# The Coccidioidal Complement Fixation and Immunodiffusion-Complement Fixation Antigen Is a Chitinase

SUZANNE M. JOHNSON AND DEMOSTHENES PAPPAGIANIS*

*Department of Medical Microbiology and Immunology, School of Medicine,
University of California, Davis, California 95616*

Culture filtrates and autolysates of *Coccidioides immitis* have provided suitable crude antigens for the serodiagnosis and prognosis of coccidioidomycosis. One of these, a heat-labile antigen which participates in the immunodiffusion reaction corresponding to the complement fixation reaction (IDCF), has been characterized as a 110-kDa native protein that, when subjected to reducing conditions and heat, yields a 48-kDa component. The present report provides serologic and biochemical evidence that this antigen is a chitinase. This chitinase, isolated from 48-h culture filtrate of the spherule-endospore-phase *C. immitis* by affinity adsorption to chitin, formed a line of identity with the IDCF reference antigen and participated in the complement fixation reaction with human serum. It lost its enzymatic as well as antigenic activity when heated, but when not heated it retained its enzymatic activity even when precipitated with coccidioidal antibody present in human serum. This chitinase represents a significant serodiagnostic substance and may be important in the morphogenesis of *C. immitis*.

*Coccidioides immitis*, the etiologic agent of a systemic fungal disease of humans and other species, grows as a hyphal-arthroconidial form in nature and under usual laboratory conditions. In the host and under specialized laboratory conditions, it grows as an endosporulating spherule. Following its inhalation or parenteral introduction, the infectious arthroconidium becomes rounded, sheds an outer wall layer, and enlarges. It becomes an immature spherule undergoing cytoplasmic and nuclear division to produce the mature spherule, which contains endospores. These endospores are then released by the rupture of the spherule, perhaps discharged through an ostiole (15). The liberated endospores can enlarge into new endosporulating spherules to repeat the cycle.

Both spherule-endospore (SE) and hyphal growth phases contain chitin, a linear polymer of β-(1-4)-linked N-acetylglucosamine, within the cell wall (10, 32). However, the relative chitin content within the wall varies with both morphological state and age (10, 32). Chitinase activity within the culture filtrate of SE-phase *C. immitis* has been demonstrated and has been previously associated with a thinning of the wall chitin layer (11). Chitinase, therefore, may be associated with weakening of the mature spherule wall prior to discharge of the contained endospores.

We have recently isolated a protein from SE-phase culture filtrate which exhibits chitinase activity. The present report provides some characterization of this protein, including its use as a serodiagnostic antigen in the complement fixation (CF) and immunodiffusion tests.

## MATERIALS AND METHODS

**Organism and culture conditions.** The Silveira strain of *C. immitis* (ATCC 28868) was grown in virtually synchronous SE-phase culture in modified Converse medium by the method of Levine et al. (18). Briefly, small endospores obtained by differential centrifugation (9) were used to inoculate flasks of medium that were then incubated at 37°C with shaking. Forty-eight hours after inoculation, the cul-

tures were centrifuged at 400 × g for 15 min. The supernatant was sterilized by passage through a 0.2-μm-pore-size Nalge cellulose acetate filter unit (Nalgene, Rochester, N.Y.). Phenylmethylsulfonyl fluoride (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to the culture filtrate to a final concentration of 1 mM to inhibit endogenous proteases, and thimerosal (Lilly, Indianapolis, Ind.) was added to a final concentration of 1:10,000 to prevent microbial contamination.

**Enzyme isolation.** The crude filtrate was concentrated 200-fold by using an Amicon stirred pressure cell with a Diaflo YM-10 filter ($M_r$ exclusion, 10,000; Amicon Division, W. R. Grace and Co., Danvers, Mass.). Affinity purification of the chitinase was carried out by a modification of the affinity adsorption-desorption procedure previously described for purification of chitinase from *Serratia marcescens* (4). This procedure, described below, is based on the binding of the soluble enzyme to the insoluble chitin substrate. The adsorbed chitinase is recovered by allowing the enzyme to digest the substrate, with subsequent release of the enzyme. Regenerated chitin (reacetylated chitosan) was prepared from crab shell chitosan (Sigma Chemical Co., St. Louis, Mo.) as described by Cabib (3), by using unlabeled acetic anhydride. The chitin was added to the crude concentrated culture filtrate at 3 mg (dry weight) per ml of filtrate, and the mixture was incubated overnight at 4°C to permit adsorption of the enzyme to the chitin substrate. The suspension was then centrifuged with refrigeration at 1,100 × g, and the supernatant was decanted. The pellet was washed twice with 50 mM potassium phosphate buffer (pH 6.3), resuspended in an equal volume of the same buffer, and incubated overnight at 37°C. Residual chitin after the digestion by the chitinase was removed by centrifugation (1,100 × g, 15 min). The soluble reaction products were removed by passing the supernatant through a Sephadex G-25 column (Pharmacia, Piscataway, N.J.).

**Enzymatic activity.** Enzymatic activity was measured semiquantitatively with 4-methylumbelliferyl (4-Muf)-conjugated substrates (Sigma Chemical Co.) (2). The substrates tested were 4-Muf-*N*-acetyl-β-D-glucosaminide (4-Muf-GlcNAc), 4-Muf-β-D-*N*,*N*'-diacetylchitobioside (4-Muf-GlcNAc$_2$), 4-Muf-

* Corresponding author.

β-D-$N$,$N'$,$N''$-triacetylchitotriose (4-Muf-GlcNAc$_3$), 4-Muf-β-D-glucoside (4-Muf-Glc), and 4-Muf-$p$-guanidinobenzoate (4-Muf-GB), a substrate for trypsinlike proteases. Stock solutions, 50 mM, were prepared in dimethylformamide. Prior to use, the stock solution was diluted 1:100 (final concentration, 0.5 mM) in 50 mM phosphate buffer, pH 6.3. Stock and unused working solutions were stored at −20°C. A 10-μl volume of the working substrate (0.5 mM) was added to 10 μl of the enzyme in a microtiter plate and mixed by gentle tapping. The plate was incubated at 37°C for 10 min. Activity, demonstrated by the release of the free 4-Muf, was observed as a light blue fluorescence when viewed with a UV transilluminator (Fotodyne, New Berlin, Wis.).

Chitinase activity was similarly demonstrated in situ following immunodiffusion and gel electrophoresis. Immunodiffusion plates containing precipitated antigen-antibody complexes were washed three times for 10 min with 50 mM phosphate buffer, pH 6.3, to remove diffused unreacted antigen and antibody as well as other extraneous serum components. One milliliter of the 4-Muf-GlcNAc$_3$ working substrate was poured into the immunodiffusion plate and incubated at 37°C for 10 min. Fluorescence was observed as before. Likewise, following electrophoresis, the polyacrylamide gel was soaked in 50 mM phosphate buffer, pH 6.3, for 5 min. A 10-ml volume of working substrate was poured over the gel and incubated at 37°C for 10 min. Both gels and immunodiffusion plates were photographed through a Wratten no. 3 gelatin filter (Kodak, Rochester, N.Y.).

**Protein estimation.** The protein concentration was estimated by the method of Lowry et al. (19), with bovine serum albumin as the standard.

**Gel electrophoresis and blot analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the discontinuous buffer system of Laemmli (17) as previously described (33). Prior to loading, the samples were reduced by being boiled for 4 min in the presence of mercaptoethanol. Proteins in their native state were similarly electrophoresed, except that all buffers were free of SDS and mercaptoethanol and the samples were not heated. Gels were stained with silver as described by Merril et al. (21). The molecular weights of the relative mobility markers (Bio-Rad, Richmond, Calif.) ranged from 200,000 to 14,000. Western blot (immunoblot) analysis was performed by the method of Towbin et al. (31) with 100 mM 3-cyclohexylamino-1-propane sulfonic acid (CAPS) buffer, pH 11, in 10% methanol as previously described (1, 33). Blots were blocked for 2 h at room temperature with 5% skim milk in phosphate-buffered saline (0.1 M phosphate–0.15 M NaCl, pH 7.2)–0.5% Tween 20 (BLOTTO). Pooled human serum containing coccidioidal antibodies detected by the immunodiffusion tests corresponding to the tube precipitin and complement fixation tests (IDTP and IDCF, respectively), as well as pooled normal serum, was used as the primary antibody, diluted 1:50 in BLOTTO. Pooled normal serum consisted of specimens negative by immunodiffusion for coccidioidal antibodies after eightfold concentration. Goat anti-human immunoglobulin G (IgG) and anti-human IgM horseradish peroxidase conjugates (Cappel Lab, Durham, N.C.) were used as the secondary antibodies, diluted 1:1,000 in BLOTTO, and 3,3'-diaminobenzidine tetrahydrochloride was used as the indicator dye as previously described (33). Prior to blocking, parallel strips containing molecular size markers were stained with 0.1% Coomassie blue in 40% methanol–1% acetic acid and destained with 50% methanol (1).

**Antigens.** F171, a pooled mycelial-phase culture filtrate of

TABLE 1. Enzymatic activities of coccidioidal antigens

| Antigen | Fluorescence intensity with the following substrate[a] | | | | |
|---|---|---|---|---|---|
| | 4-Muf-GlcNAc | 4-Muf-GlcNAc$_2$ | 4-Muf-GlcNAc$_3$ | 4-Muf-Glc | 4-Muf-GB |
| C. immitis chitinase | − | +++ | ++ | − | − |
| MFS | − | ++ | + | − | − |
| Heated MFS | − | − | − | − | − |
| Heated F171 | − | − | − | − | − |
| SF 77 | − | ++ | + | − | − |
| Immuno-Mycologics immunodiffusion antigen | − | ++ | + | − | − |

[a] Abbreviations: 4-Muf, 4-methylumbelliferyl; GlcNAc, glucosaminide; GlcNAc$_2$, diacetylchitobioside; GlcNAc$_3$, triacetylchitotriose; Glc, glucoside; GB, guanidinobenzoate. Intensities were rated on a scale of − to +++, with +++ being the most brilliant.

22 strains of C. immitis, and MFS, a mixed filtrate of C. immitis Silveira prepared in 1971, were used for antigenic comparison. F171, a previously characterized antigen, contains IDCF as well as IDTP activities and is routinely used in our laboratory to detect coccidioidal antibodies (24, 34). IDCF activity can be eliminated from the preparation by heating at 60°C for 30 min. In the present study, we have noted that heating at 56°C for 30 min suffices to inactivate the antigen, as was noted previously for the CF antigen (23). Heated F171 is routinely used to differentially detect IgM precipitins by IDTP (24). MFS also contains IDCF activity and is used in our laboratory to detect IgG by IDCF. Other antigens utilized for enzymatic comparison were SF 77, a Silveira strain filtrate antigen also used in our laboratory to detect IgG, and Immuno-Mycologics Coccidioides immunodiffusion antigen (courtesy of S. Bauman, Immuno-Mycologics, Norman, Okla.), which contains both IDCF and IDTP activities.

**Immunodiffusion.** Antigenic reactivity was assessed by double immunodiffusion with pooled human sera which are utilized in diagnostic testing by IDTP and IDCF for coccidioidal antibodies corresponding to IgM and IgG, respectively (22, 27). The serum was allowed to prediffuse for 2 h before the addition of antigen (13).

**Complement fixation.** Affinity-purified chitinase from C. immitis was tested for complement-fixing ability by the modified Kolmer CF test of Smith et al. (28, 29). The affinity-purified chitinase was prepared at protein concentrations of 10, 20, 40, and 80 μg/ml, and a box titration was carried out with complement-fixing serum from a human with coccidioidomycosis.

## RESULTS

**Affinity purification.** Chitinase was removed from the 48-h culture filtrate by adsorption to insoluble chitin at 4°C and was released by digestion of the chitin at 37°C. Approximately 2.2 mg of isolated protein per liter of culture filtrate was recovered following affinity purification.

**Enzyme activity.** Enzyme activity was detected by a semiquantitative assay using 4-Muf-conjugated substrates (2). Fractions were scored (−, +, ++, or +++) according to the fluorescence intensities observed (Table 1). All antigenic
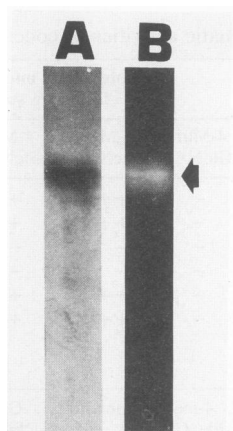
FIG. 1. Nonreduced, non-SDS PAGE of affinity-purified chitinase (12% gel). Lane A, silver stain; lane B, fluorescence of 4-Muf-GlcNAc$_3$ chitinase activity stain.

preparations known to contain IDCF activity, as well as the purified chitinase, were capable of liberating free 4-Muf from 4-Muf-GlcNAc$_2$ and 4-Muf-GlcNAc$_3$ substrates. No glucosidase or protease activity was observed. When previously heated, the coccidioidal antigens (MFS and F171) exhibited no enzymatic activity. Chitinase activity against regenerated chitin was inferred from the visual disappearance of chitin during the last step of the purification.

**PAGE.** PAGE of affinity-purified chitinase not exposed to mercaptoethanol and SDS yielded a single diffuse band (Fig. 1). This band contained chitinase activity, demonstrated in situ as fluorescence when 4-Muf-GlcNAc$_3$ was overlaid and the gel was briefly incubated. When the same affinity-purified chitinase was subjected to reducing SDS-PAGE, it yielded a doublet band with a mobility corresponding to a molecular weight of 48,000 (Fig. 2). It was not possible to demonstrate activity associated with either one or both of these bands, as enzymatic activity was destroyed by the required boiling. Furthermore, activity was lost when the chitinase preparation was heated for 30 min at 56°C (as indicated above, this treatment also destroys the antigenic activity in the IDCF test).

Following electrophoresis, the reduced, purified protein was transferred to a nitrocellulose membrane. The 48-kDa
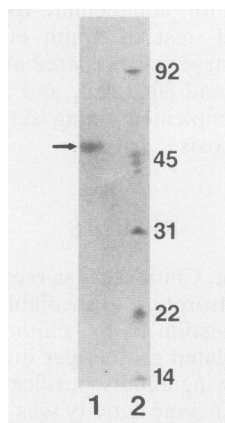


FIG. 2. Reduced SDS-PAGE (12% gel). Lane 1, affinity-purified chitinase; lane 2, molecular size markers (in kilodaltons). The arrow indicates a 48-kDa doublet.
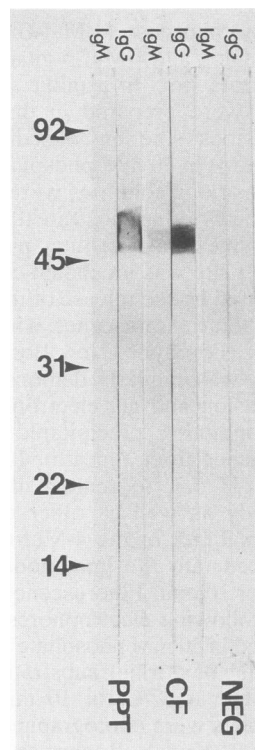


FIG. 3. SDS-PAGE immunoblot of affinity-purified chitinase (12% gel). Lanes: CF, CF control pooled human serum; PPT, precipitin control pooled human serum; NEG, negative pooled human serum; IgM, anti-IgM secondary antibody; IgG, anti-IgG secondary antibody. Molecular size markers (in kilodaltons) are on the left.

bands reacted intensely as a single unit when probed with pooled human serum positive for coccidioidal CF antibody, detected with peroxidase-labeled anti-IgG secondary antibody (Fig. 3). This area also stained when probed with human serum positive for IDTP and anti-IgG secondary antibody, indicating that CF antibodies were present in that pooled serum also. Such antibodies were demonstrated in this serum pool by IDCF (Fig. 4).

**Serologic activity.** Serologic activity was determined by double immunodiffusion and CF. Immunodiffusion produced an antigen-antibody reaction yielding a line of identity between the affinity-purified chitinase and the IDCF reference antigen MFS (Fig. 4A). This line of precipitation was not observed when the purified chitinase was heated for 30 min at 56°C (Fig. 4B) or boiled for 4 min (not shown). This heat treatment had previously been observed to inactivate enzymatic activity. Furthermore, chitinase activity, demonstrated with the 4-Muf-GlcNAc$_3$ substrate, was associated with the precipitate line of identity produced between the reference IDCF antigen and the purified chitinase when reacted with the IDCF-positive serum (Fig. 4C). No fluorescence was associated with the IDTP line, and no fluorescence was manifested by the chitinase-antibody precipitate in the absence of the 4-Muf-GlcNAc$_3$ substrate.

To ascertain whether the affinity-purified chitinase could also serve as a complement-fixing antigen, the chitinase was substituted for the coccidioidin in a box titration CF test with human serum of known CF antibody titer. CF comparable to that shown by our reference antigen occurred at chitinase protein concentrations of 10 to 80 μg/ml (the range tested),
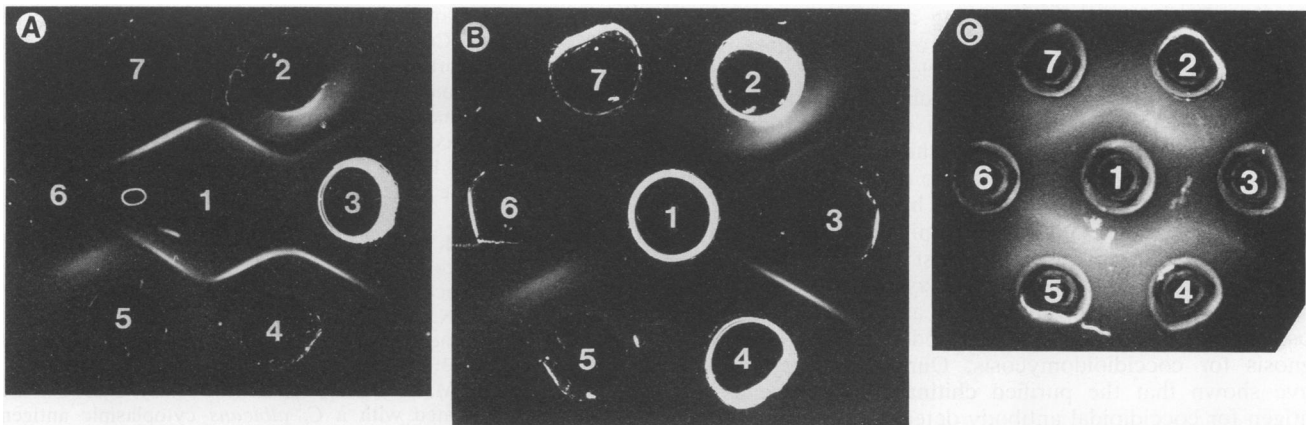
FIG. 4. (A) Immunodiffusion reactivity of affinity-purified chitinase (well 1). Note the line of identity which forms with the IDCF control serum (wells 4 and 7) and the CF reference antigen MFS (well 3). IDTP control antiserum (wells 2 and 5) also contains some IDCF antibody; however, the heated F171 (well 6) shows no reactivity with IDCF antibody. (B) Loss of immunodiffusion reactivity of heated affinity-purified chitinase (well 1). Other wells are as described for panel A. (C) Immunodiffusion plate of affinity-purified chitinase showing fluorescence when overlaid with the 4-Muf-GlcNAc$_3$ chitinase substrate. Wells are as described for panel A.

with optimal reactivity shown at 10 to 20 μg/ml. No anti-complementary activity was observed at any concentration tested.

## DISCUSSION

The important diagnostic and prognostic value of the CF antibody is well established (16, 28, 29). Naturally, the corresponding CF antigen has generated considerable interest and research (6, 13, 24, 34). When it was demonstrated that antibody detected by IDCF correlated with antibody detected by CF, the antigen producing the precipitate in the IDCF test was assumed to be the same antigen which participated in CF (13). Additionally, the participating antigen was found to be inactive in both IDCF and CF when the antigenic mixture had been previously heated (14, 22, 28). The present study provides direct evidence that an antigenic protein with chitinase activity participates in both the CF and the IDCF reactions.

The chitinase, isolated from SE culture filtrate by its affinity for chitin, shares biochemical as well as serologic activity with the IDCF antigen. After reduction with mercaptoethanol and separation by SDS-PAGE, a doublet of approximately 48,000 Da was observed. A size of >100 kDa was inferred for the native protein from its exclusion from a gel filtration column with a molecular size exclusion of 100 kDa (data not shown). The doublet band that is observed following SDS-PAGE is most likely the result of degradation of the native protein, as the two migrate in close proximity and the distance between the two appears to be unaffected by acrylamide concentration. These properties conform to those demonstrated by Zimmer and Pappagianis (34) for the IDCF antigen.

The immunodiffusion line of identity indicates that antigenic epitopes are shared by the IDCF antigen and the affinity-purified chitinase. Therefore, this chitinase is, or is a significant part of, the IDCF antigen. Since the chitinase activity was retained by the IDCF antigen-antibody precipitate, the epitopes for the antibody reaction are likely distinct from the enzymatically active site(s) of the chitinase. Finally, the lines of identity between the SE-phase chitinase and the mycelial-phase reference antigen demonstrate similarity between chitinases of both morphological phases.

Finally, the complement-fixing ability of the purified chitinase indicates that the antigen which participates in the IDCF reaction is the same antigen which participates in CF. Interestingly, however, it has been shown that complement is not required for the formation of the antigen-antibody precipitate detected by immunodiffusion (22, 24).

It has been noted that while the heat treatment required for SDS-PAGE (boiling, 4 min) is not sufficient to eliminate protein blot immunoreactivity, it does, however, eliminate antigen-antibody precipitation detected by immunodiffusion. We have similarly observed that the boiled and reduced antigen remains reactive when examined by the enzyme-linked immunosorbent assay. Presumably, the boiling denatures the protein sufficiently to inhibit formation of a visible antigen-antibody complex, yet some antigenic epitopes that can be detected by the more sensitive immunoassays remain. The sensitivity of horseradish peroxidase conjugates utilized for indirect immunoblotting assays is between 0.1 and 1 ng of target protein (7). In contrast, immunodiffusion detects antibody in the range of 30 to 60 μg of protein (12).

The chitinase appears to be active against polymers 2 N-acetylglucosamine units or more in length. Since glucosidase activity was not observed, the specificity may be limited to N-acetylglucosamine-containing carbohydrates, although other substrates remain to be examined. In addition, all examined antigenic preparations containing IDCF activity also exhibited chitinase activity.

Enzymatic activity associated with antigenic proteins is not uncommon. Recently, the M antigen of Histoplasma capsulatum was identified as a catalase (8). Cole et al. (5) isolated three proteolytic antigens (unknown serodiagnostic activity) from the mycelial phase of C. immitis, and Resnick et al. (25, 26) isolated a 21-kDa protein which had proteolytic activity and tentatively appeared to correspond to the IDTP antigen. Indeed, there are other examples, including the enolase of Candida albicans, which is the 48-kDa immunodominant protein (20, 30).

Hector et al. (11) suggested that endogenous chitinase may lyse part of the spherule wall, liberating the internal contents (endospores and additional components). In vivo, the liberated endospores would produce a second generation of mature spherules with the subsequent chitinase formation and further rounds of fungal replication. This might explain

why the titer of complement-fixing and IDCF antibodies rises with increasingly severe and progressive disease. Presumably, each fungal replication cycle occurring within the host would increase the quantity of chitinase, which would further stimulate CF (antichitinase) antibody production. Thus, the antibody titer may reflect the antigenic (chitinase) load, which would be proportional to the fungal load.

Whether the chitinase-CF antigen has any role in pathogenesis, e.g., toxicity, other than to enhance the liberation of propagules of *C. immitis* in the host remains to be seen. Meanwhile, the isolation of the enzymatically active CF antigen may permit its application as a purified antigen, possibly enhancing the sensitivity and specificity of serodiagnosis for coccidioidomycosis. Our preliminary results have shown that the purified chitinase can serve as an antigen for coccidioidal antibody detection by the enzyme-linked immunosorbent assay.

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