

Purification and Amino-Terminal Sequence Analysis of the Complement-Fixing and Precipitin Antigens from *Coccidioides immitis*

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Two proteins (21 and 48 kilodaltons) purified from endospore-spherule culture filtrates of *Coccidioides immitis* are identified as precipitin and complement-fixing antigens, respectively. To allow specific structural comparison to antigens identified by other investigators and as a first step to eventual serodiagnostic antigen production by recombinant DNA technology, amino-terminal amino acid sequences were determined for these antigens.

Serologic responsiveness to infection with *Coccidioides immitis* has been the basis for several tests employed in the diagnosis of coccidioidomycosis (8). The early immunoglobulin M (IgM) response can be detected by the tube precipitin (TP) test or by identification of a characteristic precipitin band (ID-TP) in the immunodiffusion (ID) test. The IgG response can be detected in the complement fixation (CF) test or by detection of a corresponding band in the ID test (ID-CF). These serodiagnostic tests rely on the use of crude fungal extracts or autolysates as antigens to assay for antibodies in sera or other body fluids. The purification and identification of specific antigens would enhance both the specificity and standardization of these serodiagnostic tests.

We have been studying proteins secreted by endospore-spherule phase cultures of *C. immitis*. Previously, we isolated and purified by fast protein liquid chromatography two protein components of crude endospore-spherule filtrates, which had molecular sizes of 18 to 21 kilodaltons (kDa) (hereafter called 21 kDa) and 48 to 50 kDa (hereafter called 48 kDa) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 6). The 21-kDa protein is a serine proteinase (9). The 48-kDa protein had no known enzymatic activity, but it migrated at a similar position on SDS-PAGE as a major antigen secreted by endospore-spherule cultures that had been associated with the ID-CF reactivity (11). This latter antigen in turn corresponds to a larger molecular species (110 kDa) seen on unreduced SDS-PAGE (11).

We now present data confirming that both the 21- and 48-kDa proteins are recognized by the human immune response to *C. immitis* and their identification with ID-TP and ID-CF antigenic activities, respectively. To allow more accurate comparison with other antigens of similar molecular sizes identified by other investigators and as a first step toward production of recombinant serodiagnostic antigens for *C. immitis*, an amino acid sequence was obtained for each of these proteins.

The procedures used to purify the 21- and 48-kDa proteins have been described in detail previously (9). Briefly, *C. immitis* endospore filtrate was collected 60 h after inoculation of cultures (7) and filter sterilized. The filtrate was concentrated by precipitation with 70% ammonium sulfate,

and applied to a Mono P HR 5/20 chromatofocusing column (Pharmacia, Uppsala, Sweden) in 20 mM Tris-acetate, pH 8.3. Samples were eluted by pH gradient with 70% Polybuffer 74-acetate (Pharmacia) and 30% Polybuffer 96-acetate (Pharmacia), pH 5.0, at a flow rate of 0.5 ml/min. For purification of the 21-kDa serine proteinase, fractions were assayed for elastase activity with purified, tritium-labeled elastin, a substrate of the 21-kDa protease (9). The peak of elastase activity eluting at a pI of 5.5 to 6.0 was further purified by ion-exchange high-pressure liquid chromatography, using a Mono Q HR 5/5 column (Pharmacia) in 20 mM Tris hydrochloride, pH 8.5. Samples were applied at 0.25 ml/min and eluted with a linear gradient from 0 to 500 mM NaCl (total volume of 30 ml at a rate of 0.5 ml/min). This procedure yielded >95% pure 21-kDa protein as assessed by silver-stained SDS-PAGE (9). Purity was sufficient to allow an unequivocal 22-residue, amino-terminal amino acid sequence to be determined from 10 pmol of purified protein. No secondary peaks were seen on an analysis of products of Edman degradation. Therefore, either the second high-molecular-weight band noted on SDS-PAGE (9) was so minor relative to the 21-kDa species that it was not detected or it corresponds to a dimer of the 21-kDa species with an identical amino terminus.

Purification of the 48-kDa protein was carried out in a manner similar to that of the other antigen, except that the chromatofocusing peak eluting at a pI of 6.5 to 7.0 was selected for the subsequent ion-exchange high-pressure liquid chromatography step. Purification was monitored with SDS-PAGE, and purity was approximately 90% (9). For protein sequencing, a final purification step to remove minor contaminants was carried out by reversed-phase high-pressure liquid chromatography on an Altex C3 column in 0.1% trifluoroacetic acid. Protein was eluted by a 0 to 50% acetonitrile gradient, and the major protein peak, representing the 48-kDa antigen, was collected for sequence analysis. As with the 21-kDa antigen, only one signal was detected after Edman degradation, indicating that the sample contained only a single amino terminus.

Amino acid sequencing of purified proteins was performed by automated Edman degradation, using an ABI 470A gas phase protein sequenator with an on-line 120A PTH analyzer

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TABLE 1. Reactivity of purified antigens versus human sera^a

Antigen	Secondary antibody	ELISA values (A_{414}) for pool of:	
		Positive sera ^b	Negative sera ^c
48 kDa	Anti-IgG	1.64	0.015
	Anti-IgM	0.508	0.010
21 kDa	Anti-IgG	0.498	0.010
	Anti-IgM	0.550	0.015

^a One microgram of antigen per well was used.

^b From nine patients with *C. immitis* CF titers of >1:32.

^c From five healthy controls negative for coccidioidal antibody by CF and, after eightfold concentration, by ID.

as described in Applied Biosystem Sequencer Notes 470A-0A (27 August 1987).

As an initial test of whether the fast protein liquid chromatography-purified proteins were antigens, nine human sera which had coccidioidal CF titers of 1:32 to 1:256 and were positive for CF (IgG) antibody by ID were pooled for use in an enzyme-linked immunosorbent assay (ELISA). Four human sera, negative by CF and ID, were pooled as the negative serum control. Purified 21- or 48-kDa protein (1 μ g) in 50 μ l of phosphate-buffered saline was used to coat 96-well immunoassay plates (Costar, Cambridge, Mass.). ELISA was run according to standard procedures, using human antiserum diluted 1:100 in wash buffer (1% bovine serum albumin plus 0.2% Triton X-100 in phosphate-buffered saline) and peroxidase-conjugated goat anti-human (IgG or IgM) antisera (Zymed) diluted 1:250 in wash buffer. At 20 min after the addition of substrate, the reaction color was measured spectrophotometrically on an ELISA reader (Tertek) at 414 nm.

Both the 21- and 48-kDa proteins reacted with the pooled positive serum from patients with documented *C. immitis* infections and did not react with control sera (Table 1). While both antigens showed some reactivity with IgM and IgG, the 48-kDa antigen was clearly more reactive with IgG, while the 21-kDa antigen was slightly more reactive with IgM. Because of its greater immune reactivity, the purified preparation containing the 48-kDa antigen was then tested (double-blind) against five individual patient serum samples previously assayed for standard CF titer. All five positive CF sera showed significantly higher A_{414} values than did the controls (Fig. 1). Immunoblots (Western blots) of the purified 21- and 48-kDa antigens were also prepared and reacted with CF-positive, pooled human sera. Only the 48-kDa preparation was reactive with antisera after electrophoresis and immunoblotting (Fig. 2). The failure of the purified 21-kDa antigen to react on immunoblot is probably due to the loss of major epitopes when the protein is boiled, reduced, and reacted with SDS during electrophoretic separation.

The purified 21- and 48-kDa antigens were available in sufficient quantity to be used as antigens in the standard ID (Ouchterlony) plate assay. Human sera submitted for serodiagnoses were tested by ID, using modifications of the method of Huppert and Bailey (5). The modifications consisted of concentrating the specimen eightfold by evaporation and using heated and unheated coccidioidin to test for the presence of precipitin (IgM) and CF (IgG) antibodies, respectively (8). The 48-kDa antigen had identity with the ID-CF antigen, as assessed by precipitation with sera known to be positive by ID-CF (Fig. 3, top). The 21-kDa antigen also precipitated with immune sera but formed a line of identity with the ID-TP band (Fig. 3, bottom).

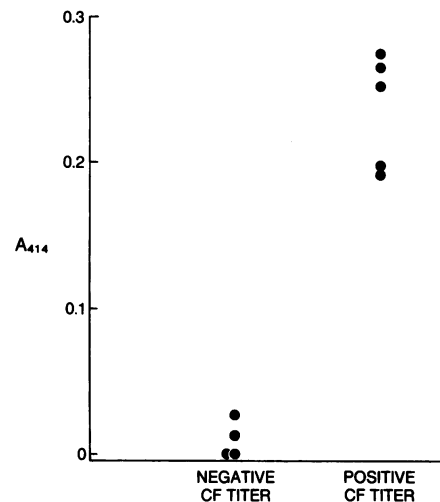


FIG. 1. ELISA titer versus purified 48-kDa antigen of sera from patients showing positive or negative complement-fixing reactivity.

The 48- and 21-kDa proteins were sufficiently pure to be further characterized by amino-terminal amino acid sequencing of 10 pmol of protein. The 48-kDa protein had the following amino-terminal sequence: Tyr-Tyr-Pro-Val-Pro-Glu-Ala-Pro-Ala-Glu-Gly-Gly-Phe-Arg-Ala-Val-Val-Tyr-Phe-Val-Asn-Arg-Ala-Ile-Tyr-Gly-Arg. The 21-kDa serine protease had the following amino-terminal sequence: Thr-Pro-Leu-Ala-Ser-Thr-Ala-Asp-Leu-Ser-Tyr-Asp-Gly-Pro-Ser-Leu-Pro-Leu-Ser-Gly-Val-Thr. Neither of these sequences showed significant homology with any protein sequences in the Dayhoff Protein Sequence Data Base.

Identifying these two fast protein liquid chromatography-purified proteins as ID-TP and ID-CF antigens, coupled with

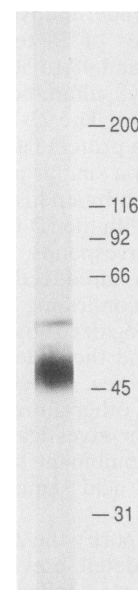


FIG. 2. Western blot of pooled CF-positive sera versus purified 48-kDa antigen. A minor higher-molecular-weight band is also visible, possibly representing a precursor or a more glycosylated form of the 48-kDa antigen. No reaction was seen with a pool of CF-negative sera.

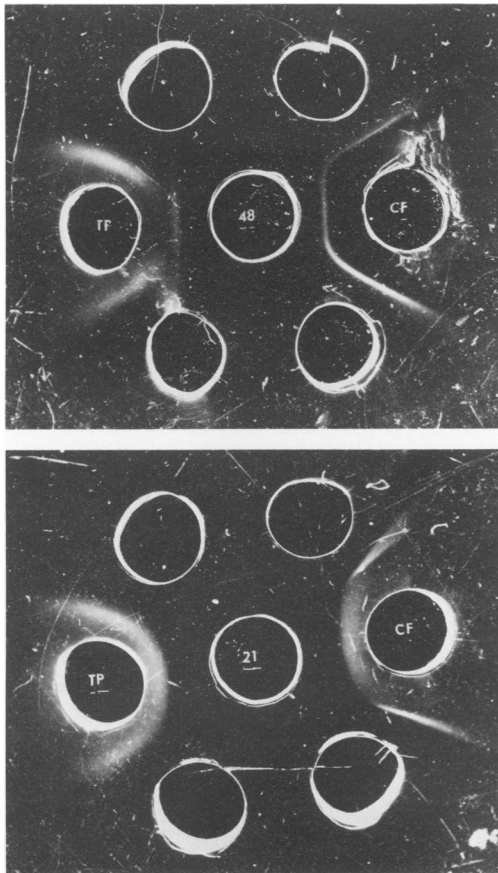


FIG. 3. (Top) ID reactivity of the 48-kDa antigen (center well). Note that the 48-kDa antigen forms a line of identity with the ID-CF antibody (middle row, right-hand well) and a line of nonidentity with serum containing ID-TP antibody (middle row, left-hand well). (Bottom) ID reactivity of the 21-kDa antigen (center well). Note that the 21-kDa antigen shows a line of identity with the ID-TP antibody (middle row, left-hand well) and partial identity with the ID-CF antibody (middle row, right-hand well). The top and bottom wells on the left contain the control (heated coccidioidal antigen) which retains reactivity with IgM (ID-TP) antibody but not CF (IgG) antibody. The top and bottom wells on the right contain unheated antigen which can react with both IgM (ID-TP) and CF (IgG) antibodies.

recent work of other investigators, helps to unravel the complex mixture of antigens used for the serodiagnosis of coccidioidomycosis. A previous study of crude mycelial and spherule-endospore filtrate by SDS-PAGE and immunoblot (11) demonstrated the presence of immunoreactive antigens in the ranges of 18 to 21 and 40 to 70 kDa, respectively. Silver-stained SDS-PAGE of both heated (60° for 30 min) and unheated filtrates indicated that several of the antigens recognized by immunoblot were clearly proteins and at least one of the proteins (21 kDa) was stable to the heat treatment. The 18- to 21-kDa antigens also did not appear to be significantly glycosylated because there was no binding by concanavalin A (11). These observations on the lower-molecular-weight species in crude filtrate are consistent with the characteristics of the 21-kDa antigen we have purified. Serine proteases are not significantly glycosylated and, in fact, concanavalin A columns have been used to separate fungal proteases (which do not bind) from other fungal proteins which are more glycosylated (1). An antigen in the

18- to 21-kDa range was detected on immunoblot with anti-IgG antibodies but not anti-IgM antibodies (11). We could not detect the purified 21-kDa antigen on immunoblot with mixed (IgG, IgM, and IgA) secondary antibody. This could mean either that epitopes on the 21-kDa proteinase were altered during purification or that another antigen of similar molecular size is present in the crude filtrate.

Several antigens are present in the 48- to 50-kDa range in crude filtrate (11). Protein blots followed by binding with concanavalin A indicated that some but not all of the antigens in this range were heavily glycosylated (11). To determine whether the purified 48-kDa antigen was heavily glycosylated, we examined its mobility on SDS-PAGE before and after reaction for 24 h with 2 U of endoglycosidase F (Sigma Chemical Co., St. Louis, Mo.). While this treatment significantly affected the mobility of glycosylated RNase B (Sigma), it had no effect on the mobility of the purified 48-kDa antigen.

Yuan and Cole (10) have identified and purified a 36-kDa protease from the mycelial phase of *C. immitis*. This protein, identified as antigen 11 by two-dimensional electrophoresis, does not appear related to either of the proteins described in the present study. Cox and Britt (3) have speculated that their "HS" antigen of coccidioidin corresponds to antigen 11. Cox et al. (3, 4) have also fractionated coccidioidin and isolated antigens that they suggest correspond to the ID-TP and ID-CF, and they raise the question whether the latter activity (F antigen) is similar to that of the 48-kDa antigen of Zimmer and Pappagianis (11) and the 45- to 48-kDa antigen of Calhoun et al. (2). The determination of an amino-terminal amino acid sequence for these purified *C. immitis* antigens now allows more direct structural comparisons with different antigen preparations which have similar electrophoretic mobilities and come from different laboratories. Sequence determination also makes it possible to use oligonucleotide probes to isolate antigen genes and to express recombinant antigens, using recently constructed genomic (S. Resnick, G. Apodaca, G. Newport, C. Halde, and J. McKerrow, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D-48, p. 80) and cDNA expression libraries (L. Yuan, J. H. McKerrow, and G. T. Cole, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, F-22, p. 395) for *C. immitis*.

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