Isolation and Characterization of an Extracellular Proteinase of *Coccidioides immitis*

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A proteinase isolated from the respiratory pathogen, *Coccidioides immitis*, was shown to have collagenolytic and elastinolytic activity, as well as the ability to cleave human serum immunoglobulin G and secretory immunoglobulin A. Proteolytic activity was demonstrated with a bovine casein digestion assay in conidial culture exudates, mycelial and spherule culture filtrates, conidial and spherule wall material, and Sephacryl S-300 fractions of the isolated soluble conidial wall material described previously. One of the latter fractions (fraction 2) demonstrated high proteolytic activity. The proteinase was purified from this chromatographic fraction by cold acetone extraction followed by Sephadex G-50 gel filtration and was identified as a polypeptide band of 36,000 M_r by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By means of tandem two-dimensional immunoelectrophoresis, the proteinase was identified as antigen 11 on the basis of its reaction in the coccidioidin/anticoccidioidin reference system. The proteinase is characterized by a broad substrate specificity, optimal activity at 35 to 40°C (pH 8.0) in the presence of human collagen, elastin, or hemoglobin, an isoelectric point of pH 4.5, and inhibition by organofluorides, N-tosyl-L-phenylalanine chloromethyl ketone, chymostatin, and α-1-antitrypsin. These features of the enzyme are comparable to those of chymotrypsinlike serine proteinases. Demonstration that the proteinase can cleave human immunoglobulins and digest ubiquitous tissue structural proteins (e.g., collagen and elastin) suggests that it may play a role in the virulence of the fungal pathogen.

Coccidioides immitis is a fungal respiratory pathogen of humans and certain other terrestrial mammals (26). Initiation of the disease (coccidioidomycosis) typically involves inhalation of dry, air-dispersed conidia which are small enough to pass down the respiratory tract and reach the alveoli (6, 32). From these foci of infection, the pathogen can disseminate to almost all organs of the body (1). Little is known about the role of this fungal pathogen and the role of the host during initial contact and subsequent stages of coccidioidomycosis (9). Filamentous fungi which successfully colonize substrates in nature typically do so by the action of specific, digestive exoenzymes (11). It is logical, therefore, to look for such extracellular products in cultures of saprobic and parasitic cells of *C. immitis*, as well as in infected tissue. In recent studies, both collagenolytic and elastinolytic activity of the pathogen were demonstrated in vitro when the fungus was exposed to the substrates in liquid suspension culture (20, 23). The enzyme(s) responsible for such digestion has not been isolated.

In this report, we characterize a single proteinase from *C. immitis* with both collagenolytic and elastinolytic activity. The enzyme was isolated from the culture supernatant and cell wall fractions of the saprobic and parasitic phases of two virulent strains. We also demonstrate that this extracellular and wall-associated proteinase is capable of degrading human immunoglobulins. The enzyme may represent an important virulence factor in the development of coccidioidomycosis.

MATERIALS AND METHODS

Cultivation. Two strains of *C. immitis* (C634 and C735) recently isolated from patients with disseminated coccidioidomycosis were used in this study. Conidia were produced in plate cultures on glucose-yeast extract agar and vacuum harvested from 40- to 60-day-old cultures as reported previously (2, 3, 7). During this isolation procedure, a liquid exudate was collected from the surface of the sporulating mycelium which accumulated in plate cultures during conidial morphogenesis. The exudate was separated from the conidia by filtration through an Acrodisc filter assembly (Gelman Sciences, Inc., Ann Arbor, Mich.; pore size, 0.2 μm) attached to a 10-ml disposable syringe. The filtrate was frozen and stored at −20°C until analyzed in the proteolysis assay described below.

The mycelial phase was grown in a liquid medium which was completely defined. The following components were successively dissolved in 950 ml of distilled water: NH_4SO_4 (5.0 g), KH_2PO_4 (2.5 g), NaCl (5.0 g), $MnCl_2 \cdot 4H_2O$ (19.6 μg), and three amino acids (Sigma Chemical Co., St. Louis, Mo.), including leucine (1.3 g), methionine (0.1 g), and phenylalanine (0.5 g). The pH was adjusted to 7.0. Aliquots (100 ml) were dispensed in 250-ml Erlenmeyer flasks, and the medium was autoclaved (15 lb/in² for 20 min). Filter-sterilized biotin (Sigma) was prepared separately (0.02 g/100 ml of distilled water) and added to the medium (1 ml/100 ml of medium). Glucose stock (40% [wt/vol]) was autoclaved separately and added to the medium (5 ml/100 ml of medium). The culture medium was inoculated with a suspension of conidia which had been grown on glucose-yeast extract agar plates as previously described (3, 7). The flasks were incubated in a gyratory shaker (100 rpm) at 30°C for 9 days.

Spherules (parasitic phase) were grown in modified liquid Converse medium (18, 19) at 39°C in the presence of 20% CO_2–80% air for 3 to 8 days. The cultures were inoculated with arthroconidia (approximately 10^8 cells per 125 ml of medium) which had been grown on glucose-yeast extract agar.

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Identification of proteolytic activity. Detection of proteolysis was conducted using Bio-Rad Protease Substrate Gel Tablets (Bio-Rad Laboratories, Richmond, Calif.), which produce a 1% agar gel containing bovine casein in a Tris-buffered physiological saline solution at pH 7.2. Sample wells were cut from the gel, and 250 μg (dry weight) of fungal material in 10 μl of distilled water was added to each well. If proteinase was contained in the sample, it diffused from the wells into the substrate gel, accompanied by digestion of the casein. This formed a transparent ring around the sample wells in the turbid gel. The width of the ring relative to the amount of sample in the well is a crude measure of proteolytic activity. A trypsin solution containing 2.5 to 20 μg/ml of 0.05 M Tris hydrochloride (pH 7.5) plus 0.05 M $CaCl_2$ was added to wells as a positive control. Buffer alone added to wells served as a negative control. The plate was covered and incubated at 25 or 37°C for 12 h. Termination of the proteinase digestion and enhancement of the cleared rings were accomplished by overlaying the plate with a solution of 3% acetic acid followed by a rinse with distilled water. A comparison of the proteolytic activity of selected fractions of C. immitis was based on the relative widths of the transparent rings surrounding the sample wells. The same dry weight of sample was used for each fraction tested.

Isolation of proteolytic fractions. In addition to the crude conidial exudate described above, conidial and spherule wall fractions and culture filtrates were also tested for proteolytic activity. A water-soluble conidial wall fraction (SCWF) isolated by passage of an aqueous suspension of conidia through a Ribi cell fractionator (Model RF-1, refrigerated; Ivan Sorvall, Inc., Norwalk, Conn.) as previously described (2, 3, 7) was tested in the assay described above. The only modification of the previously reported wall isolation procedure was that no inhibtor of proteinase activity (i.e., phenylmethylsulfonyl fluoride [PMSF]) was used during the fractionation process. Chromatographic fractions of the SCWF obtained from a Sephacryl S-300 (Pharmacia, Inc., Piscataway, N.J.) column (bed volume, 318 ml) as described previously were also examined for proteolytic activity.

The mycelial culture filtrate was examined for proteolytic activity. After incubation of the mycelial phase for 9 days as described above, the culture was filtered through two layers of Whatman no. 1 filter paper (Whatman, Inc., Clifton, N.J.), and the filtrate was then passed through a membrane filter (pore size, 0.2 μm; Millipore Corp., Bedford, Mass.). The final filtrate was dialyzed against distilled water (three changes over 72 h at 4°C), and the retentate was lyophilized and stored at −20°C until tested in the proteolysis assay.

The filtrate of 8-day-old spherule cultures and isolated whole spherule wall from 72-h-old cultures were tested for proteolytic activity. The culture filtrate was dialyzed as described above. The spherule wall was prepared as previously described (4). These preparations were also lyophilized and stored at −20°C until tested in the assay.

Purification of proteinase. The Sephacryl S-300 fraction 2 (Fr2) was subjected to a two-step procedure for proteinase purification involving cold acetone extraction followed by gel filtration. This fraction was chosen for the purification procedure because of its high proteolytic activity. The total protein and antigen composition of this Sephacryl fraction has been described (2).

(i) Acetone precipitation. Lyophilized Fr2 (approximately 4 mg) was dissolved in 200 μl of filtered distilled water (4°C) to which 100 μl of cold absolute acetone (−20°C) was added. The suspension was left on ice for 10 min and then centrifuged (10,000 × g for 10 min) in a Microfuge II (Beckman

Instruments, Inc., Fullerton, Calif.). The supernatant was further extracted by the addition of 100 μl of cold acetone and treated as described above. The supernatant obtained from this 50% acetone cut was again extracted by the addition of 400 μl of cold acetone. The pellet obtained after centrifugation of this extract (75% acetone cut) was evaporated to dryness under N_2 and then resolubilized in an enzyme buffer which consisted of 100 mM Tris hydrochloride (pH 8.0) containing 50 mM KCl. The solubilized acetone precipitate was tested for proteolytic activity in the Bio-Rad assay.

(ii) Gel filtration. The dried acetone precipitate resolubilized as described above was applied to a Sephadex G-50 column (Pharmacia; bed volume, 7.5 ml; 200 μg of sample) which was equilibrated at 4°C with enzyme buffer. Fractions were monitored at 280 nm and tested for proteolytic activity in the Bio-Rad assay. After the active fractions from several acetone precipitate fractionations on the Sephadex G-50 column were pooled, the material was dialyzed against distilled water (three changes over 48 h at 4°C), lyophilized, and stored at −20°C until further examination.

Quantitative determination of proteolytic activity. A reaction mixture (total volume, 30 ml) consisting of enzyme buffer (pH 8.0), 10 mg of bovine casein (Sigma) per ml, and 10 μg of the proteinase per ml was incubated at 37°C. Samples (0.5 ml) were removed at various times, and the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid (TCA). After centrifugation of the sample at 10,000 × g for 10 min, the A_{280} of the supernatant was measured and compared with that of a control mixture containing the same components less the proteinase.

Substrate analysis. The specificity of the proteinase was determined by its proteolytic activity on several substrates (Sigma), including human collagen, elastin, and hemoglobin. The proteinase (3 μg in 10 μl of phosphate buffer [pH 7.2]) was added to 200 μl of the enzyme buffer (pH 8.0) containing 200 μg of the protein substrate. An equal volume of distilled water was substituted for the proteinase plus phosphate buffer as a control. After incubation of the mixture at 37°C for 3 h, the reaction was quenched by the addition of an equal volume of 10% TCA, and the sample was centrifuged (10,000 × g for 10 min). The A_{280} of the reaction and control mixtures was compared.

pH profile. The pH optimum of the C. immitis proteinase was measured at 37°C in either 50 mM sodium acetate buffer (pH 4.0 to 6.0) or 50 mM Tris hydrochloride buffer (pH 7.0 to 9.0), with bovine casein, collagen, or elastin as the substrate. The assay mixture consisted of 500 μl of enzyme buffer, 500 μg of substrate, and 25 μg of proteinase. After 1 h of incubation at each pH (4.0 to 9.0), an equal volume of 10% TCA was added and the A_{280} of the supernatant was measured. For each pH step, blanks were measured separately.

Isoelectric focusing. Isoelectric focusing was performed in vertical slab gels by the method of O'Farrell et al. (24). The proteinase (approximately 10 μg) was dissolved in isoelectric focusing sample buffer consisting of 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, and 2% ampholyte (pH 3.5 to 10; LKB Instruments, Inc., Rockville, Md.). The gel (1.5 mm thick) was subjected to 1 W/cm of gel length. Isoelectric focusing standards (pH 3.5 to 9.3; Sigma) were used for calibration.

Inhibitors. Several enzyme inhibitors were surveyed for their ability to decrease or abolish the activity of the proteinase (see Table 2). In the assay, 10 μg of proteinase dissolved in 500 μl of enzyme buffer (pH 8.0) was preincubated for 1 to 2 h at room temperature with a specific

inhibitor (see Table 2 for the final concentration and solvent used for solubilization of each inhibitor). A longer incubation time for PMSF was necessary to assure completion of the reaction (22, 34). The substrate (collagen, 0.5 mg) was subsequently added to the preincubated reaction mixture followed by an additional 1-h incubation at room temperature. As controls, each inhibitor was replaced with the solvent alone. The reaction was terminated by the addition of an equal volume of 10% TCA, and proteinase activity was measured spectrophotometrically as described above.

Amino acid analysis. Approximately 500 μg of purified proteinase was hydrolyzed in 0.4 ml of constant-boiling HCl at 110°C for 24 h in an evacuated and sealed ampoule. Amino acid analysis was performed with a Beckman 121 MB autoanalyzer. Norleucine was injected as an internal standard.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12.5% slab gel and the discontinuous buffer system described by Laemmli (17). Dried preparations of proteinase were resolubilized in a buffer containing 2% SDS, 0.4 M 2-mercaptoethanol, 0.01% bromophenol blue, 10% glycerol, and 60 mM Tris hydrochloride (pH 8.3) and then heated for 3 min at 100°C. Electrophoresis was conducted using a Hoefer mini-gel unit (Hoefer Scientific Instruments, San Francisco, Calif.) and constant current (18 mA) at room temperature for 2 h. Approximately 10 μg of sample was applied to each lane of the gel, which was 1.5 mm thick. The polypeptide bands were revealed by using Coomassie blue R-250 stain (Sigma). Bio-Rad molecular mass standards (14,400 to 92,500) were used for calibration of each gel.

Samples of purified human serum immunoglobulin G (IgG) and purified human secretory IgA (sIgA) (both from Sigma) subjected to proteinase digestion as described below were examined by electrophoresis on a nondenaturing polyacrylamide gradient gel (3 to 10%) by the methods of Margolis and Wrigley (21). Electrophoresis was performed with constant power (2 W) for 7 h at room temperature. Approximately 60 μl of sample was applied to each lane, and the gels were stained with Coomassie blue R-250.

IEP. Tandem two-dimensional immunoelectrophoresis (2D-IEP) was used for identification of the purified proteinase in a previously described coccidioidin/anticoccidioidin (CDN/anti-CDN) antigen reference system (2, 3, 13, 15). Different concentrations of proteinase in the anodal well (1 to 5 mg/ml) and a range of immunoglobulin dilutions in the upper gel (1:5, 1:10, 1:15, or 1:20 dilutions of precipitated immunoglobulin reconstituted to 5 mg of protein per ml in electrophoresis buffer) were used to resolve all possible tandem peaks.

A modification of the 2D-IEP technique was used to examine the humoral reactivity of the proteinase to sera of patients with coccidioidomycosis, as previously described (G. T. Cole, S. H. Sun, J. Dominguez, L. Yuan, and T. N. Kirkland, in E. Drouhet, G. T. Cole, L. De Repentigny, and J. P. Latge [ed.], Proceedings of the First International Symposium on Fungal Antigens, in press). The intermediate gel of the IEP plate was composed of 1 part serum to 1 part 2% agarose. The sera used were obtained either from coccidioidomycosis patients with high complement fixation (CF) antibody titers, as determined by the immunodiffusion assay (12, 14), or from normal human volunteers. The latter served as controls.

Immunoglobulins as substrates. Purified human serum IgG and sIgA were purchased from Sigma. Mixtures containing 5 μg of either IgG or sIgA and 2 μg of purified proteinase in 5

TABLE 1. Proteolytic activity of conidial, mycelial, and spherule fractions of *C. immitis*[a]

Sample (strain)	Relative activity (%)[b]
SCWF (C634)	100
Sephacryl S-300[c]	
Fr1	20
Fr2	80
Fr3a	40
Fr3b	20
Fr4	20
Exudate of sporulating mycelia (C634)	40
Mycelial culture filtrate (C634 and C735)[d]	60
Whole spherule wall (C634)[e]	40
Spherule culture filtrate (C634 and C735)[f]	40

[a] The proteolytic assay was conducted by solubilizing the same dry weight of each sample in distilled water and testing for digestion of bovine casein after incubation at 37°C for 12 h.

[b] The relative activity of each sample was based on the width of the transparent ring surrounding the sample well in a bovine casein-containing agar test plate (Bio-Rad) compared with that produced by the SCWF, which was designated as 100%.

[c] Fr1 to Fr4 were obtained from fractionation of the SCWF (strain C634) on a Sephacryl S-300 column as previously described (2).

[d] Filtrate from 9-day-old mycelial cultures in defined liquid medium.

[e] The spherule wall was isolated by cell homogenization (4) of 3-day-old cultures.

[f] Filtrate from 8-day-old spherule cultures in liquid Converse medium (18, 19).

μl of enzyme buffer were incubated at 37°C for 1, 2, and 6 h. The reactions in the mixture were stopped at the end of the incubation period by the addition of an equal volume of acetate buffer at pH 4.0. Inhibition of hydrolysis of immunoglobulins by the proteinase was examined by incubation of the enzyme and buffer in the presence of 1.0 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (see Table 2) for 1 h followed by incubation of the resultant mixture with either IgG or sIgA as described above for 1 h. All samples were analyzed by electrophoresis with a nondenaturing gradient gel (described above).

RESULTS

Identification of proteinase-containing fractions. The procedure for isolation of the SCWF, which has been described (2, 3, 7), is extremely mild, and the components are not denatured or degraded during the preparatory steps. Fractionation of the SCWF by gel filtration, a procedure which has also been reported, produced reproducible elution profiles, and isolated fractions from successive fractionations reveal the same chemical composition and similar immunoreactivity in both cellular and humoral immunoassays (2). We have also presented evidence, based on immunoelectron microscopy (2), that the SCWF is a complex of macromolecules associated with the cell envelope which can be released from the conidium. For these reasons, we first examined the proteolytic activity of the SCWF and products of its fractionation derived from the Sephacryl S-300 gel filtration column in the Bio-Rad assay. The relative activity of each chromatographic fraction compared with that of the SCWF is given in Table 1. Slightly higher activity (i.e., greater width of the transparent ring) was noted for all fractions incubated at 37°C than at 25°C. Note that Fr2 and the SCWF showed the highest activities. In addition Fr3a showed greater proteolytic activity than did Fr3b. Other samples tested revealed less activity than the SCWF or Fr2. Indication of proteolysis by the Bio-Rad assay upon incuba-
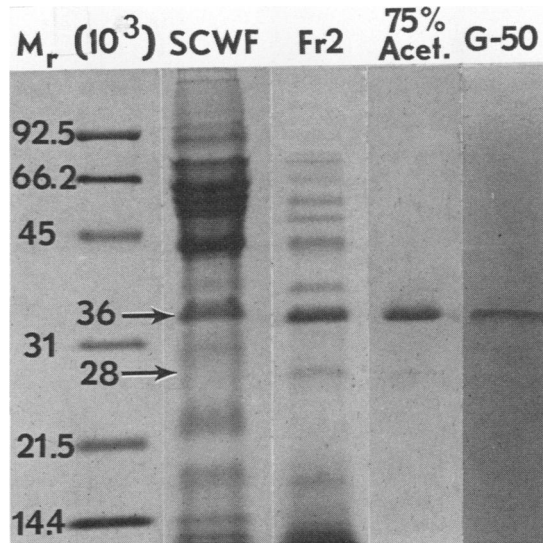
FIG. 1. SDS-PAGE with Coomassie blue stain of SCWF, Fr2 from Sephacryl fractionation of SCWF, 75%-acetone-extracted precipitate of Fr2, and proteolytic fraction obtained from Sephadex G-50 column. Molecular weight standards are also shown.
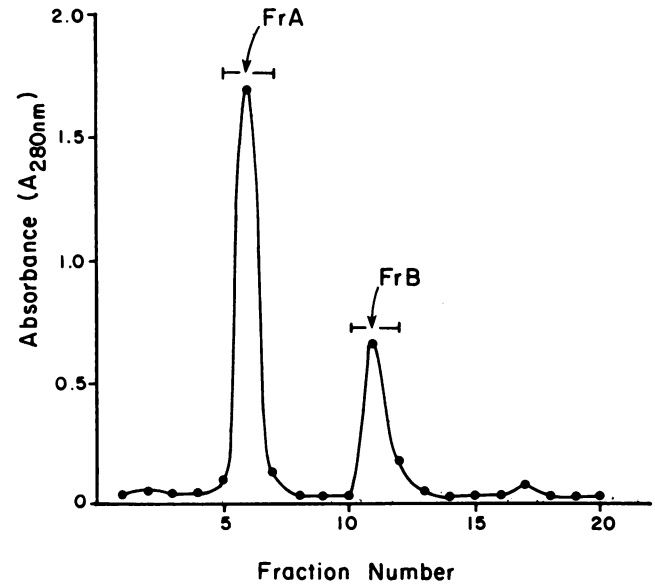

Fraction Number

FIG. 2. Elution profile of Sephadex G-50 gel filtration chromatography of acetone extract of Sephacryl S-300 Fr2. Sample fractions were monitored at 280 nm and eluted with enzyme buffer.

tion of the sporulating mycelial exudate and culture filtrates, as well as isolated wall fractions, with the protein substrate is consistent with the production of an extracellular proteinase.

Isolation and purification of proteinase. SDS-PAGE of the SCWF revealed polypeptide bands corresponding to a M_r range of approximately 10,000 to 100,000 (Fig. 1). A major component was recognized with a M_r of 36,000. After Sephacryl S-300 fractionation of the SCWF, the protein composition of each fraction was compared by SDS-PAGE (2). The 36,000-M_r component was most concentrated in Fr2 (Fig. 1), which also demonstrated the greatest proteolytic activity of the fractions tested (Table 1).

Gel electrophoresis of the pellet obtained by cold acetone extraction of Fr2 revealed the 36,000-M_r polypeptide band and a minor 28,000-M_r band (Fig. 1) after Coomassie blue staining. When tested in the Bio-Rad assay for proteolytic activity, the resolubilized acetone precipitate of Fr2 demonstrated the presence of a proteinase. A typical elution profile resulting from Sephadex G-50 fractionation of the acetone precipitate of Fr2 is shown in Fig. 2. Pooled FA (tubes 5 to 7) and FrB (tubes 10 to 13) were each tested for proteolytic activity, and only FrA was positive. SDS-PAGE of FrA showed a single 36,000-M_r band stained by Coomassie blue (Fig. 1), whereas FrB showed a single 28,000-M_r band (results not shown).

Characteristics of proteinase activity. The activity profile of the purified proteinase with bovine casein as the substrate is shown in Fig. 3. Proteolytic activity showed a linear increase for the first 3.5 h of incubation at 37°C and then gradually reached a plateau during the subsequent 1 to 1.5 h. Very similar activity profiles were revealed when the proteinase was incubated in the presence of human collagen, elastin, and hemoglobin (results not shown). The optimum temperature for activity of the enzyme in the presence of these substrates was 35 to 40°C, and loss of activity was demonstrated when the enzyme was incubated at 60°C for 15 min.

The pH profiles of the enzyme over a range of 4.0 to 9.0 with bovine casein, collagen, or elastin as the substrate were comparable (Fig. 4). High enzymatic activity was demon-

strated between pH 7.5 and 8.5, with an optimum at pH 8.0. Digestion of human hemoglobin demonstrated a similar pH profile and optimum. No significant change in the pH profiles was observed over a temperature range of 25 to 40°C.

The isoelectric point (pI) of the proteinase was localized at pH 4.5. Superior results were obtained in the isoelectric focusing experiments with slab gels as compared with tube gels.
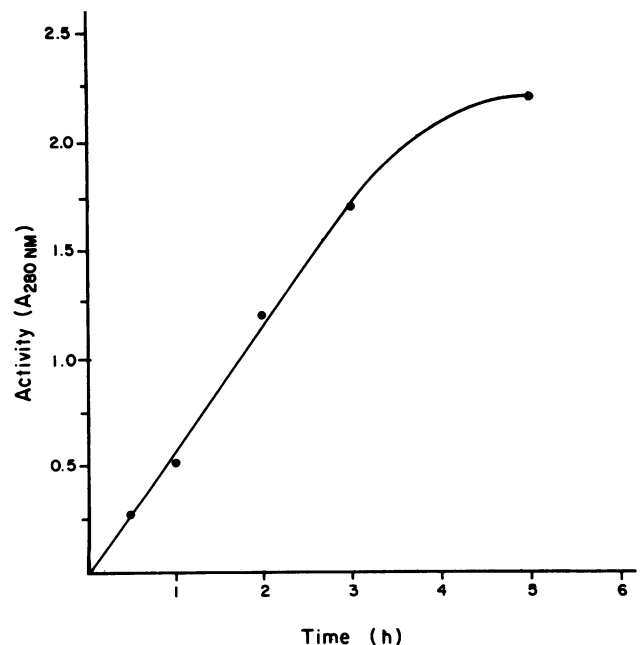

Time (h)

FIG. 3. Activity profile of proteinase in presence of bovine casein (30 µg of proteinase per 300 mg of substrate) incubated at 37°C (pH 8.0).
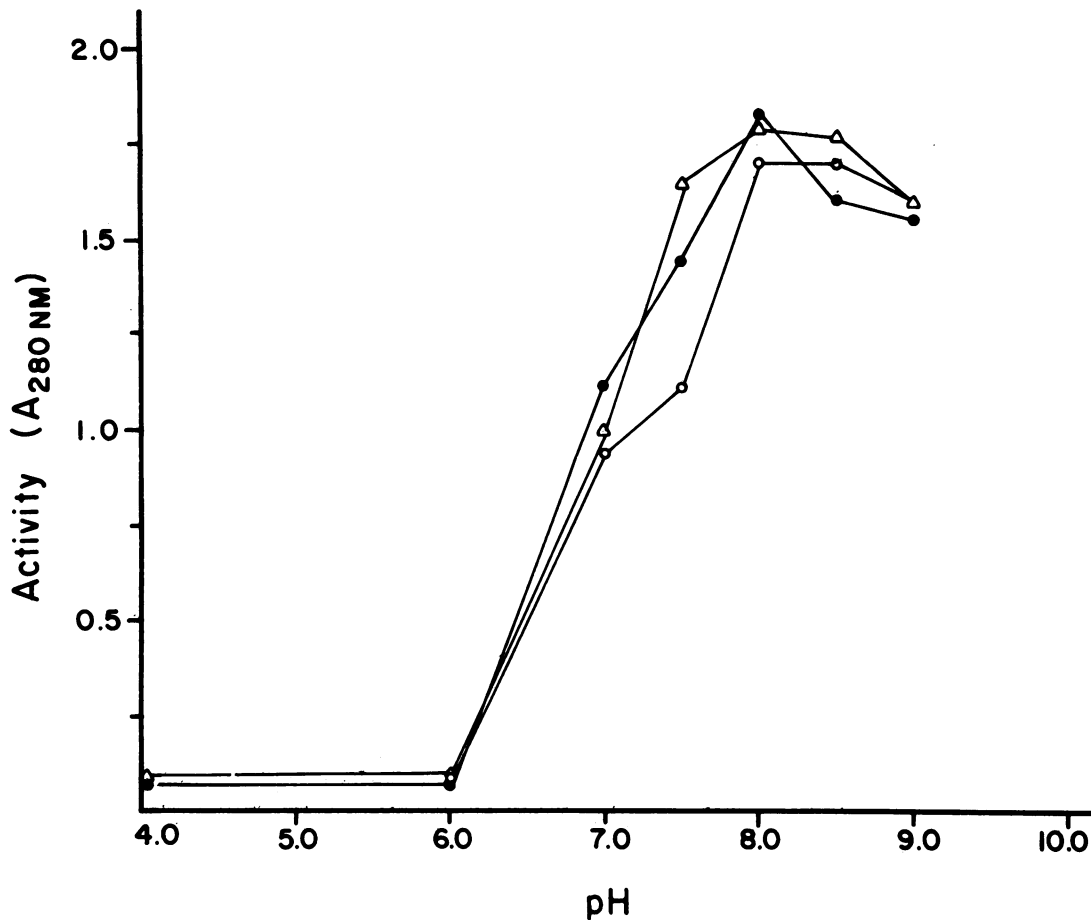
FIG. 4. pH-dependent activity profile of proteinase in presence of bovine casein (●), elastin (○), and collagen (△). Samples were monitored at 280 nm.

TABLE 2. Effect of inhibitors on activity of
C. immitis proteinase[a]

Inhibitor (concn [mM])[b]	Solvent	Incubation time (min)	% Activity of control[c]
None	H₂O	60	100
	0.9 and 1.8% 1-propanol	60	99[d]
		120	99[d]
	0.9% DMSO[e]	60	96[d]
DFP (1.0)	1.8% 1-propanol	60	0
TPCK (1.0)	H₂O	60	0
α-1-Antitrypsin (0.01)	H₂O	60	0
PMSF (1.0)	0.9% 1-propanol	60	60
		120	0
Chymostatin (0.01)	H₂O	60	0
Iodoacetamide (1.0)	H₂O	60	99
N-Ethylmaleimide (1.0)	H₂O	60	100
EDTA (1.0)	H₂O	60	100
Pepstatin (0.01)	0.9% DMSO	60	96
TLCK (1.0)	H₂O	60	100

[a] Proteinase obtained from a Sephadex G-50 column was dialyzed, lyophilized, and resolubilized in enzyme buffer.

[b] DFP, Diisopropylphosphorofluoridate; TLCK, N-p-tosyl-L-lysine chloromethyl ketone. DFP, TPCK, PMSF, EDTA, and TLCK were obtained from Sigma.

[c] Proteinase dissolved as indicated in footnote a and incubated with collagen at room temperature served as the control.

[d] Compared spectrophotometrically with H₂O blank.

[e] DMSO, Dimethyl sulfoxide (Sigma).

Inhibition of activity. The effects of a wide range of inhibitors on the activity of the proteinase are presented in Table 2. Note that all solvents used had virtually no effect on enzyme activity. Inhibition of activity was clearly demonstrated by diisopropylphosphorofluoridate, TPCK, α-1-antitrypsin, chymostatin, and PMSF but not by the other inhibitors tested.

Amino acid composition. The amino acid composition of the proteinase is presented in Table 3. Note the relatively high content of aspartic acid, threonine, glutamic acid, proline, and glycine and low content of methionine, tyrosine,

TABLE 3. Amino acid composition of C. immitis proteinase[a]

Amino acid	No. of residues	Amino acid	No. of residues
Lysine	19	Alanine	25
Histidine	9	Cysteine	3
Arginine	13	Valine	19
Aspartic Acid	36	Methionine	2
Threonine	31	Isoleucine	13
Serine	24	Leucine	19
Glutamic Acid	38	Tyrosine	5
Proline	36	Phenylalanine	11
Glycine	35	Tryptophan	ND[b]

[a] Proteinase was obtained from Sephadex G-50 column, dialyzed, and lyophilized, and 500 μg was hydrolyzed for amino acid analysis.
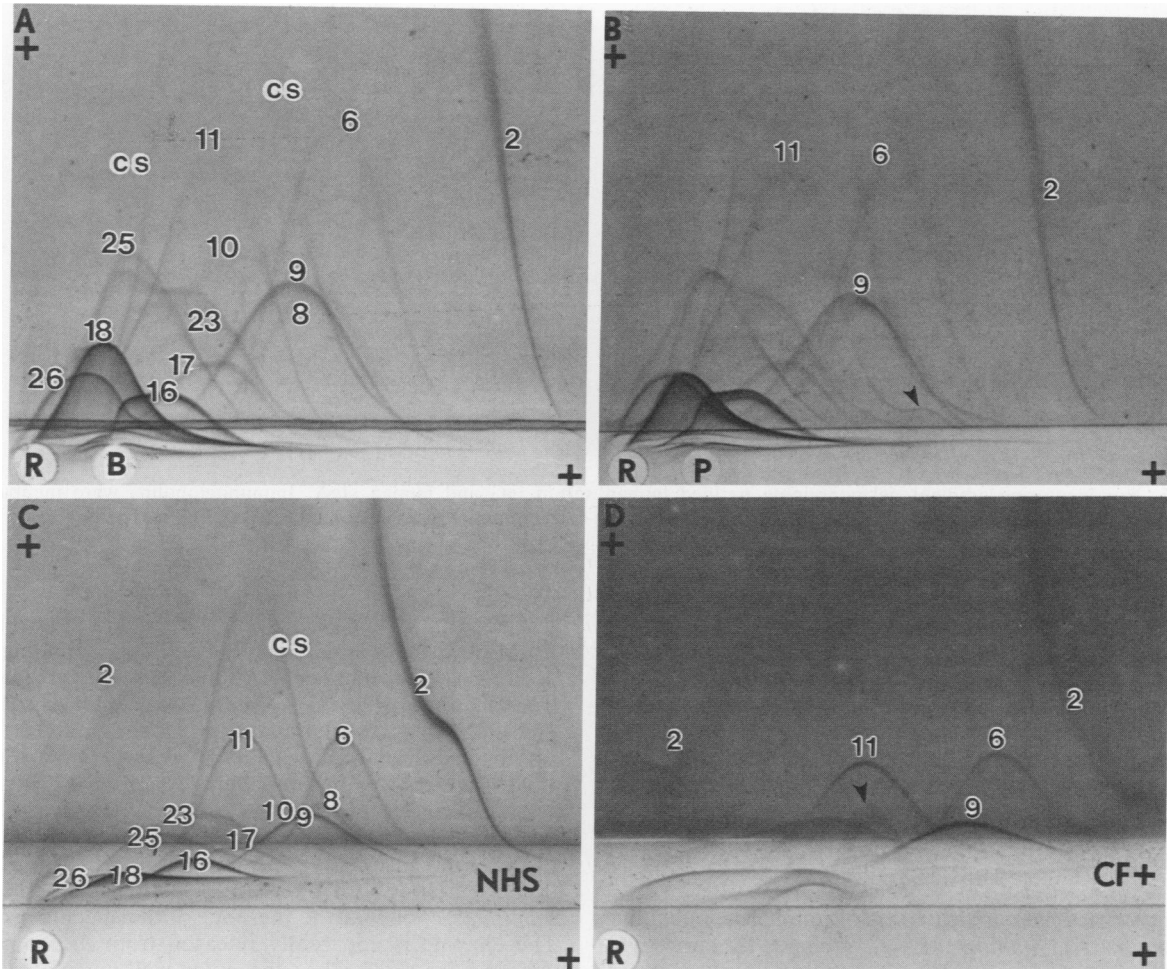
[b] ND, Not detected.

FIG. 5. (A and B) Tandem 2D-IEP gels showing precipitin peaks of reference antigen (R) and phosphate buffer (B) compared with reference antigen and proteinase (P). The arrowhead in panel B indicates single tandem peak of Ag11. Precipitin peaks are designated in accordance with a previously described reference system (13, 15). The upper gel contained reference immunoglobulin diluted 1:15 in electrophoresis buffer. (C and D) Intermediate gels of 2D-IEP plates containing either normal human serum (NHS) or serum from CF$^+$ patient. The upper gel contained reference immunoglobulin diluted 1:10 in electrophoresis buffer. The arrowhead in panel D locates the precipitin peaks of AgCS. The anodes (+) indicate the direction of migration in each dimension.

and cystine. The absence of tryptophan may have been due to destruction during hydrolysis.

Identification of proteinase in CDN/anti-CDN reference system. A tandem 2D-IEP gel containing reference antigen (CDN) in the cathodal well and buffer (phosphate-buffered saline) in the anodal well is shown in Fig. 5A. The immunoglobulin (anti-CDN) dilution in the upper gel was 1:15 in electrophoresis buffer. The precipitin peaks of the reference antigen are designated in accordance with previous reports (2, 5, 13, 15). When the proteinase was added to the anodal well, a single tandem peak with reference antigen 11 (Ag11) was observed (Fig. 5B). No other tandem peaks were observed with the complete range of immunoglobulin dilutions indicated above.

In Fig. 5C, reference Ag11 has migrated through an intermediate gel containing normal human serum. Note the location of the other precipitin peaks in this control plate (e.g., Ag2 and AgCS). In Fig. 5D, the intermediate gel contained serum from a coccidioidomycosis patient which reacted positively in the immunodiffusion test for CF anti-

body (CF$^+$). Little change in the position of the Ag11 peak is visible, in contrast to marked reductions in the heights of the Ag2 and AgCS peaks. Whereas Ag2 and AgCS were partially precipitated in the intermediate gel as a result of adsorption to specific antibody from the patient, only minor precipitation of Ag11 occurred in the intermediate gel, suggesting the presence of little antibody against this antigen in the serum of the patient. Five different CF$^+$ sera from patients tested in this manner revealed comparable results.

Proteolysis of human immunoglobulins. The nondenaturing gradient gel containing the purified human sera IgG and sIgA samples (Fig. 6) shows distinct bands for the untreated immunoglobulins (IgG and sIgA) and immunoglobulins which were incubated with the proteinase pretreated with TPCK. On the other hand, immunoglobulins incubated with the proteinase for 1 to 6 h at 37°C demonstrated the absence of these distinct bands. Instead, lower-M_r bands were revealed after 1 to 2 h of incubation with the proteinase, and virtually no bands were visible after 6 h of incubation. Human sera were also collected from healthy volunteers
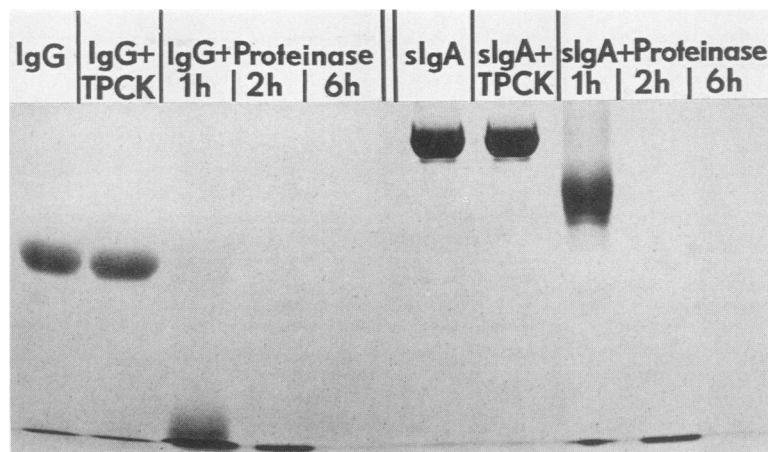
FIG. 6. Nondenaturing gradient gel containing purified human serum IgG and human sIgA. Immunoglobulins were incubated with proteinase which had been pretreated with TPCK or incubated with active proteinase for 1, 2, and 6 h (pH 8.0) at 37°C.

who were shown to be CF⁻ by the immunodiffusion assay (14). Serum samples were incubated with the proteinase as described above. After analysis of the reaction mixture by electrophoresis as described above, followed by Western blotting with goat anti-human IgG (heavy and light chains) and anti-goat IgG alkaline phosphatase conjugate (Promega Biotec, Madison, Wis.), evidence for the digestion of IgG by the *C. immitis* proteinase was also obtained (data not shown).

DISCUSSION

Recent studies have indicated that the inhaled, infectious arthroconidia of *C. immitis* are well equipped for survival in the hostile environment of the host. It has been suggested that the hydrophobic, outer conidial wall layer provides protection to the fungal cell against phagocytosis and biochemical attack (8). The outer wall layer has also been shown to be immunosuppressive in an immune lymph node proliferation assay (T. N. Kirkland and G. T. Cole, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 787, 1986; 2), and efforts are under way in our laboratory to isolate and characterize the immunosuppressive factor(s). The conidial envelope has been shown to be the source of immunoreactive, water-soluble macromolecules which can elicit both cellular and humoral responses in the host (2). In the present investigation, we showed that this same pool of water-soluble, conidial wall-associated macromolecules (i.e., the SCWF) includes a proteinase capable of digesting elastin and collagen (major structural proteins of the lungs; 10), as well as sIgA (immunoglobulin that provides antibody defense to mucosal surfaces; 25). The arthroconidium of *C. immitis* is the vanguard of the invasive process, and the nature of its interaction with host tissues during initial insult may significantly influence subsequent events of the fungal disease.

Of the wall and culture filtrate fractions tested for proteolysis in the Bio-Rad assay, the highest activity was found in the SCWF, obtained by mechanically stripping arthroconidia of their hydrophobic, surface wall component. The possibility exists that more than one proteinase may be present in these crude fractions. In addition, the content of the medium may be a factor influencing induction of the proteinase (16, 31); this may be particularly significant in mycelial and

spherule cultures. Both aspects are under investigation in our laboratory. Each sample, however, was shown by SDS-PAGE to contain a polypeptide band corresponding to a M_r of 36,000 which characterizes the isolated enzyme. After fractionation of the SCWF by size exclusion chromatography, the most proteolytic fraction (Fr2) contained the highest concentration of the 36,000-M_r protein. Fr3a, Fr3b, and Fr4, which showed progressively less of the 36,000-M_r band on the basis of comparative SDS-PAGE (2), revealed a corresponding decrease in proteolytic activity. Identification of the proteinase as Ag11 in the CDN/anti-CDN reference system provides an alternative method of screening *C. immitis* fractions for the presence of the specific proteinase. The enzyme is apparently released from the conidial and spherule envelope when the outer wall layer of the cell is mechanically removed (4). The proteinase was also released from mycelia and parasitic cells (i.e., spherules) of both strains tested when they were grown in vitro (Table 1).

Immunoelectron-microscopic examinations of thin sections of arthroconidia, which were reacted with anti-SCWF rabbit antibody, have demonstrated that the antigenic components of the SCWF are, for the most part, trapped within the thin, hydrophobic, outer wall layer (2). The labeled macromolecules were mainly located in the inner conidial wall and the intermural space between the inner and outer wall layers. However, the fragile, sleevelike outer wall of the conidium may be dislodged during inhalation by the host and has been shown to fracture as the conidium swells during initial stages of transformation into a young spherule (4, 6, 22). It is suggested, therefore, that soon after inhalation of arthroconidia the proteinase is released from the hydrophobic envelope and interacts with the host surface. Immunoelectron-microscopic examinations, which are in progress involve determination of the exact location of Ag11 in different cell types of *C. immitis*, both in vitro and in vivo, by using monospecific antibody raised against the purified proteinase.

Isolation and purification of the proteinase from the conidia of *C. immitis* by cell fractionation, acetone extraction, and gel filtration procedures are relatively straightforward. Preparative PAGE of the cold acetone precipitate of Fr2 was also used to purify the proteinase. The recovered 36,000-M_r polypeptide band demonstrated proteolytic activity in the Bio-Rad assay. However, the amount of enzyme

recovered was low, and therefore, gel purification was abandoned in this investigation. Preparative 2D PAGE of FrA from the Sephadex G-50 column has revealed a single spot in the gel. The purified proteinase has been isolated from the 2D gel for amino acid sequence studies, which are in progress.

The characteristics of the C. immitis proteinase are as follows: M_r, 36,000 (based on SDS-PAGE with appropriate standards); pH optimum, 8.0 to 9.0; temperature optimum, 35 to 40°C at pH 8.0; stability, pH 4.0 to 9.0 and temperature to 50°C; pI, 4.5. Substrates were human collagen, elastin, hemoglobin, IgG, sIgA, and bovine casein. Inhibitors were diisopropylphosphorofluoridate, TPCK, α-1-antitrypsin, PMSF, and chymostatin. The enzyme has a broad pH and temperature range, and near optimal activity was demonstrated under simulated, physiological conditions. The alkaline pH optimum, inhibition of activity by organofluorides (e.g., DFP and PMSF), as well as by TPCK, chymotastin, and α-1-antitrypsin, and the ability to hydrolyze characteristic chymotrypsin substrates are features of a chymotrypsinlike serine proteinase (33–35). However, additional studies of substrate specificity using natural and synthesized peptides of known sequence are necessary to identify the sites of enzyme hydrolysis (35).

Evidence is presented in this report that the proteinase of C. immitis can cleave both human serum IgG and sIgA in vitro. Production of proteinases by pathogenic bacteria and yeast which cleave various human immunoglobulins, including IgG and sIgA, is well documented (25, 28, 30). Cleavage of secretory immunoglobulins by Candida albicans and Candida tropicalis has been correlated with the potential of these yeasts to colonize the mucosa and cause thrush (27, 29). Some inhaled arthroconidia of C. immitis probably become lodged in bronchioles and associate with the mucosal surface. Release of proteinase from the pool of water-soluble macromolecules located within the outer sleeve of the conidial wall may play an important role in early host-parasite interaction in these regions of the respiratory tract. In addition, release of the same proteinase from parasitic cells located in systemic foci of infection may result in interaction of the proteinase with circulating immunoglobulins and thereby compromise host defense against the fungal pathogen. The apparent low levels of antiproteinase (Ag11) antibody in sera of CF+ patients requires further investigation. Although it is tempting to speculate that release of the enzyme identified in this study represents a virulence factor, further in vivo studies comparing animals challenged with the proteinase-producing strain and the derived nonproteolytic mutant and proteolytic revertant (16) are necessary before conclusions can be drawn.

ACKNOWLEDGMENTS

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