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Isolation of Antigens with Proteolytic Activity from *Coccidioides immitis*

GARRY T. COLE,[1]* SHIWEN ZHU,[1] SHUCHONG PAN,[1] LING YUAN,[1] DAVID KRUSE,[1] AND S. H. SUN[2]

Department of Botany, University of Texas, Austin, Texas 78713-7640,[1] and Veterans Administration Hospital, San Antonio, Texas 78284[2]

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Three antigens with proteolytic activity have been isolated from crude, water-soluble fractions of the saprobic phase of the fungal pathogen *Coccidioides immitis*. Two proteinases, identified in our immunoelectrophoresis reference system as Ag11 and AgCS, were isolated from the soluble conidial wall fraction (SCWF). Ag11 was previously shown to be a serine proteinase and was characterized in this study as a 60-kilodalton (kDa) fraction by gel filtration (GF). The purified proteinase demonstrated little or no reactivity with 21 serum samples from coccidioidomycosis patients in the enzyme-linked immunosorbent assay; this may be due to limited presentation of this antigen to the host during the course of coccidioidomycosis. AgCS was separated by GF chromatography into two fractions identified by molecular masses of 39 and 19 kDa. Most proteolytic activity was shown by substrate gel electrophoresis to be associated with the lower-molecular-mass fraction. AgCS was reactive with 18 of the 21 serum samples and shown to be the major component of a heat-stable antigen previously reported to be immunospecific for *C. immitis*. The third antigen with proteolytic activity was isolated from the 5-day mycelial culture filtrate and identified by GF as a 56-kDa fraction. Uniformly high levels of immunoreactivity between 18 of the 21 patient sera and the 56-kDa antigen were demonstrated. Antigens with proteolytic activity may play important roles in fungus-host interactions as well as morphogenesis of the pathogen.

Immunological examinations of crude and purified antigens isolated from *Coccidioides immitis*, a primary fungal pathogen of humans (8) which causes a rare but sometimes fatal respiratory mycosis (12), have identified reactive fractions which appear to play significant roles in host cellular and humoral immune responses against coccidioidomycosis (1, 2, 5, 10). Few studies of these antigenic fractions, however, have been directed toward elucidation of the biological nature and function of the immunoreactive macromolecules (11). Several recent investigations have focused on characterization of the proteinases of the saprobic and parasitic phases of *C. immitis* and the possible roles of these products in morphogenesis of the pathogen and host tissue invasion (24, 28, 30). We have isolated one such proteinase from the soluble wall fraction of the infectious conidia (SCWF [5]) and identified it in our coccidioidin–anti-coccidioidin (CDN–anti-CDN) immunoelectrophoresis reference system as antigen 11 (Ag11) (29). This product was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a protein with relative molecular weight (M_r) of 36,000 under reducing conditions (29). The enzyme was classified as a serine proteinase, shown to be capable of digesting human collagen and elastin (29), and suggested to participate in sporulation during the parasitic cycle (30). Because of the recognized proteolytic activity of the crude SCWF and mycelial culture filtrate of *C. immitis* (24, 29), we decided to use these soluble fractions as sources of additional proteinases. In this study we further characterize the protein composition and immunoreactivity of Ag11 and compare these aspects with features of two additional antigens of *C. immitis* that have proteolytic activity. These two antigens are shown by our CDN–anti-CDN reference system and SDS-PAGE (under reducing conditions) to correspond to AgCS and a protein with M_r 66,000, respectively. The 66,000-M_r proteinase was not identified as a component of the CDN–anti-CDN reference antigen system and, therefore, cannot be given a numerical designation. The two newly isolated proteinases were demonstrated earlier to be antigenic components of an immunoreactive, detergent soluble fraction obtained from the envelope of *C. immitis* spherules grown in vitro (4, 7). Characterization of antigens that have proteolytic activity and are derived from crude, soluble fractions of the cultured pathogen may permit the recognition of products which significantly influence interactions of host and pathogen, as well as the identification of macromolecules which could potentiate morphogenetic changes.

MATERIALS AND METHODS

Cultivation. Arthroconidia of *C. immitis* 634 and 735 (recent isolates from patients with disseminated coccidioidomycosis) were produced in plate culture and isolated by vacuum harvesting (5). The mycelial phase was grown in liquid medium consisting of 1% glucose plus 0.5% yeast extract. The flasks were incubated in a gyratory shaker (100 rpm) at 30°C for 5 days.

Isolation of crude antigenic fractions. The water-soluble SCWF was isolated by a previously described method (6). The mycelial culture filtrate was collected as previously reported (30). The heat-stable (HS) antigen, which has been reported as a *C. immitis*-specific antigen identified in immunodiffusion (ID) tests (21, 22), is referred to in this report as the ID-HS antigen and was kindly provided by L. Kaufman, Centers for Disease Control, Atlanta, Ga. Its isolation from the mycelial culture filtrate of *C. immitis* has been described previously (21, 22). The preparation of the ID-HS antigen used in this study is a multicomponent antigen designated as

* Corresponding author.

CIA-1-82 and cited in an earlier study which evaluated the immunodiagnostic potential of this antigen (22).

2D-IEP. Tandem two-dimensional immunoelectrophoresis (2D-IEP) was used in this study to identify the antigenic components of crude and purified fractions by using a CDN–anti-CDN reference system. The latter was recently developed in our laboratory and is based on the system reported by Huppert et al. (16, 20). The reference antigen (CDN) was prepared as reported earlier (5). The burro anti-CDN immunoglobulin in the upper gel was diluted 1:10 in electrophoresis buffer (16). Precipitin arcs were identified in the 2D-IEP plate by following the established numbering and lettering code of the CDN–anti-CDN reference system (16, 20).

Antigen purification. The serine proteinase (Ag11) was first concentrated by cold-acetone extraction of the SCWF as previously reported (29) and then isolated by immunoaffinity chromatography as described below. The monospecific antiserum used for isolation of Ag11 was raised against the purified proteinase obtained by gel filtration as described in our earlier reports (29, 30). AgCS was also first concentrated by cold-acetone extraction of the SCWF. The concentrate was subjected to electrophoresis separation under nonreducing conditions, and AgCS was isolated by electroelution (14, 26) as described below. The 66,000-M_r fraction had been shown by SDS-PAGE to be a major component of the crude proteolytic mycelial culture filtrate. We decided to examine this immunoreactive component for proteolytic activity. The 66,000-M_r fraction was concentrated by cold-acetone extraction of the 5-day mycelial culture filtrate and then isolated by electrophoretic separation and electroelution. The acetone-precipitated 66,000-M_r fraction, however, lacked proteolytic activity when examined by the substrate gel electrophoresis procedure outlined below. Nevertheless, the electroeluted fraction was used to immunize rabbits for production of monospecific antiserum. Subsequent isolation of the active 66,000-M_r fraction from the 5-day mycelial culture filtrate involved a two-step process. The culture filtrate was first separated by anion-exchange chromatography, and the 66,000-M_r proteinase was then isolated from selected fractions by immunoaffinity chromatography with the specific rabbit antiserum. Homogeneity of the Ag11, AgCS, and 66,000-M_r preparations was determined by SDS-PAGE under reducing and nonreducing conditions. The specificity of the rabbit antiserum raised against the electroeluted 66,000-M_r fraction was demonstrated by immunoblotting as described below.

(i) Acetone precipitation. To concentrate Ag11 and AgCS, we subjected the lyophilized SCWF (approximately 10 mg) to successive steps of cold absolute acetone extraction as described previously (29). The final precipitate obtained (75% acetone cut) was evaporated to dryness under N_2 and stored at −20°C. The lyophilized 5-day mycelial culture filtrate (approximately 4 mg) was dissolved in 400 μl of distilled water (4°C), and the suspension was centrifuged (10,000 × *g* for 10 min) in a Microfuge II (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was extracted by the addition of 200 μl of cold absolute acetone. The mixture was left on ice for 10 min and then centrifuged as above. The pellet resulting from this extraction (33% acetone cut) was dried as above and stored at −20°C.

(ii) Electroelution of SDS-PAGE gels. Initial separation of components of the acetone precipitates obtained by extraction of the SCWF and 5-day mycelial culture filtrate was performed by SDS-PAGE under nonreducing conditions. Electrophoresis was conducted separately with a 14% slab gel and the discontinuous buffer system described by Laemmli (23). The dried acetone precipitates were resolubilized in a buffer containing 0.4% SDS, 0.025% bromophenol blue, 20% glycerol, and 0.025 M Tris hydrochloride (pH 6.8) and applied to the gel. The conditions used for electrophoresis were the same as described previously (29). After completion of electrophoretic separation, the lane containing the standards and the adjacent lane to which the sample had been added were cut from the gel and stained with Coomassie blue R-250 (Bio-Rad Laboratories, Richmond, Calif.). The gel bands of interest were located in the unstained gel by their careful realignment next to the stained lanes. The bands were excised from the former, minced, and subjected to electroelution in an electroseparation system (Elutrap; Schleicher & Schuell, Inc., Keene, N.H.) by modifications of the procedure described by Hunkapiller et al. (14). Electroelution was conducted with a constant voltage (200 V) at room temperature for 3 h. The elution buffer consisted of 0.05 M Tris acetate (pH 9.0) plus 0.02% SDS. The dialysis membrane disks used in the Elutrap had molecular mass cutoff values of 3 to 5 kilodaltons (kDa). The electroeluted sample was removed from the Elutrap and dialyzed against distilled water (cellulose dialysis tubing; molecular mass cutoff of 6 to 8 kDa; Medical Industries Inc., Los Angeles, Calif.) at 4°C for 48 h (three changes of dialysate). The retained material was lyophilized and stored at −20°C.

(iii) Ion-exchange chromatography. Anion-exchange chromatography was performed on DEAE-Sephacel (Pharmacia, Inc., Piscataway, N.J.). The lyophilized, 5-day mycelial culture filtrate (100 mg) was solubilized in 20 mM citrate buffer (pH 6.5). The sample was applied to the column (bed volume, 286 ml) and eluted with the same buffer until the A_{280} of the effluent returned to base-line values. The bound fractions containing the 66,000-M_r proteinase were then eluted with 200 mM citrate buffer (pH 6.2) and monitored by A_{280}.

(iv) Production of rabbit antisera. New Zealand White rabbits (two females, approximately 2.5 kg each) were immunized subcutaneously with the 66,000-M_r fraction isolated by electroelution as described above. The immunization protocol was the same as described previously (30).

(v) SDS-PAGE and immunoblotting. The procedures for SDS-PAGE conducted under reducing conditions and immunoblot analysis have been described previously (30). The rabbit antiserum raised against the isolated 66,000-M_r fraction was used at a 1:200 dilution in phosphate-buffered saline (PBS; pH 7.4). After being washed, the nitrocellulose membrane (Promega Biotec, Madison, Wis.) was incubated with goat anti-rabbit immunoglobulin G (IgG; heavy and light chain [H+L] specific) conjugated to peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) at a 1:200 dilution in PBS. Nitrocellulose membranes to which proteins had been transferred and then reacted with either the preimmune rabbit serum, or secondary antibody-conjugate alone, served as controls.

(vi) Immunoaffinity chromatography. Solid-phase immunoadsorption was performed by modifications of the method of Cox and Britt (9). The immunoglobulin fractions (anti-Ag11 or anti-66,000 M_r) were covalently coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.). The dried acetone precipitate of the SCWF (4.0 mg), or the pooled DEAE fraction of the 5-day mycelial culture filtrate (4.0 mg), was solubilized in PBS (pH 7.4) and applied to the anti-Ag11 and anti-66,000-M_r immunoaffinity columns, respectively. The antibody-

bound fraction was eluted with 0.1 M acetate buffer (pH 4.0). The eluate and effluent fractions were dialyzed against distilled water (4°C for 48 h, with four changes of dialysate) and lyophilized separately.

Characterization of antigens. The protein composition of the purified antigens (Ag11, AgCS, and 66,000-M_r) was examined by SDS-PAGE under nonreducing and reducing conditions as described above. Additional methods used in characterizing these antigens are described below.

(i) Interrupted gel electrophoresis. The procedure of Gurusinghe et al. (13) was used for detection of disulfide-bonded polypeptide components in the immunoaffinity-purified 66,000-M_r fraction. SDS-PAGE was performed with a 12.5% slab gel. Approximately 250 μg of sample was applied to each of three adjacent lanes, and electrophoresis was initially conducted at 18 mA until the dye front had just entered the separating gel. The power was disconnected, and 10 μg of 20% 2-mercaptoethanol was added to the outer two lanes only. After 5 min, electrophoresis was resumed at 18 mA until the tracking dye reached the bottom of the gel. Gels were stained with Coomassie blue R-250.

(ii) Gel filtration by high-pressure liquid chromatography. Each purified antigen preparation (approximately 200 μg) was resolubilized in filtered distilled water, applied to a Superose 12 HR 10/30 gel filtration (GF) column (10 by 300 mm; Pharmacia), and subjected to high-pressure liquid chromatography in a model HP1090 liquid chromatograph equipped with a diode array detector (Hewlett-Packard Co., Palo Alto, Calif.). GF standards (Sigma) (see Fig. 5) were prepared in the same manner and separated by high-pressure liquid chromatography. All samples were eluted with 0.025 M Na_2HPO_4 buffer (pH 7.4) at a flow rate of 0.25 ml/min. The fractions were simultaneously monitored at 214, 254, and 280 nm. Molecular weight estimates of the nonreduced antigens were based on comparison of retention times between samples and standards.

(iii) Substrate gel electrophoresis. Simultaneous detection of proteolytic activity and determination of molecular mass of the crude and purified antigen fractions were performed by modifications of the method reported previously (28). Polyacrylamide (7.5 to 12%) was copolymerized with 1% gelatin (type B from bovine skin; Sigma). Approximately 10 μg of each sample was solubilized separately in 15 μl of sample buffer (0.125 M Tris hydrochloride [pH 6.8], 0.025% bromophenol blue, 0.4% SDS, 20% glycerol). Electrophoresis was conducted at 12.5 mA for 1.5 h at room temperature. The gel was washed in 100 ml of 2.5% Triton X-100 for 1 h to remove the SDS and transferred to the incubation buffer (10 mM Tris hydrochloride, 5.0 mM $CaCl_2$, 1.0 mM $MgSO_4$), which was adjusted to pH 6.8, 7.0, 7.4, or 8.0 to test for the ability of each isolated fraction to digest gelatin. The gel was incubated at 37°C for 12 h and finally stained with Coomassie blue R-250.

(iv) Immunoglobulins as substrates. The ability of AgCS and the chromatographically purified 66,000-M_r fraction to digest purified human IgG and secretory IgA (sIgA) was tested by using the method described in an earlier report (29). Ag11 and AgCS were incubated at pH 8.0 and 7.2, respectively. The 66,000-M_r fraction was tested for its ability to digest immunoglobulins over a pH range of 6.8 to 8.0.

(v) ID. ID tests were performed by the method of Huppert and Bailey (17–19) for detection of complement-fixing (CF) and tube-precipitin (TP) antibodies in human serum samples which react with the *C. immitis* reference antigens. The reference antisera and antigens used were the same as previously reported (4).

(vi) ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed with an indirect screening kit (Kirkegaard and Perry) by the method described previously (4). Serum samples from 21 coccidioidomycosis patients (from the Veterans Administration Hospital, San Antonio, Tex.) and 21 control serum samples from hospital admissions with no systemic or pulmonary mycoses (provided by Athis da Silva, Brackenridge Hospital, Austin, Tex.) were tested. All coccidioidomycosis patient samples used in this study were determined to be CF positive and four were TP positive on the basis of the standardized ID test described above. All control sera were determined to be negative in the ID-CF and ID-TP tests.

The products examined in the ELISA included the SCWF, 5-day mycelial culture filtrate, immunoaffinity-purified Ag11 and 66,000-M_r proteinase, electroeluted AgCS, and the ID-HS antigen. The procedures for incubation of antigens, application of blocking reagents, and washing were the same as previously reported (4). Optimal antigen concentrations were determined by block titration (4). An antigen concentration of 10 μg/ml of buffer and a 1:200 dilution of test sera (control and patient serum samples) in blocking solution were chosen as the standards for comparative purposes. The antigen concentration used was determined to be optimal for each test antigen. The particular serum dilution was chosen because it fell in the midpoint of the optimal dilution range of the test sera which were titrated in the ELISA. Test sera with an absorbance higher than the mean of the control sera plus twice the standard deviation were considered to be positive (4). Assays with sera in the absence of antigens and with antigens in the absence of sera served as controls. All sera were tested in triplicate wells.

The possible influence of proteolysis of immunoglobulins by the serine proteinase (29) on the binding capacity of patient antibody to Ag11 in the ELISA was examined. The antigen bound to wells of the microdilution plate as described above was first incubated with blocking reagent (4) plus 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) solubilized in 0.9% 1-propanol (29) for 2 h at room temperature. The wells were then washed as previously reported (4). Preincubation of antigen with PMSF prior to addition of negative sera was used as a control assay.

Competitive inhibition ELISAs were also conducted to determine whether the purified AgCS was the major immunoreactive component of the ID-HS antigen. Patient serum samples were preincubated with different concentrations of AgCS before being applied to the ID-HS antigen-coated wells (10 μg/ml) of the microdilution plate. Six patient serum samples showing a range between high and low optical density (OD) values in the ELISA when tested against the ID-HS antigen were chosen. Each serum sample (150 μl diluted 1:200 in blocking solution) was preincubated with 15 μl of either the ID-HS antigen, AgCS, or Ag11 at concentrations ranging from 0.05 to 500 μg/ml of PBS. The sera preincubated with PBS alone served as controls. Preincubation was carried out at 4°C for 14 h. Subsequent steps of the ELISA were conducted as described above. The percent inhibition of patient serum binding to the ID-HS antigen in the ELISA was calculated as follows: [(OD of serum preincubated with PBS − OD of serum preincubated with Ag)/OD of serum preincubated with PBS] × 100.

RESULTS

Composition and proteolytic activity of crude antigenic fractions. The anodal well of the tandem 2D-IEP plate in Fig.

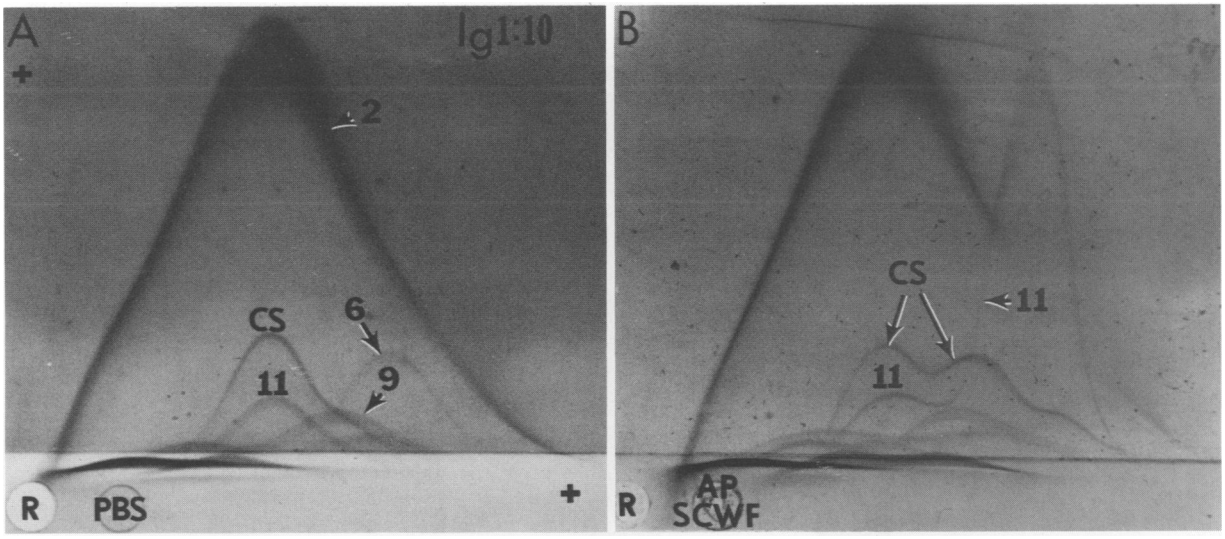

FIG. 1. Tandem 2D-IEP gels of reference antigen (CDN) (R) and acetone precipitate of the soluble conidial wall fraction (AP-SCWF). The cathodal well contained the reference antigen (10 μg in 20 μl of PBS), and the anodal well contained either 20 μl of PBS (A) or 200 μg of the test antigen (AP-SCWF) in 20 μl of PBS (B). The upper gel contained burro anti-CDN immunoglobulin diluted 1:10 in electrophoresis buffer. The plus signs indicate the anodes and directions of migration in each dimension. Precipitin peaks are labeled according to the CDN–anti-CDN reference system (8, 17, 21). Prominent tandem peaks with AgCS and Ag11 are shown in panel B.

1B contained the acetone precipitate (75% acetone cut) of the SCWF. On the basis of comparison of the pattern of precipitin arcs in this plate with that of the control (Fig. 1A), the two prominent tandem peaks shown in Fig. 1B were identified as Ag11 and AgCS.

The results of examination of the protein composition of the SCWF acetone precipitate in SDS-PAGE gels under nonreducing and reducing conditions are shown in Fig. 2. Two prominent polypeptide bands in the nonreduced sample are of M_r 62,000 and 38,000, whereas the reduced sample was characterized by bands with M_r 36,000 and 19,000. The acetone precipitate of SCWF demonstrated proteolytic activity when examined by substrate gel electrophoresis (Fig. 2). Unstained, digestion bands with M_r ca. 66,000 and 19,000 were revealed after electrophoresis of the sample and incubation of the substrate gel at pH 8.0.

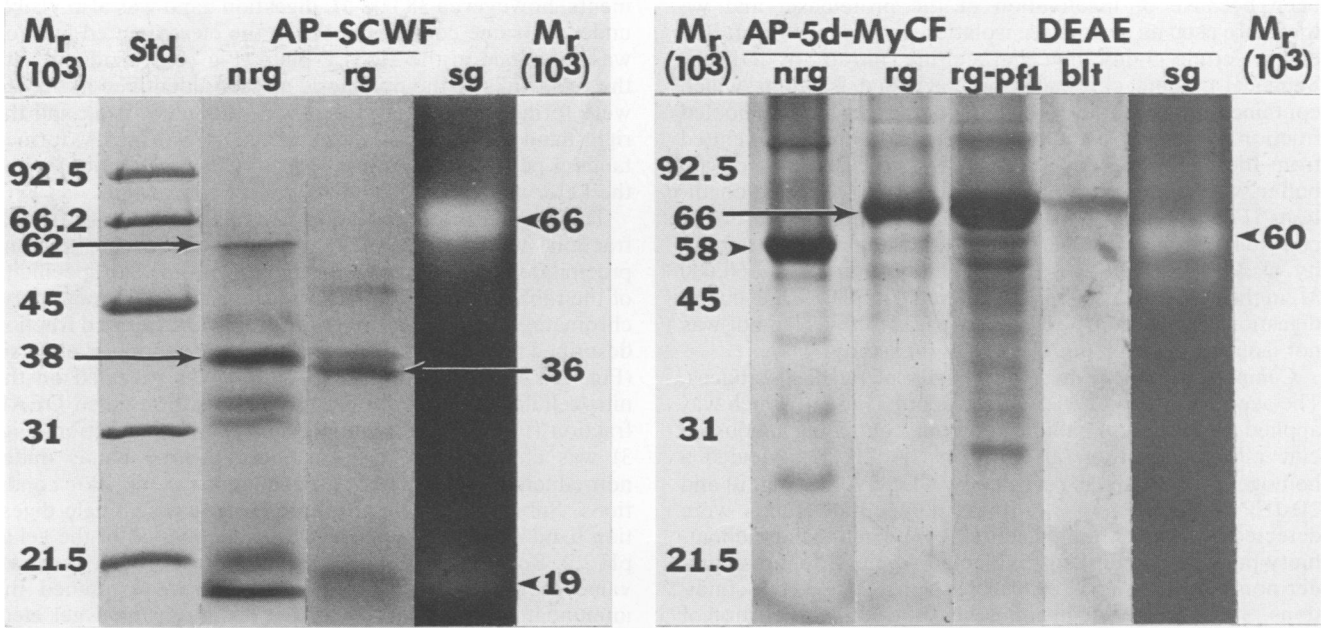

FIG. 2. Nonreducing and reducing SDS-PAGE gels (lanes nrg and rg, respectively) and substrate gels (lanes sg) with Coomassie blue stain of the acetone precipitate of SCWF (AP-SCWF), acetone precipitate of the 5-day mycelial culture filtrate (AP-5d-MyCF), and DEAE fraction of the same culture filtrate. Also shown is an immunoblot (lane blt) of the reducing-gel separation of the DEAE fraction which was obtained from the first peak eluted with 200 mM citrate buffer (lane rg-pf1). The gel components were electrotransferred to nitrocellulose and reacted with monospecific, anti-66,000-M_r rabbit antibody. Estimated M_r of test samples and standards (lane Std.) are indicated.

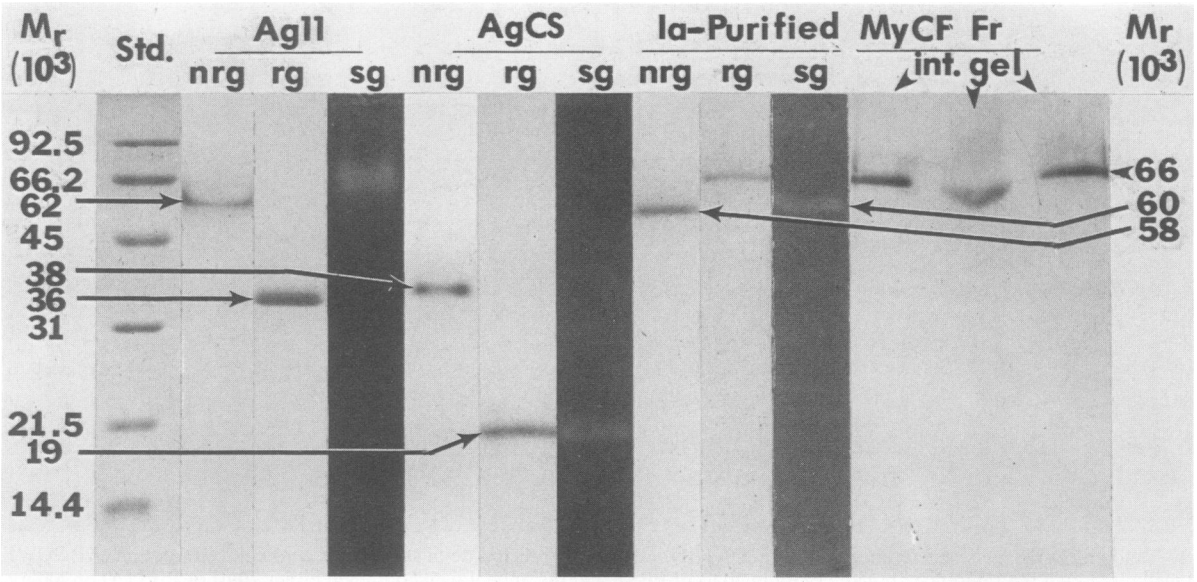

FIG. 3. Nonreducing, reducing, substrate, and interrupted SDS-PAGE (lanes nrg, rg, sg, and int. gel, respectively) with Coomassie blue stain of purified Ag11, AgCS, and the immunoaffinity-purified mycelial culture filtrate fraction (Ia-Purified MyCF Fr). Estimated M_r of samples and standards (lane Std.) are shown.

Cold-acetone precipitation of the 5-day mycelial culture filtrate resulted in the concentration of a major polypeptide band of M_r 58,000 and 66,000 in SDS-PAGE gels under nonreducing and reducing conditions, respectively (Fig. 2). The M_r 58,000 polypeptide band was excised from the polyacrylamide gel, isolated by electroelution, and examined by SDS-PAGE under reducing conditions. A single 66,000-M_r band was revealed, but the isolated fraction lacked proteolytic activity when examined by substrate gel electrophoresis. We reasoned that the absence of proteolytic activity in the 66,000-M_r-containing acetone precipitate may have been because of inactivation of the proteinase, and we therefore used an alternative isolation procedure. Separation of the crude 5-day mycelial culture filtrate by DEAE-Sephacel column chromatography yielded fractions which contained mainly the 66,000-M_r component. The pooled fraction, which consisted of the first absorbance peak eluted from the anion-exchange column with the 200 mM citrate buffer, was examined by SDS-PAGE under reducing conditions (Fig. 2). In contrast to the acetone precipitate of the culture filtrate, the DEAE fraction showed proteolytic activity, as demonstrated by a prominent digestion band at 60,000 M_r in the substrate gel (Fig. 2) incubated at pH 7.0. A second digestion band with M_r ca. 50,000 was also revealed but was not isolated or examined further in this study.

Composition and proteolytic activity of purified antigens. The acetone-precipitated SCWF fraction (Fig. 2), which was applied to an immunoaffinity column containing monospecific rabbit antiserum raised against Ag11 (30), yielded a homogeneous eluate as revealed by SDS-PAGE (Fig. 3) and 2D-IEP (Fig. 4A and B). Single polypeptide bands were detected in polyacrylamide gels (Fig. 3) when the immunoaffinity-purified antigen was subjected to electrophoresis under nonreducing (M_r 62,000) or reducing (M_r 36,000) conditions. Substrate gel electrophoresis showed the formation of a single digestion band with M_r ca. 66,000 (Fig. 3) after the gel was incubated at pH 8.0. A single precipitin peak was shown in tandem with Ag11 of the reference antigen in the 2D-IEP plate (Fig. 4B).

A homogeneous fraction was also obtained by electroelution of the 38,000-M_r polypeptide band excised from the SDS-PAGE gel of the acetone-precipitated SCWF. The electroeluted fraction was characterized by single polypeptide bands with M_r 38,000 and 19,000 under nonreducing and reducing conditions, respectively (Fig. 3). However, if the dried acetone precipitate was suspended in buffer and allowed to remain at room temperature for 8 to 12 h, both M_r 38,000 and 19,000 bands were revealed in a nonreducing gel (results not shown). Results of substrate gel electrophoresis of the 38,000-M_r protein (Fig. 3) revealed a single 19,000-M_r digestion band after incubation at pH 8.0. In some experiments, however, a 38,000-M_r digestion band was also visible under the same conditions. When the electroeluted fraction was examined in the 2D-IEP plate (Fig. 4C), tandems with the two peaks of the polymeric antigen, identified as AgCS, were formed. It appeared that both the major peak and the right-hand shoulder of the reference AgCS (Fig. 4A) formed tandem peaks with the test antigen (38-kDa fraction) when the latter was added to the anodal well (Fig. 4C).

To generate monospecific antibody against the 66,000-M_r fraction, we used the electroeluted band of the acetone precipitated culture filtrate for immunization. The specificity of the rabbit antiserum, which was used for immunoaffinity-chromatographic separation of the DEAE-separated fraction described above, was demonstrated by immunoblot analysis (Fig. 2). A single band of M_r 66,000 was revealed on the nitrocellulose membrane blotted against the pooled DEAE fraction (Fig. 2). The immunoaffinity-purified fraction (Fig. 3) was characterized by single polypeptide bands under nonreducing (M_r 58,000) and reducing (M_r 66,000) conditions. Substrate gel electrophoresis showed a single digestion band of M_r 60,000 (Fig. 3) after incubation of the gel at pH 7.2. Because the nonreduced fraction showed a lower M_r value then the denatured sample did, we examined the immunoaffinity-purified proteinase by interrupted gel electrophoresis (Fig. 3) for the occurrence of intramolecular disulfide bonds. The samples applied to the two outer lanes exhibited horizontal bands, each with M_r 66,000. The fraction in the center lane, to which no reductant was added, showed a distinctly concave band. When the 66,000-M_r

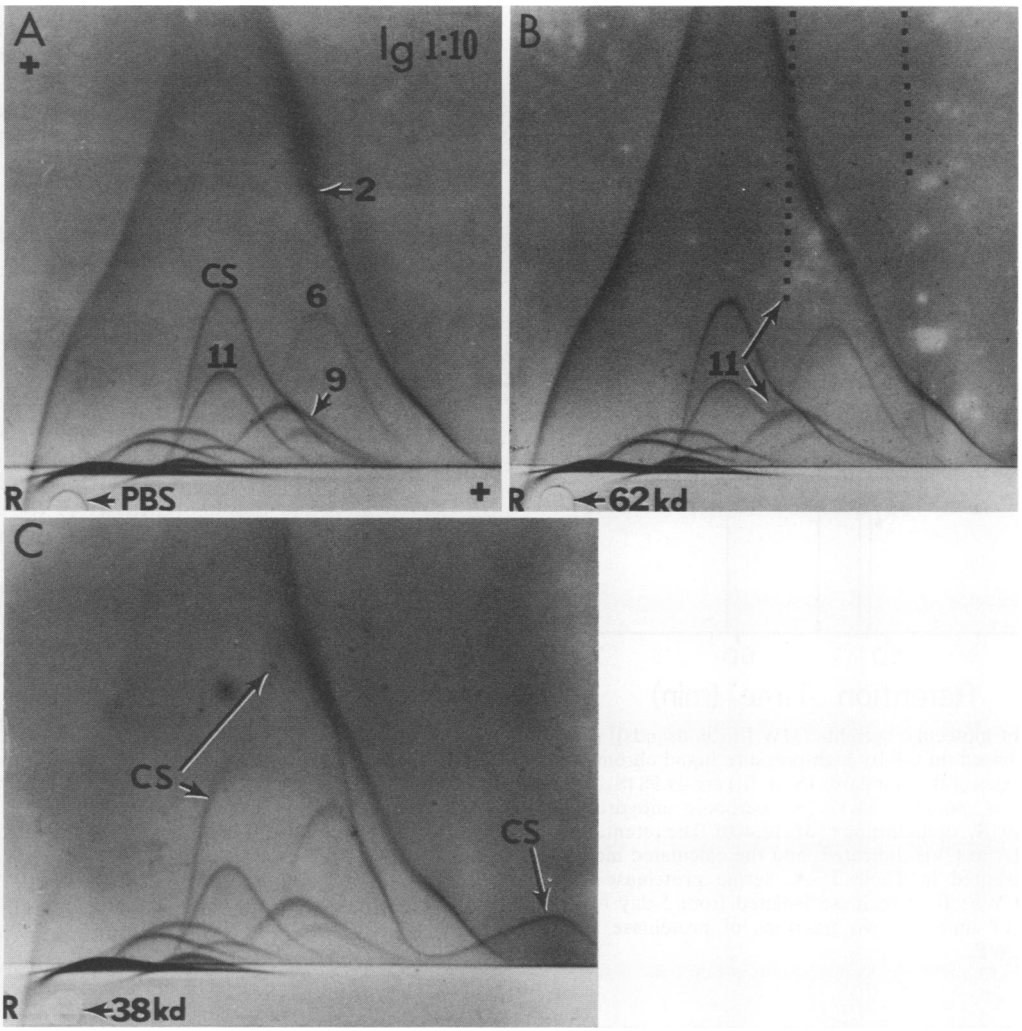

FIG. 4. Tandem 2D-IEP gels of reference antigen (CDN) (R) and purified proteinases obtained from an acetone precipitate of SCWF. The cathodal well contained the reference antigen (25 μg in 20 μl of PBS), and the anodal well contained PBS (A), the nonreduced serine proteinase isolated by immunoaffinity chromatography (62 kDa) (B), or the nonreduced proteinase isolated from SCWF by electroelution (38 kDa) (C). Immunoelectrophoresis conditions are the same as described in the legend to Fig. 1. The single tandem peak with Ag11 is indicated by dots in panel B. Two tandem peaks are shown with AgCS in panel C.

fraction was examined by tandem 2D-IEP with CDN, no fusion peaks with precipitins of the reference antigen were visible, and the 66,000-M_r fraction, therefore, was not recognized in our CDN-anti-CDN reference system.

GF estimates of the molecular masses of purified antigens. Results of the molecular mass determinations of the purified antigens based on GF analysis are presented in Fig. 5. The immunoaffinity-purified antigens (Ag11 and antigen derived from mycelial culture filtrate fraction) each eluted as single chromatographic peaks with estimated molecular masses of 60 and 56 kDa, respectively. AgCS eluted from the size exclusion column as two distinct peaks with estimated molecular masses of 39 and 19 kDa. A summary of the molecular mass data obtained from SDS-PAGE and GF examinations of the purified antigens is presented in Table 1.

Proteolysis of human immunoglobulins. A summary of results of the incubation of each proteinase in the presence of purified human serum IgG and sIgA is also presented in Table 1. Both Ag11 and AgCS were capable of digesting immunoglobulins when incubated with the substrates at pH 8.0 and 7.2, respectively. The immunoaffinity-purified proteinase isolated from the mycelial culture filtrate, on the other hand, was unable to use the human immunoglobulins as substrates under the incubation conditions employed.

ID. All patient serum samples showed reactivity with the CF reference system and demonstrated a wide range of titers (1:1 to 1:128). Only four samples showed reactivity with the TP reference system, and this occurred at low titers (1:1 to 1:2). All control serum samples were negative for ID tests in both reference systems.

ELISA. Results of adsorption of patient and control serum samples to the crude and purified antigenic fractions are shown in Fig. 6. All test antigens were added to wells of the plate at the same concentration (10 μg/ml), the dilution of each antiserum was the same (1:200), and antibody adsorption to each of the test antigens was examined by using the same goat anti-human IgG (H+L)-peroxidase conjugate. The OD values for these assays, as well as values for the respective assay of control serum samples, are plotted. The data are mean values for triplicate determinations.

The range of OD values was similar for the two crude antigenic preparations (SCWF and 5dMyCF [Fig. 6]). How-

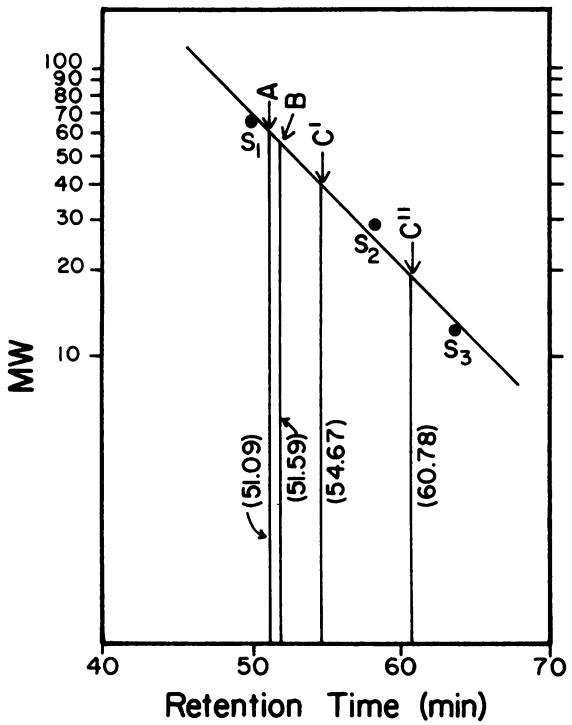

FIG. 5. Plot of molecular weights (MW [in thousands]) of purified proteinases based on GF by high-pressure liquid chromatography. Retention times of the standards (S_1 to S_3) are 49.98 (S_1, bovine serum albumin; M_r 66,000), 58.37 (S_2, carbonic anhydrase; M_r 29,000), and 62.39 (S_3, cytochrome *c*; M_r 12,400). The retention time of each sample (A to C) is indicated, and the calculated molecular weights are presented in Table 1. A, serine proteinase (Ag11) isolated from SCWF; B, proteinase isolated from 5-day mycelial culture filtrate; C′ and C″, two fractions of proteinase (AgCS) isolated from SCWF.

ever, when the reactivity of individual patient serum samples was compared between the SCWF and mycelial culture filtrate, little correlation was found (data not shown). All serum samples from coccidioidomycosis patients tested showed a positive reaction with the 66,000-M_r fraction, and 18 of the 21 samples showed high levels of binding in the ELISA. The similarities of range of OD values for patient serum samples tested with the crude mycelial culture filtrate (5dMyCF) and AgCS (Fig. 6), as well as similarities of OD values for the same individual sera tested (not shown), suggest comparable reactivity to these two antigenic fractions. Such a result is likely because a major antigenic component of this culture filtrate has been shown to be AgCS, on the basis of its examination in our CDN–anti-CDN reference system. Low levels of adsorption of antibody to Ag11 were revealed by the patient serum samples tested, 13 (62%) of which were recorded as negative (Fig. 6).

TABLE 1. Summary of data on molecular weight and substrate activity for isolated proteinases of *C. immitis*

Proteolytic fraction	Estimated mol wt (10^3) by:				Proteolytic activity[a]		
	SDS-PAGE (nonreducing)	SDS-PAGE (reducing)	Substrate gel	GF	Gelatin	IgG	sIgA
Ag11	62	36	66	60	+	+	+
AgCS	38	19	19 (38)[b]	39, 19[c]	+	+	+
IA-5d-MyCF[d]	58	66	60	56	+	−	−

[a] Ability or inability of proteinases to digest gelatin and human immunoglobulins is indicated by + and −, respectively.
[b] Major proteolytic fraction appeared to be 19,000-M_r fraction; digestion band at 38,000 M_r also observed.
[c] Two absorbance peaks were recorded by gel filtration for AgCS with estimated M_r 39,000 and 19,000.
[d] IA-5d-MyCF, Immunoaffinity-purified 5-day mycelial culture filtrate fraction.

Because Ag11 was shown to be a proteinase capable of cleaving immunoglobulins, we asked whether the addition of a serine proteinase inhibitor would reveal increased antibody binding in the ELISA. The addition of PMSF to the blocking reagent during preincubation of Ag11 resulted in no difference in the apparent binding capacity of patient antibody to this antigen in the ELISA. No difference was found in OD values between control serum samples incubated with Ag11 which had or had not been preincubated with PMSF.

Composition and immunoreactivity of the ID-HS antigen. The protein composition of the ID-HS antigen was examined by SDS-PAGE under reducing conditions (Fig. 7), which revealed a major polypeptide band with M_r 19,000, and minor bands with M_r from 17,000 to 46,000. When the ID-HS antigen was examined by 2D-IEP in tandem with CDN, a prominent fusion peak formed with AgCS of the reference antigen (Fig. 7).

The immunoreactivity of the ID-HS antigen was examined by using the same patient and control serum samples in the ELISA as were used for testing the other crude and purified antigens. The range of OD values and reactivity of individual patient serum samples was comparable for the mycelial culture filtrate, AgCS, and the ID-HS antigen (Fig. 6). Serum samples from coccidioidomycosis patients were preincubated with purified AgCS in a range of concentrations to determine the inhibitory effects of this antigen on the binding of patient antibody to the ID-HS antigen in the ELISA. The data presented in Fig. 8 are representative of the serum samples tested. AgCS demonstrated inhibition of antibody binding which was approximately equal to the inhibition resulting from preadsorption of serum with the homologous antigen (i.e., antigen bound to the wells) when the same concentration of the two antigens was tested. Ag11 showed essentially no effect on binding of patient serum samples to the ID-HS antigen.

DISCUSSION

The *C. immitis* SCWF is a reservoir of immunoreactive macromolecules, some of which may play important roles in host response to the invasive pathogen (5). The SCWF has been shown to contain a multiplicity of antigens which were identified as components of our CDN–anti-CDN reference system (6). It is reactive with both human anti-*C. immitis* CF and TP antibodies and has been shown to stimulate the proliferation of lymph node cells derived from mice immunized with attenuated spherules (5). The SCWF showed strong proteolytic activity and was the source of the serine proteinase described in our earlier studies (29, 30), and we speculated that it probably contained additional proteinases. In this study, we have described a second proteinase of SCWF which was identified by substrate gel electrophoresis and by 2D-IEP as AgCS. We believe that the 19,000-M_r protein, which represents AgCS, corresponds to the 21,000-M_r component of SCWF described in our earlier investigation (5) and the 21,000-M_r proteinase reported by Resnick et al. (28).

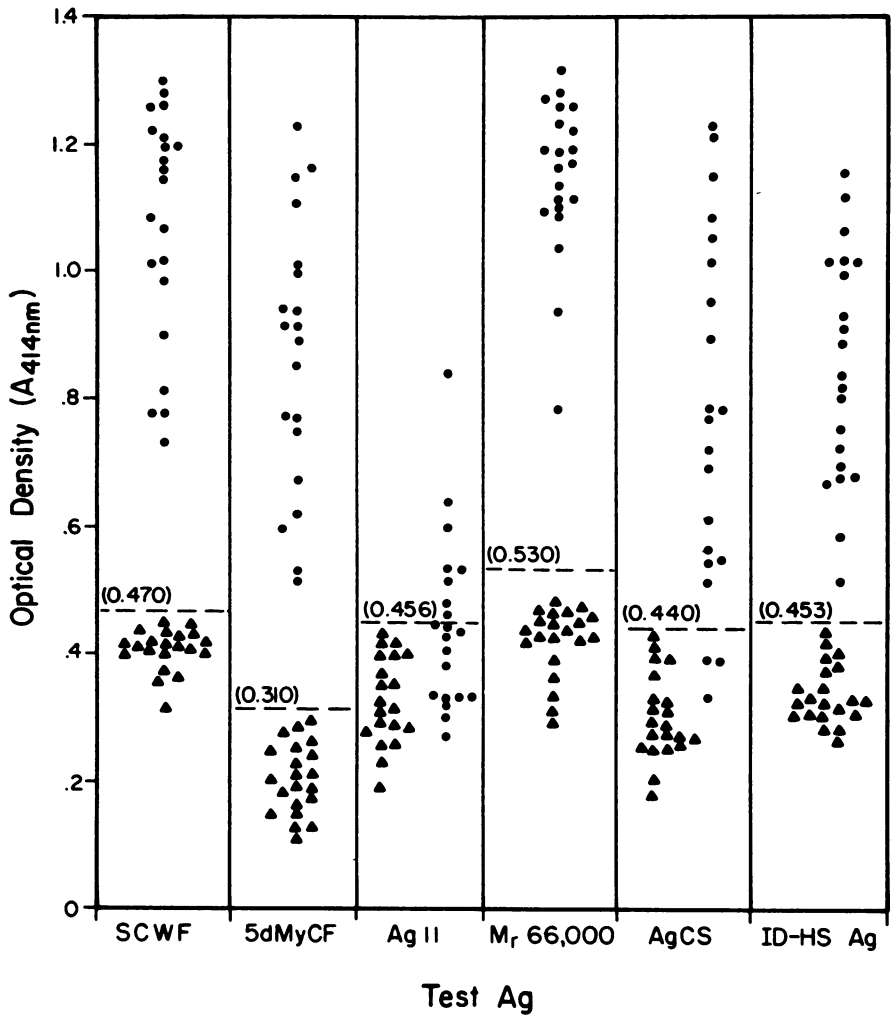

FIG. 6. Results of ELISA with control serum samples (▲) and coccidioidomycosis patient serum samples (●) adsorbed to the indicated crude or purified test antigens (Ag) (10 μg/ml) bound to wells of microdilution plates. Goat anti-human IgG (H+L) conjugated to peroxidase was used for the detection of adsorbed antibody. The value for the mean OD of the control serum sample plus twice the standard deviation is shown above the dashed line.

Acetone precipitation of the SCWF facilitated the concentration of two antigenic proteinases examined in this study. The immunoaffinity-purified Ag11 was identified by substrate gel electrophoresis as a 66,000-M_r digestion band. The estimated molecular mass of this same fraction based on GF analysis was 60 kDa. The interaction of enzyme and substrate during electrophoresis probably results in the delay of migration in substrate gels and could account for the difference in apparent molecular weight of the proteinase in nonreducing and substrate gels (Table 1). AgCS appeared as a 38,000-M_r band in nonreducing SDS-PAGE gels and was well separated from other components of the acetone precipitate of SCWF, which permitted its isolation directly from preparative gels. The excised and eluted gel band was identified as AgCS by tandem 2D-IEP and as a single 19,000-M_r polypeptide band in reducing gels. When examined by GF, this sample eluted from the chromatographic column as two fractions with molecular masses of 39 and 19 kDa. The 19,000-M_r band consistently showed proteolytic activity, whereas the 38,000-M_r digestion band was occasionally observed in substrate gels. It is not known at present whether the 38,000-M_r protein is a proenzyme or a dimeric form of the enzyme with reduced proteolytic activity.

The filtrate of mycelial cultures of *C. immitis* has long been appreciated as a complex of immunoreactive macromolecules (15) and was previously shown to have proteolytic activity (24, 29). A major component of the filtrate, revealed by SDS-PAGE, is the 66,000-M_r protein. We asked whether these two observations are related. The 66,000-M_r fraction was also recognized by SDS-PAGE as a major component of the acetone precipitate of the mycelial culture filtrate. Under nonreducing conditions, a 58,000-M_r band was clearly distinguished and excised from unstained gels. Subsequent electrophoresis of the eluted sample under reducing conditions revealed a single 66,000-M_r band. The higher M_r value for the reduced form of this fraction compared with the nonreduced form suggests that the molecular weight of the former may be influenced by disulfide bonds. It is known that chemical reduction of intrachain disulfide bonds leads to an apparent increased molecular weight of the polypeptide component in SDS-PAGE gels (27). In the electrophoresis method used in this study for detection of disulfide-bonded

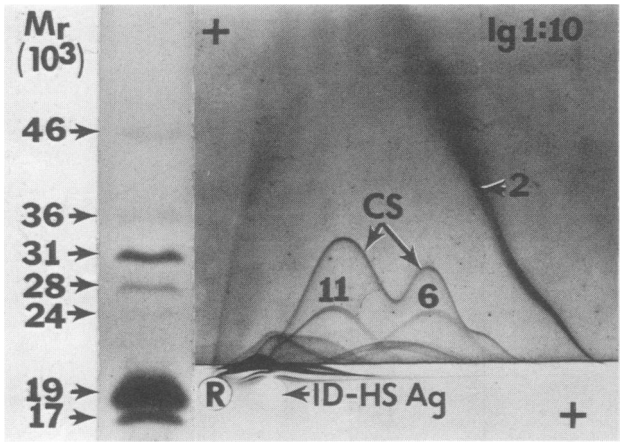

FIG. 7. SDS-PAGE with Coomassie blue stain of the HS antigen (Ag) identified in the ID-HS system and described by Kaufman et al. (22), and tandem 2D-IEP gel of reference antigen (CDN) (R) and the ID-HS antigen. The cathodal well contained the reference antigen (20 μg in 20 μl), and the anodal well contained the ID-HS antigen (diluted 1:1 in PBS). Other IEP conditions are the same as described in the legend to Fig. 1. The major polypeptide band in the SDS-PAGE gel had M_r 19,000, and a prominent tandem peak was formed with AgCS in the 2D-IEP plate.

polypeptides, the concave band of the immunoaffinity-purified proteinase was formed as a result of diffusion of reductant from adjacent lanes and is characteristic of a polypeptide with intrachain disulfides (13).

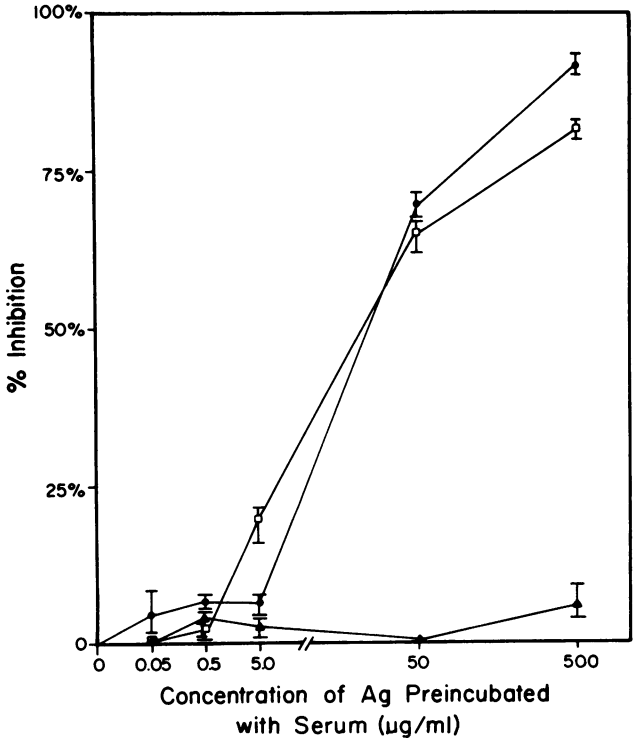

FIG. 8. Competitive-inhibition ELISA of representative serum of coccidioidomycosis patients preincubated with different concentrations of AgCS (□), Ag11 (▲), and the ID-HS antigen (●), prior to assay of antibody binding to the ID-HS antigen-coated wells of the microdilution plate.

Because we were concerned that acetone precipitation may have destroyed the proteolytic activity of the 66,000-M_r protein, we used an alternative method for fractionation of the mycelial culture filtrate which included DEAE and immunoaffinity chromatography. Monospecific antibody raised against the excised 58,000-M_r band from the preparative SDS-PAGE gels was used in the immunoaffinity column to isolate an active proteinase from a pooled DEAE fraction of the mycelial culture filtrate. The enzyme was identified by substrate gel electrophoresis as a 60,000-M_r digestion band. The molecular mass estimation of the purified fraction based on GF analysis was 56 kDa. Estimates of the molecular weight of this antigen under non-reducing conditions were very similar (Table 1).

Differences in humoral reactivity to the three purified antigens were demonstrated with serum samples from 21 CF-positive coccidioidomycosis patients. The serum samples screened in the ELISA showed a wide range of binding capacity to most of the antigenic fractions. A clear difference in the range of OD values was noted, however, for Ag11 and the 66,000-M_r fraction. One possible contributing factor to this difference is that cleavage of immunoglobulins during incubation of serum samples with the serine proteinase could influence the amount of antigen-antibody binding. We determined that Ag11 and AgCS could cleave both human serum IgG and sIgA in vitro, whereas the 66,000-M_r proteinase was unable to utilize these substrates. Preincubation of Ag11 with PMSF, which inhibits its proteolytic activity (29), had no effect on the antigen-antibody binding capacity as determined by the ELISA. Patient serum samples contain natural proteinase inhibitors (e.g., α-1-antitrypsin) which may block the activity of Ag11 (29) and AgCS. On the other hand, cleavage of immunoglobulins by the *C. immitis* proteinase may occur by a mechanism comparable to that of bacterial IgA1 proteinases, in which the Fab and Fc fragments are released from the parent molecule intact and the Fab fragments retain their antigen-binding capacity (25). However, we expect that the explanation for poor antibody response to Ag11 observed in the ELISA is due simply to the paucity of antigen presented to the host during the parasitic cycle. We have shown by immunoelectron microscopy that Ag11 is localized within the inner wall complex of sporulating spherules and that, on the basis of ELISA studies, relatively little antigen was released into the growth media of the parasitic cells (30). In support of this latter observation, Zimmer and Pappagianis (31) did not detect a 36,000-M_r band (reducing gels) in their immunoblot analysis of extracellular proteins released during growth of the spherule-endospore phase. In the same study, however, the authors did report a reactive band of approximately 66,000-M_r in immunoblots of the 28-h spherule-endospore culture filtrate when using both CF- and TP-positive patient serum samples. We have recently shown (4) that the membranous, spherule outer wall fraction of the cultured pathogen contains a 66,000-M_r component which is reactive with patient serum samples. Our interpretation of the uniformly high levels of IgG binding to the 66,000-M_r fraction in the ELISA is that this antigen is presented to the host at the surface of spherules and probably persists throughout the disease.

Antigen CS was also detected in the spherule outer wall fraction (4). In an earlier report we showed that this antigen is heat stable and may be specific for *C. immitis* (3). In a survey of the reactivity of cell wall preparations of a range of pathogenic fungi with anti-CDN in advancing-line IEP plates (3), only an alkali-soluble, water-soluble extract of isolated conidial walls of *Aspergillus fumigatus* showed apparent

cross-reactivity with anti-AgCS antibody in the reference antiserum. However, the presence of a dark pigment derived from the conidial wall material migrated from the anodal well of the IEP plate and obscured the advancing line (3, 5). Our subsequent examinations of this fraction by advancing-line IEP have suggested that AgCS is not present in wall preparations of *A. fumigatus*. We have also demonstrated that AgCS is a component of the ID-HS antigen used by Kaufman et al. for immunoidentification of *C. immitis* (21, 22). We have determined that AgCS does not bind to concanavalin A (unpublished data), which is characteristic of the *C. immitis*-specific, HS exoantigen reported by Cox and Britt (10). The same authors (10) demonstrated the reactivity of their isolated fraction in the ID-HS assay. We have also shown reactivity of AgCS in the ID-HS assay (unpublished data). The problem of correlating AgCS with the ID-HS antigen was that the latter contains multiple components, as revealed by SDS-PAGE. However, the 19,000-M_r band in the reducing gel of the ID-HS antigen was the major polypeptide, which correlated with the presence of AgCS in tandem 2D-IEP gels of the ID-HS antigen. Similar OD values were obtained in the ELISA for individual serum samples tested with AgCS and the ID-HS antigen. Binding of antibody to the ID-HS antigen was inhibited by preincubation of patient serum samples with AgCS. On the basis of results of their ID tests, Kaufman et al. (22) reported that antibodies to the ID-HS antigen are infrequently found in human serum samples from patients with coccidioidomycosis and that this reagent is thus of little serodiagnostic value. Our ELISA data, on the contrary, suggest that the crude ID-HS antigen and purified AgCS warrant further investigation to evaluate their immunodiagnostic potential.

Results of our substrate and inhibitor analyses of the 19,000-M_r and 66,000-M_r proteinases, as well as immunoelectron-microscopic data on the cellular localization of these antigenic fractions, will be presented in a future communication. Current investigations are focused on determining whether a functional interrelationship exists between these newly isolated proteolytic enzymes and the serine proteinase which was previously suggested to play a morphogenetic role in the parasitic cycle of *C. immitis* (30).

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