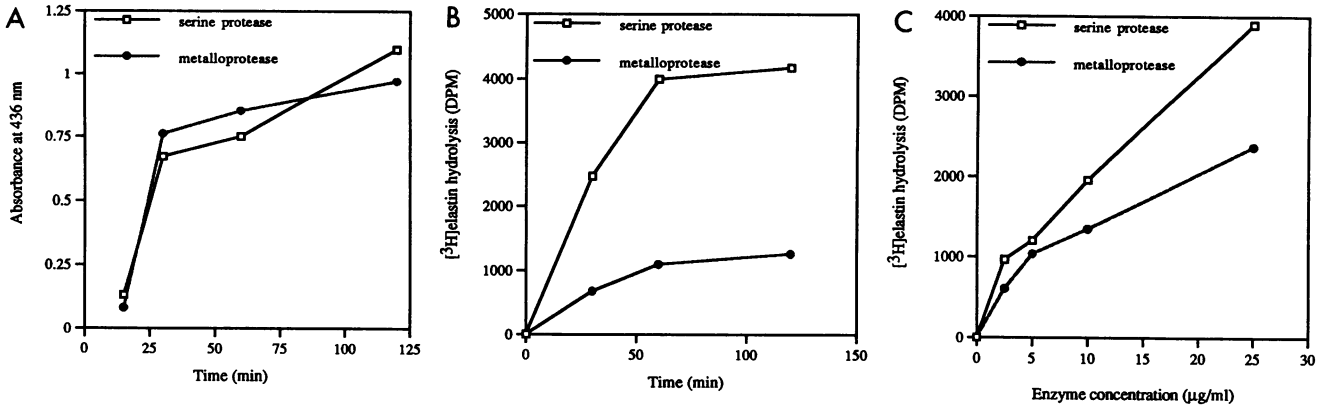

FIG. 4. Protease activity of and [^{3}H]elastin hydrolysis by serine protease and metalloprotease purified from *A. fumigatus*. (A) Protease activity. Aliquots of each purified enzyme (1.5 μg) were incubated with azocasein at 37°C, and released dye was measured as described in Materials and Methods. (B) Time course of [^{3}H]elastin hydrolysis. A 3.0-μg quantity of each enzyme was incubated with [^{3}H]elastin at 37°C, and released labeled products were measured as described in Materials and Methods. (C) Enzyme concentration-dependent [^{3}H]elastin hydrolysis. Assays were done for 60 min at 37°C, and released labeled products were measured as described in Materials and Methods.

product, indicating that the metalloprotease hydrolyzed a peptide bond. With this assay, linear dependence of the fluorescence versus time and of the rate of the hydrolysis versus enzyme concentration was determined (data not shown).

Since a previously isolated metalloprotease from *A. fumigatus* was reported to be incapable of hydrolyzing elastin (12), we tested the ability of the present metalloprotease to hydrolyze [^{3}H]elastin and azocasein and compared these activities with those of the elastinolytic serine protease purified from this organism. Under linear assay conditions of enzyme concentration and reaction time, the two enzymes shared equal hydrolytic activity on azocasein (Fig. 4A). The purified metalloprotease showed a linear relationship of [^{3}H]elastin hydrolysis to reaction time (0 to 60 min) and enzyme concentration (Fig. 4B and C). The specific activity of [^{3}H]elastin hydrolysis by the metalloprotease was about 60% of that of the serine protease.

Effect of Zn^{2+} in the growth medium on metalloprotease production. Since this enzyme is probably a zinc metalloprotease, we determined the effect of various Zn^{2+} concentrations in the growth medium on the level of the enzyme produced in the medium. The appearance of the metalloprotease was detected in the culture supernatant by using the synthetic substrate Abz-Ala-Ala-Phe-Phe-pNA. Since the serine protease can also hydrolyze this substrate, the culture supernatant was treated with PMSF and the residual activity was measured. The level of this metalloprotease activity in the medium was increased by the addition of Zn^{2+} in the growth medium; the maximal increase was obtained with 0.1 to 0.5 mM Zn^{2+}. The time course of appearance of metalloprotease activity in the elastin medium containing either no Zn^2 or 0.5 mM Zn^{2+} is shown in Fig. 5A. The same culture supernatants were evaluated by immunoblot analysis (Fig. 5B). Both methods showed that Zn^{2+} stimulated metalloprotease production.

Immunological characterization of *A. fumigatus* metalloprotease. Purified metalloprotease was used for immunization of rabbits. The specific antibodies in sera were diluted 1:200 for Western blot analysis of the *A. fumigatus* culture medium. After blot development with ECL reagents, only one stained band was obtained and this band corresponded to 43-kDa metalloprotease (Fig. 6A). The inhibition of metalloprotease activity by the antibodies was also tested. With increasing antiserum concentrations, increasing inhibition (at least up to

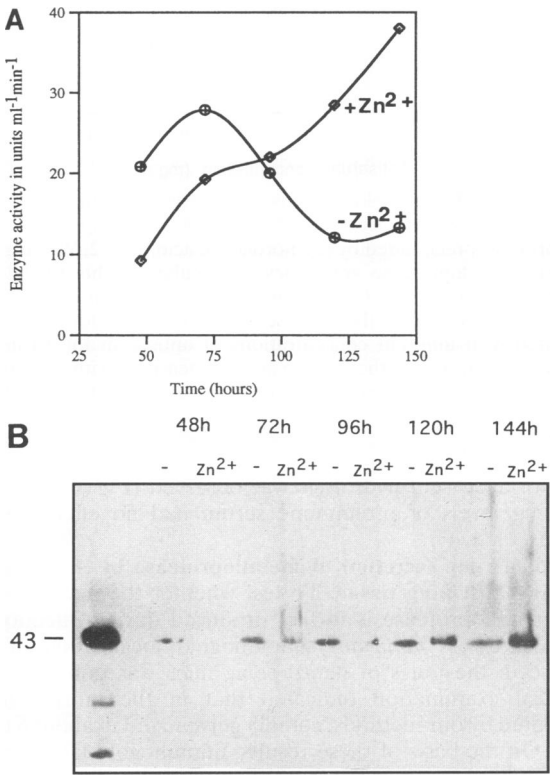

FIG. 5. (A) Time course of production of extracellular metalloprotease by *A. fumigatus* grown in elastin medium containing no Zn^{2+} or 0.5 mM Zn^{2+}. Aliquots of culture fluid (50 μl) were treated with PMSF (1 mM) and assayed for metalloprotease activity, using as a substrate Abz-Ala-Ala-Phe-Phe-pNA as described in Materials and Methods. (B) Immunoblot analysis of metalloprotease production by *A. fumigatus* with no addition (−) or with 0.5 mM $ZnSO_4$ (Zn^{2+}). The duration of culture growth is indicated above the lanes. Aliquots (100 μl) of extracellular fluid from culture for which results are shown in panel A were lyophilized and separated by SDS–13% PAGE. The proteins were then immunoblotted, and metalloprotease was detected with an antiserum produced against purified metalloprotease, diluted 1:200.

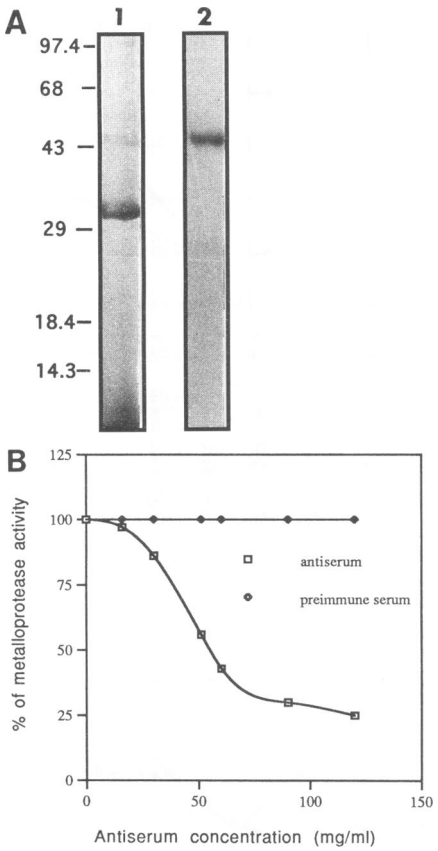

FIG. 6. (A) Immunoblot analysis of *A. fumigatus* extracellular fluid. Lane 1, Coomassie-stained SDS-PAGE of total *A. fumigatus* extracellular proteins precipitated by trichloroacetic acid; lane 2, Western blot with antimetalloprotease antibodies. Molecular weights (in kilodaltons) are shown on the left. (B) Inhibition of *A. fumigatus* metalloprotease activity by antimetalloprotease antibodies. Metalloprotease was incubated with different concentrations of antiserum for 10 min at room temperature, and the enzyme activity measured with azocasein is expressed as a percentage of the value for the control with no antibody.

75%) of azocasein hydrolysis was observed (Fig. 6B). Corresponding levels of preimmune serum had no effect on the protease activity.

Evidence for secretion of metalloprotease by *A. fumigatus* invading host lung tissue. To test whether the newly discovered metalloprotease is in fact produced during infection of the host by *A. fumigatus*, immunogold localization of the enzyme in the lungs of neutropenic mice was done. A cytochemical examination indicated that in the murine lungs inoculated by our methods, conidia germinated in about 6 to 10 h (8). On the basis of these results, immunogold labeling was done on lung tissue of neutropenic mice at 8 (Fig. 7A and B) and 20 (Fig. 7C and E) h after inoculation with conidia of *A. fumigatus*. Specific gold labeling was observed when the antiserum prepared against the metalloprotease from *A. fumigatus* was used (Fig. 7A, C, and E), but no labeling was observed with preimmune serum (Fig. 7B and D). Spores germinating in the lung appeared to secrete the metalloprotease, as the labeling was essentially confined to the translucent cell walls and label was not found within the fungal cell. Later in the infection, hyphal walls showed immunogold labeling (Fig. 7C and E) and the labeling pattern suggested that the secretion of the metalloprotease may be targeted to the growing points, as such regions appeared to show heavier gold labeling (Fig. 7C and E). These results show that *A. fumigatus* invading the lung tissue of neutropenic mice secretes the elastinolytic metalloprotease.

DISCUSSION

We report the isolation and characterization of an elastinolytic metalloprotease from the wild-type strain of *A. fumigatus*. That this is a metalloprotease is demonstrated by inhibition by EDTA and 1,10-phenanthroline. In support of this conclusion was the finding that phosphoramidon, a transition-state analog of a zinc metalloprotease (thermolysin) produced by *Bacillus thermoproteoliticus* (27), also inhibited the metalloprotease from *A. fumigatus*. The pH optimum of the *A. fumigatus* metalloprotease showed that it is a neutral metalloprotease. Reversal of inhibition by exogenous Zn^{2+} caused by the chelators suggests that this might be a Zn metalloprotease.

The substrate specificity of the elastinolytic metalloprotease from *A. fumigatus* appears to be similar to that of thermolysin. The metalloprotease hydrolyzes the same peptide bonds (His-Leu, Ala-Leu, Tyr-Leu, and Phe-Phe) as thermolysin in the B chain of oxidized insulin (15). This enzyme also released fluorescent product(s) from Abz-Ala-Ala-Phe-Phe-pNA, making this a useful substrate for spectrofluorimetric assay of this protease. During the hydrolysis of this substrate, release of free *p*-nitroaniline could not be detected but fluorescent products were generated, showing removal of the quenching *p*-nitroaniline group. From the specificity indicated by the cleavage sites in the insulin B chain, it is likely that the Phe-Phe peptide bond in the synthetic substrate is cleaved by the metalloprotease. The *A. fumigatus* metalloprotease does not hydrolyze the substrates of serine proteases, Z-Ala-Ala-Leu-pNA, Suc-Ala-Ala-Pro-Leu-pNA, Abz-Ala-Ala-Phe-Phe-pNA, and Z-Phe-Arg-AMC.

Despite the probable importance of proteases in the establishment and maintenance of aspergillosis, little is known about the environmental signals that regulate their biosynthesis. The ionic composition of lung tissues has never been correlated with the expression of *A. fumigatus* virulence factors. It is of interest to ascertain the environmental signals that could be responsible for enhancing virulence in the lung. One signal influencing expression of metalloprotease could be Zn^{2+}. This is not surprising, since the prevalence of zinc is surpassed only by iron among the transition metals in biological systems (29). In addition, *A. fumigatus* metalloprotease is likely a zinc metalloprotease and thus requires zinc for activity. We examined the influence of different Zn concentrations (0.1, 0.5, and 1 mM) in the growth medium and found that at early time points (2 or 3 days) the zinc content had a negative effect and that only after 4 days zinc promoted metalloprotease production. To test whether this increased activity represented an increased level of the metalloprotease production or its activation by Zn^{2+}, we examined the same samples by immunoblot analysis. The results showed that immunologically measured enzyme levels correlated with the metalloprotease activity levels, thus demonstrating that Zn^{2+} in the medium increased the level of secreted metalloprotease. It was found that the enhancement of metalloprotease production by Zn^{2+} in the medium increased in the following order of Zn^{2+} concentrations: 0.1 mM > 0.5 mM > 1 mM (data not shown). The present results are reminiscent of the stimulation of Zn^{2+} metalloprotease production of *Pseudomonas aeruginosa*, in which case the role of Zn^{2+} was suggested to be stimulation of production and processing (16).

The present metalloprotease appears unique, as the first 22

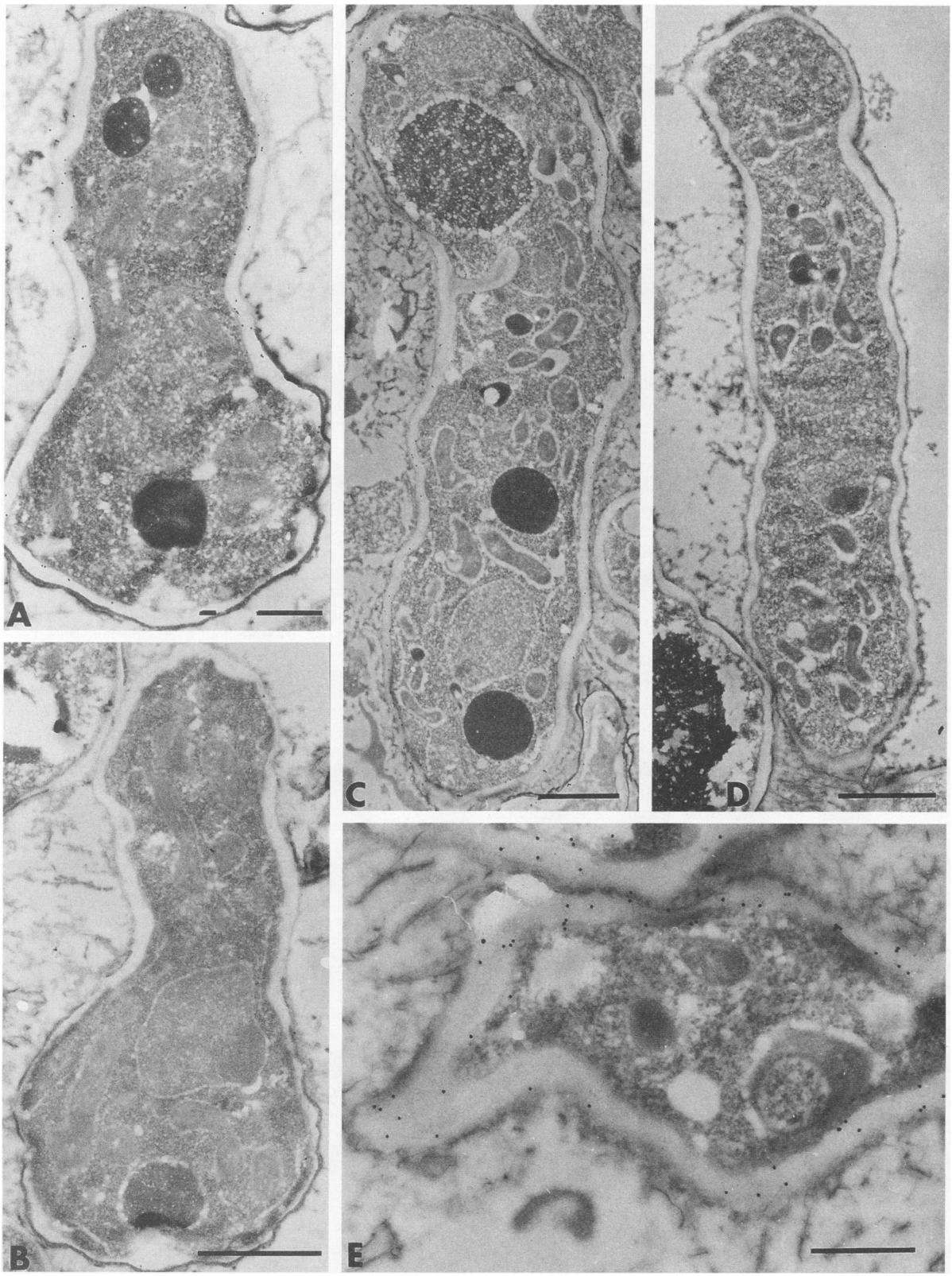

FIG. 7. Immunogold localization of metalloprotease secreted by *A. fumigatus* in the host (mouse) lung. (A and B) At 8 h after inoculation, when germination was beginning. In panel A, 400-fold-diluted antiserum was used; in panel B, similarly diluted preimmune serum was used. Bars = 1 μm. (C through E) At 20 h after inoculation, when hyphae were penetrating lung tissue. In panel C, 400-fold-diluted rabbit antiserum against metalloprotease was used; in panel D, similarly diluted preimmune serum was used. Bars = 1 μM. In panel E, the penetrating tip of a hyphae 20 h after inoculation is shown, with specific gold labeling in the wall surrounding the advancing point. Bar = 0.5 μm.

N-terminal amino acids of this enzyme did not show homology with metalloproteases available in the protein data bank. A metalloprotease of *A. fumigatus* isolated from the serine protease-deficient mutant was described recently (12). Some of its properties are different from those of the enzyme described in the present report. For example, the temperature optimum for the protease activity of the present enzyme is 15°C higher than that of the previously reported enzyme. The present enzyme retains 50% of its protease activity after 1 h at 60°C, whereas the previously reported enzyme was completely inactivated in 10 min at 50°C. The previously described metalloprotease was reported to be completely inhibited by 1 mM Zn^{2+} and Co^{2+}, whereas the present enzyme is not affected by such concentrations of the metal ions. In contrast to the previously reported enzyme, the present metalloprotease is not a glycoprotein. The metalloproteases previously isolated from aspergilli differ significantly in their molecular weight, optimum pH, pH stability, substrate specificity, thermostability, and other physicochemical properties (28).

The metalloprotease described in this paper hydrolyzes elastin, whereas the metalloprotease from the serine protease gene-disrupted mutant was reported to be incapable of hydrolyzing elastin. The time course of hydrolysis and the enzyme concentration dependence of [^{3}H]elastin hydrolysis catalyzed by the purified serine protease and purified metalloprotease from *A. fumigatus* showed comparable elastinolytic activities; on a molar basis, the metalloprotease showed about 80% of the specific activity of the serine protease. This elastinolytic activity could not be due to serine protease contamination because the metalloprotease preparation used was already treated with PMSF. Furthermore, before each elastinolytic activity determination the preparation was treated with PMSF, and the observed elastinolytic activity was completely eliminated by *O*-phenanthroline or EDTA. The extracellular proteolytic activity of *A. fumigatus* is thought to be due largely to the serine protease (8, 14). Since disruption of this serine protease gene did not significantly decrease mortality in the murine model, the role of elastinolytic activity as a virulence factor has been questioned (13, 25). The report that the metalloprotease isolated from this mutant could not hydrolyze elastin cast further doubt on the relevance of elastinolysis to virulence. However, the metalloprotease described in the present paper hydrolyzes elastin with a specific activity comparable to that of the serine protease. Furthermore, immunogold labeling showed that *A. fumigatus* spores infecting the lungs of neutropenic mice produce this elastinolytic metalloprotease. These results also suggested that the secretion of this enzyme in the host tissue may be targeted to the advancing hyphal tip, supporting the idea that this enzyme probably aids the pathogen to invade the host. *Aspergillus flavus*, the other major causative agent of aspergillosis, is also known to produce elastinolytic serine protease (18) and elastinolytic metalloprotease (20). The metalloprotease produced by *A. flavus* appears to be immunologically quite different from the metalloprotease isolated from *A. fumigatus*, as antibodies prepared against the latter showed no cross-reactivity to the former (unpublished results). We have evidence that these *Aspergillus* species produced other proteases, including acid protease, and it appears that there are families of genes that code for these enzymes (10a). Thus, single-gene disruption experiments are unlikely to reveal the role of proteases in aspergillosis. The various proteases produced during fungal invasion should be identified and characterized before the role of proteases in pathogenesis can be assessed.

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