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Purification and Characterization of Two Proteins from Culture Filtrates of *Mycobacterium tuberculosis* $H_{37}Ra$ Strain

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Two proteins have been purified from culture filtrates of *Mycobacterium tuberculosis*, $H_{37}Ra$ strain by a procedure combining gel filtration, diethylaminoethyl (DEAE)-cellulose chromatography, and zone electrophoresis. The two proteins are similar in molecular weight but differ slightly in charge. The faster migrating protein, designated a_1, is not antigenic. The slower migrating protein, designated a_2, is antigenic both with respect to antisera and as a skin-testing antigen.

The antigenic nature of culture filtrates of *Mycobacterium tuberculosis* is well known. A 10-fold concentrated filtrate and Purified Protein Derivative (PPD), the ammonium sulfate precipitable constituents of Old Tuberculin, have been studied extensively as antigens. Both of these preparations contain a variable mixture of a large number of antigenic constituents.

The purpose of this communication is to describe the purification and characterization of two proteins from culture filtrates of *M. tuberculosis*. The procedure combines gel filtration, ion-exchange chromatography, and zone electrophoresis. The two proteins have been designated a_1, the protein which moves fastest in an electrophoretic field, and a_2, the slower migrating component. Both proteins are major constituents of culture filtrates, readily identifiable as discrete bands on disc electrophoresis and, in the case of a_2, giving an easily recognized line on immunoelectrophoresis. Differing in charge but similar in molecular weight, a_2 has been found to be antigenic and a_1 nonantigenic.

MATERIALS AND METHODS

Culture filtrates. *M. tuberculosis* $H_{37}Ra$ strain was obtained from the Tuberculosis Culture Bank of the Trudeau Foundation Medical Research Laboratories, Saranac Lake, New York. Pellicle cultures were grown on the totally synthetic medium of Proskauer and Beck (11). Culture medium (300-ml volumes) in 1,000-ml glass Roux culture bottles with offset necks was inoculated from 10- to 14-day-old seed cultures and incubated in the horizontal position at 37 C for 8 to 10 weeks. Filtrates were harvested by filtration through Whatman no. 4 filter paper and filtered through 0.45-mμ pore cellulose acetate membranes. The sterile filtrate was then concentrated 10-fold by flash evaporation with a temperature gradient of 37 to 2 C. Thimerosal was added in a concentration of 1:10,000 and the concentrated filtrate was stored at 4 C.

Ammonium sulfate precipitation. Ammonium sulfate precipitation at a concentration of 50% was accomplished by slowly stirring in dry ammonium sulfate in an amount equivalent to 297 g/liter at 4 C, sodium hydroxide being added as necessary to maintain approximate neutrality.

Gel filtration. Gel filtration was carried out in glass chromatographic columns fitted with upward flow adapters with the use of Biogel polyacrylamide gels of appropriate porosity. Columns were packed by gravity with limitation of hydrostatic pressure to approximately 10 cm at all times. Saline (0.15 M) was used for elution. Biogel P-300 columns with total bed lengths of 250 to 300 cm were achieved by connecting three 100-cm columns in tandem, top to bottom, and eluting with upward flow. Hydrostatic pressure was limited to 30 cm when three columns were connected in tandem. Flow rates varied from 6 to 10 ml/hr. Analytic gel filtration for estimation of molecular weight was carried out in a column (0.9 by 28 cm) with Biogel P-30. The analytic column was calibrated by using the following proteins as reference standards: crystallized human serum albumin, assumed molecular weight 65,000; human hemoglobin, assumed molecular weight 68,000; and bovine beta lactoglobulin, assumed molecular weight 36,000 (gift of David Bing, Michigan State University, Lansing, Mich.).

Diethylaminoethyl (DEAE)-cellulose chromatography. Ion-exchange chromatography was carried out in 2.5-cm glass chromatography columns by using DEAE-cellulose. Columns were packed by gravity to bed heights of 30 to 35 cm, and elution was carried out with gravity flow. The gradient used for elution was achieved by connecting two 2,000-ml aspirator bottles in tandem, bottom to top, with a magnetic stirrer mix-

ing the buffer in the second bottle. The first bottle contained 2,000 ml of 0.5 M monobasic sodium phosphate; the second bottle, connected to the column, contained 2,000 ml of 0.01 M sodium phosphate buffer at *p*H 8.0. A 300-ml amount of 0.01 sodium phosphate buffer at *p*H 8.0 was used for elution before the gradient was started. Samples were dialyzed against the 0.01 M buffer before being chromatographed.

Zone electrophoresis. Preparative zone electrophoresis was carried out in a water-cooled electrophoresis cell with a horizontal bed measuring 21 by 33 cm, by using a matrix of powdered polyvinyl chloride (Pevikon, Mercer Chemical Corp.). The bed was divided longitudinally with glass rods so that only a central channel 2.5 cm wide and 0.5 cm deep was used for electrophoresis. A 2-amino-2-hydroxymethyl-1-3-propandiol (Tris)-glycine buffer at *p*H 8.2, prepared with 3.0 g of Tris and 14.4 g of glycine in a final volume of 1 liter, was used. The sample was dialyzed against the electrophoresis buffer and applied to the electrophoresis block by saturating four thicknesses of Whatman 3MM filter paper cut to fit the central channel of the bed with sample material and imbedding the saturated paper in the bed. Electrophoresis was carried out for 20 hr at 475 v, resulting in 7 to 10 ma current flows. At the end of the electrophoresis, the block was cut in 1-cm strips and the powdered polyvinyl chloride was removed with a spatula. The samples were then eluted with buffer.

Concentration. Concentration of eluates from columns was accomplished by vacuum dialysis against isotonic saline by using cellulose dialysis tubing when small volumes were involved. Large volumes were concentrated by pressure ultrafiltration by using a Diaflo UM-1 membrane in a Diaflow model 50 ultrafiltration cell (Amicon Corp., Lexington, Mass.) This membrane is capable of excluding solutes with a molecular weight greater than 10,000.

Immunodiffusion and immunoelectrophoresis. Double diffusion studies in agar gel and immunoelectrophoresis were carried out as described by Crowle (6). Antisera for these studies was prepared by the repeated injection into rabbits and goats of culture filtrates and protein-containing fractions emulsified in complete Freund's adjuvant containing mycobacteria of the $H_{37}Ra$ strain.

Disc electrophoresis. Disc electrophoresis in 7% polyacrylamide gel at *p*H 9.5 was performed by using a Canalco model 12 disc electrophoresis apparatus. Reagents supplied by the manufacturer were used, and the manufacturer's directions were followed. Samples, to which sucrose had been added, were layered on the gels just prior to electrophoresis. Gels were routinely stained for protein with Coomassie Brilliant Blue R 250 as described by Chrambach et al. (5).

Density gradient ultracentrifugation. Ultracentrifugation studies were carried out in gradients of 5 to 20% sucrose formed as described by Britten and Roberts (2). Samples of 0.2 ml volume were layered on the gradients and centrifuged in a model L-2 preparative ultracentrifuge by using the SW-50 rotor (17) for 25 hr at 35,500 rev/min (average radial centrifugal force = 100,000 × *g*) at 0 C. Under these conditions the sedimentation constant is linearly related to the distance of migration during centrifugation (13). Sedimentation constants were estimated by comparison with a standard of crystallized human serum albumin for which a sedimentation constant of 4.5 was assumed.

Protein estimation. Protein was measured by the method of Lowry et al. (12) or by comparing absorption at 280 nm with that of a crystallized human serum albumin standard prepared gravimetrically.

Skin tests. Intradermal skin tests to estimate tuberculin activity were performed on the flanks of adult male albino guinea pigs previously sensitized by the subcutaneous injection of heat-killed mycobacteria, $H_{37}Ra$ strain, emulsified with incomplete Freund's adjuvant. PPD-S was used as a reference standard in skin testing (gift of Lydia Edwards, U.S. Public Health Service). All skin tests were performed by using disposable plastic syringes and read by measuring induration at 48 hr. Serial dilutions of PPD-S and the a_1- and a_2-containing preparations were always tested simultaneously in groups of 10 guinea pigs. Relative potency was determined by plotting the diameter of reaction size against the concentration of protein on semilogarithmic graph paper.

RESULTS

Protein purification. The most satisfactory purification was achieved by combining fractionation methods serially as shown in Fig. 1. Volumes (100 ml) of 10-fold concentrated culture filtrate were passed through a Biogel P-30 column (54 by 5 cm). The entire first peak, including all of the fractions comprising the down slope portion, was collected, pooled, and brought to 50% saturation with ammonium sulfate. Two such ammonium sulfate precipitates were combined and taken up in 30 to 35 ml of 0.15 M saline. This

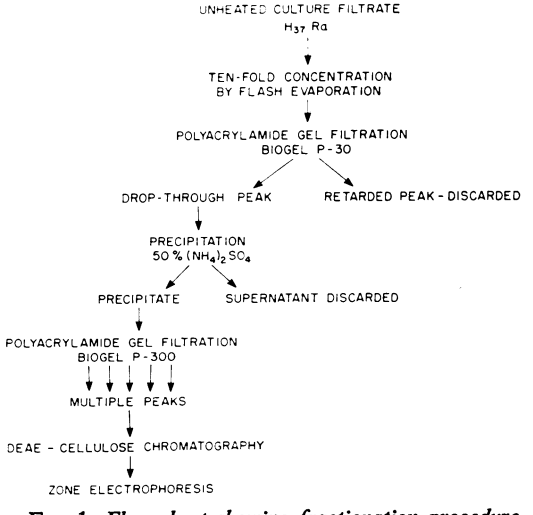

FIG. 1. *Flow sheet showing fractionation procedure employed in the isolation of proteins a_1 and a_2 from mycobacterial culture filtrates.*

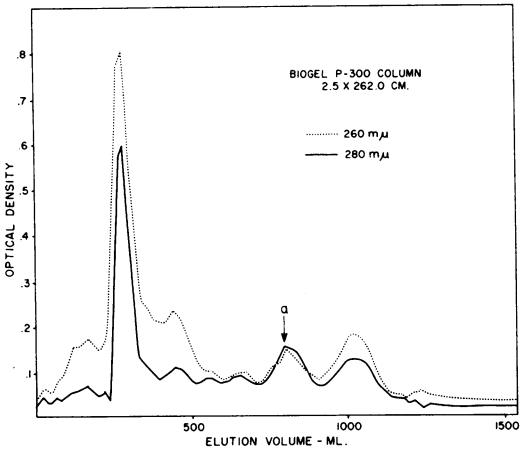

FIG. 2. *Elution chromatogram from Biogel P-300 gel filtration column. The peak labelled a contained both a_1 and a_2 proteins. Note that at this point in the chromatogram the optical density at 280 nm (solid line) exceeded that at 260 nm (dotted line), suggesting the presence of protein constituents.*

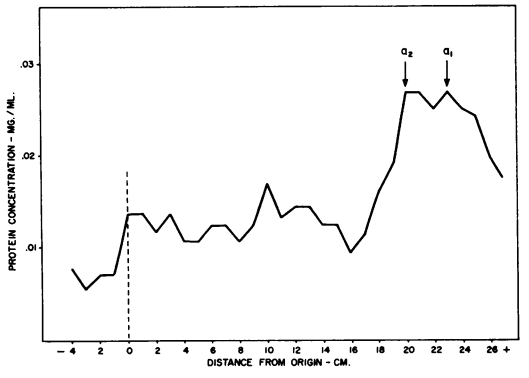

FIG. 3. *Electrophoretic pattern obtained in zone electrophoresis. Protein a_1 was present maximally in the fraction 23 cm from the origin. Protein a_2 was present maximally in the fractions 20 and 21 cm from the origin and was the only protein identifiable in the 20 cm fraction.*

material was then passed through Biogel P-300 column (262 by 2.5 cm), the chromatogram from which is shown in Fig. 2. The peak labelled a in Fig. 2 could be identified as containing the a_1 and a_2 proteins by disc electrophoresis and immunoelectrophoresis. The fractions comprising the central portion of this peak were pooled, concentrated by ultrafiltration to approximately 30 to 35 ml, and subjected to chromatography on a column (31 by 2.5 cm) of DEAE-cellulose. The fractions from the center of the major peak eluted in the mid-portion of the gradient were pooled, concentrated approximately fivefold, and stored at −20 C. The final step of purification was zone electrophoresis. Stored material from the DEAE-cellulose column was subjected to electrophoresis just prior to its further use. The electrophoretic pattern obtained is shown in Fig. 3. Separations between the a_1 and a_2 proteins were probably not complete, although in general at least one fraction contained each protein in sufficiently pure a state so that only a single line could be seen on disc electrophoresis (Fig. 4).

Characterization of a_1 and a_2 proteins. Both a_1 and a_2 lines in polyacrylamide disc electrophoresis columns could be stained readily with a variety of protein stains including Coomassie Blue, amido schwartz, thiazine red, and ponceau-S. Neither could be stained with Alcian blue 8GX or the periodic acid-Schiff reaction, although other components of culture filtrates could be stained thus. These staining characteristics indicate that the a_1 and a_2 components are largely protein without major polysaccharide moieties. The protein concentration of final preparations was usually in the range of 0.15 to 0.25 mg/ml.

The molecular weights of the a_1 and a_2 proteins were estimated both by density gradient ultracentrifugation and by analytic gel filtration. By neither technique could separation of the two components be achieved, indicating nearly identical sedimentation constants and molecular weights for the two proteins. The sedimentation constant determined from the ultracentrifugation

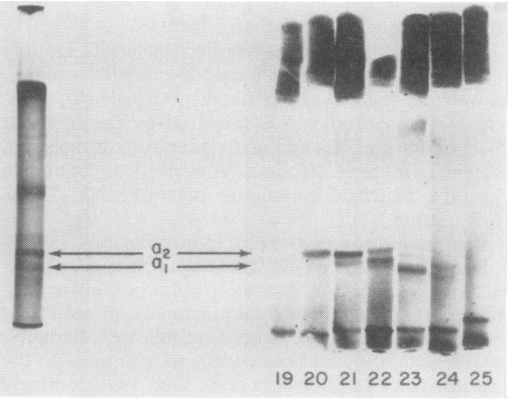

FIG. 4. *Disc electrophoresis gels from a 50% ammonium sulfate precipitate of an unfractionated culture filtrate (left) and consecutive electrophoresis fractions (right). The lines representing proteins a_1 and a_2 are indicated. Electrophoresis fractions are numbered according to the distance in centimeters from the origin at which they were taken. Gels were stained with Coomassie blue; the dense band at the bottom of each gel represents tracking dye which is not removed by the Coomassie Blue staining technique.*

studies was 2.8, and the molecular weight estimated from the analytical gel filtration was 45,000 to 48,000.

The antigenicity of the two proteins was studied separately with respect to their ability to react with serum antibody and to elicit delayed hypersensitivity skin reactions. By embedding an unstained disc electrophoresis gel column in agar and placing antiserum in a parallel trough, it was possible to show that the slower migrating component, designated a_2, reacted with antisera from 14 separate rabbits and goats. The a_1 component did not react with any of the antisera tested. In guinea pig skin testing experiments a_2 was found to be consistently skin test active, exhibiting approximately 20% of the potency of an equal amount of protein of PPD-S. Preparations of protein a_1 were invariably inactive or only very weakly active. Since preparations of a_1 were usually contaminated with small amounts of a_2, it seems reasonable to assume that most of the weak skin test activity observed with preparations of a_1 was due to contaminating a_2.

By methods of immunodiffusion and immunoelectrophoresis, protein a_2 was compared with proteins A, B, and C of Seibert (gift of Lewis Affronti, George Washington University, Washington, D.C.). The results of a gel diffusion study are shown in Fig. 5. It can be readily seen that a_2 is identical with a major component of Seibert's A protein and with minor components of B and C proteins.

By using the technique of immunoelectrophoresis, the a_2 antigen was identified in culture filtrates of *M. kansasii* as well as filtrates of *M. tuberculosis*. It was not identified in filtrates from a limited number of strains of other *Mycobacteria*.

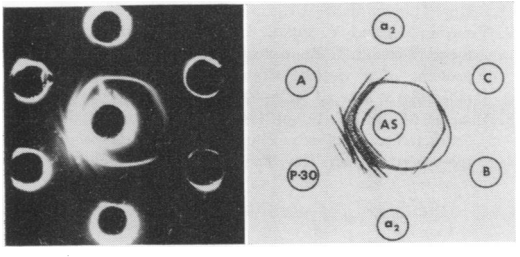

FIG. 5. *Double-diffusion analysis in agar gel. A double-diffusion pattern is shown photographically on the left and by artist's drawing on the right. The center well (labelled AS) contained polyvalent rabbit antiserum. The peripheral well labelled P-30 contained ammonium sulfate precipitated material from the first peak of the Biogel P-30 gel filtration. The two peripheral wells labelled a_2 contained purified a_2 protein. Peripheral wells labelled A, B, and C contained A, B, and C proteins of Seibert, respectively. Note the line of identity between the single line formed with protein a_2, the major line formed with A protein, and components of B and C proteins.*

DISCUSSION

Many investigators have sought to purify individual protein constituents from mycobacterial culture filtrates. Protein a_2 was demonstrated by immunodiffusion to be a major constituent of Seibert's protein A (16). Both proteins a_1 and a_2 would be expected to be recovered as the alpha protein in the purification method described by Yoneda and Fukui (8, 17, 18). The molecular weight of 30,000 which they estimated for their alpha protein was smaller than that observed in these studies, and a_2 also differs from the alpha protein by virtue of its reactivity as a skin test antigen. Similarly proteins a_1 and a_2 would be expected to fall in the GB fraction of Chaparas and Baer (4). The GB fraction was observed to be tuberculin-active with a potency similar to that of PPD-S and to be antigenic with respect to rabbit antisera. Its sedimentation constant was 1.3, denoting a smaller molecule than a_2. It is difficult to relate the a_2 antigen to the antigens characterized by Lind (10), but examination of the immunoelectrophoretic patterns which he has published suggest that the a_2 antigen may be the same as the antigen which Lind designated C. Castelnuovo and co-workers also studied culture filtrates by immunoelectrophoresis (3), but they did not isolate individual components and it is not possible to relate their antigens to the ones described in this communication. Fractionation of culture filtrates by DEAE-cellulose chromatography alone has been carried out by Kniker and LaBorde (9) and Diena et al (7). Both a_1 and a_2 proteins of the present study would be expected to occur in Kniker and LaBorde's fraction A and in either the F4 or F5 fraction of Diena et al (7). Pickett and co-workers recovered many antigens by using ammonium sulfate precipitation followed by DEAE-cellulose chromatography (14). They identified antigens by immunoelectrophoresis, obtaining patterns similar to those which we have obtained. Protein a_2 is probably similar to the antigen which they labelled i when obtained from cell cytoplasm and f when obtained from culture filtrates. Comparison of the behavior of a_2 antigen in polyacrylamide disc electrophoresis with the published results of others suggests that it may be the same protein as that assigned an R_F value of 40.0 by Affronti et al. (1). The nonantigenic a_1 protein is probably the same as that assigned a value of 37.5 in the same study. According to the scheme of Roszman et al. (15), proteins a_2 and a_1 probably correspond to the fractions designated 13 and 14, respectively.

The isolation procedure used in this study is a tedious one. It can, however, be applied on a preparative scale. The yield is probably small, but the degree of purity is high enough to allow study of the two proteins separately. The isolation of two proteins of essentially identical molecular size, apparently differing slightly in charge and markedly diverse in antigenicity, raises interesting questions. Foreign proteins are in general antigenic, and it is unusual to find a material such as a_1 which is not. The possibility should be considered that a_1 did not exist or was not accessible in the whole tubercle bacilli used to immunize animals. Such a situation might exist if a_1 were a product of autolytic denaturation occurring during purification or during the prolonged incubation of the original culture filtrates. Perhaps a_1 might be a denaturation product of a_2. Investigation of the differences between a_1 and a_2 might then prove fruitful in elucidating the nature of the antigenic site of a_2. It is also worth noting that both skin test antigenicity and serological antigenicity are missing in a_1, supporting the suggestion of Chaparas and Baer that the two types of antigenicity are related to the same molecular structure (4).

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