THE ISOLATION AND PURIFICATION OF TWO ANTIGENS FROM CRUSHED CELLS OF *BACILLUS MEGATERIUM* ATCC 14581

by

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ABSTRACT

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A sonicated cell mass of Bacillus megaterium ATCC 14581, extracted with buffered salts pH 7.0, yielded four precipitin lines when assayed for serological activity by immunodiffusion against homologous high titer precipitating antisera; six precipitin lines were observed with immunoelectrophoresis. Passive hemagglutination gave a titer of 512.

Cell walls were isolated from a cell suspension of B. megaterium crushed by decompression rupture in the French pressure cell and purified by sucrose density gradient centrifugation followed by repeated washings in distilled water. Acid extracts of cell walls failed to produce precipitin lines when tested by immunodiffusion against homologous whole cell and cell wall sera.

Hot HCl extracts of crushed cells yielded one heavy and two faint precipitin lines. Alcohol precipitates of the acid extracts yielded two precipitating antigens, designated A and B. The major antigen, A, was eluted by chromatography on a Dowex-1-Cl anion exchange resin at a linear gradient concentration of 0.2 M ammonium formate. The minor antigen,

B, was eluted by gel-filtration on Sephadex G-100 in fractions 9 and 10.

Preliminary studies showed that antigen B is mainly carbohydrate in nature, with a small amount of protein and nucleic acid present, in an approximate ratio of 14:2:1 respectively.

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INTRODUCTION

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Recent advances in biochemical analysis have enabled research workers to localize, isolate, purify and characterize many types of microbial antigens. Gel-filtration and ion exchange chromatography on DEAE cellulose and Dowex resins have yielded purified antigen fractions from mycobacteria, staphylococci and several other bacterial species (Haukenes, 1962; Keeler and Pier, 1965; Hofstad, 1965; Lind, 1965). Agar gel diffusion and immuno-electrophoresis provide a method for the assay and the identification of multiple antigenic specificities. This kind of antigenic analysis can be used for the localization of structural antigenic components, as well as for the investigation of the nature of antigen-antibody reactions.

In this study, a method for the extraction of antigens from a crushed cell mass with large quantities of materials is described. Hot acid extraction and separation by column chromatography yield purified antigen fractions. The serological activity of the isolated components is measured by gel diffusion. Preliminary chemical analysis is carried out.

HISTORICAL

HISTORICAL

Bacteria are composed of many different chemical constituents, some of which are antigenic in nature. The varied chemical nature of these antigens enables them to be differentiated and characterized by a combination of biochemical and serological techniques.

Bacterial antigens may be composed of proteins, polysaccharides or polysaccharide-phospholipid complexes (Carpenter, 1965). The antigenicity of these compounds is due to the presence of certain specific determinant groups. Thus, it can be seen that serological analyses can be a valuable tool to aid in classifying bacteria since related organisms would be expected to possess similar antigenic components.

The serological activity of cell walls, capsules, protoplasts and other isolated structures is, for the most part, highly specific. Vennes and Gerhardt (1956, 1959), using complement fixation tests, found that the polysaccharide components extracted from cell walls of B. megaterium KM were immunologically distinct from polypeptide antigens.

Methods used for the extraction of antigens from micro-organisms are designed to recover an antigenic fraction from large quantities of cells without altering its original antigenic specificity. Extraction of whole cells with hot HCl was introduced by Lancefield in 1928 as a method of isolating heat stable antigens from streptococci species. She found three antigens: two proteins and a species-specific carbohydrate (C) which was used as a basis for the serological grouping of Streptococci. However, Lancefield (1933) stated that this treatment may alter the structure of certain antigens.

Hot acid extraction of staphylococci by Julianelle and Wieghard (1934) yielded a type-specific carbohydrate antigen A, which has been further investigated by Haukenes (1962). Maxted and Frazer (1964), using hot acid extraction of group D streptococci cells followed by ethanol treatment, found an alcohol-soluble, acetone-insoluble substance which combined with type-specific antibody but did not form a visible precipitate. Slade (1965) has also used hot trichloroacetic acid to extract the polysaccharide antigens from cell walls of various streptococci species.

Two antigens were isolated from Bacillus "M", a

strain of Bacillus megaterium, by boiling the organisms in water (Geux-Holzer and Tomcsik, 1956). A polysaccharide hapten was separated from a polypeptide antigen by ethanol fractionation. The polysaccharide was further purified by repeated ethanol precipitation and gave high titers when tested against homologous antisera. A unique modification of phase contrast microscopy was employed to show the relationship of the polysaccharide to the capsule and cell wall of Bacillus "M". The addition of anti-polypeptide serum to suspensions of whole cells produced a uniform halo surrounding the cell; whereas the addition of anti-polysaccharide serum resulted in a dense precipitate located at the septa and the polar caps. These workers concluded that the polysaccharide was present in both the cell wall and the capsular framework of Bacillus megaterium.

Gel diffusion methods of immunologic analysis are especially suitable for qualitative analysis of highly complex antigenic reactants as they allow the direct observation of the number of antigenic fractions present (Ouchterlony, 1958; Lind, 1965). Several modifications of Ouchterlony's original method (1949) have been devised, each providing a variation which enables one to exploit

fully the usefulness of immunodiffusion for serological analysis.

The antigenic analysis of culture filtrates of Mycobacterium tuberculosis has been carried out by Lind (1965) using the technique of gel diffusion. He stresses the importance of using a reference system to aid in the identification of different antigenic fractions.

The immunoelectrophoretic technique devised by Grabar and Williams (1953) is an important method by which the antigenic character of soluble precipitating antigens can be studied. Baillie and Norris (1964) used immuno-electrophoresis to follow changes in the types and levels of antigens present during spore formation in B. cereus. In the course of their studies, they found vegetative extracts to contain several antigens which were resistant to 80^{o} C for 10 minutes.

Using Ouchterlony's technique as an assay system, Hofstad (1965) purified and characterized a major polysaccharide antigen from the 80/81 complex of S. aureus. This antigen proved to be the main precipitating antigen in the organism studied. A crude preparation was obtained by buffer extraction at an acidic pH followed by repeated

ethanol precipitation. The polysaccharide was further purified by chromatography on a DEAE cellulose anion exchange column, using a linear gradient of sodium chloride. The purified antigen was shown to consist of a polysaccharide and a polypeptide moiety that were firmly linked together. The carbohydrate portion of the antigen was identified as a glucosaminyl ribitol teichoic acid, similar in nature to the polysaccharide A antigen isolated from a strain of S. aureus by Haukenes (1962).

Haukenes considers polysaccharide A to be a teichoic acid-mucopeptide complex similar to that released from the cell walls of Bacillus megaterium KM by lysozyme treatment (Ghuysen, 1961, 1964). The teichoic acid portion of the B. megaterium complex was associated with a residue of glucose, N-acetyl hexosamine and amino acids. The polysaccharide could not be extracted by cold trichloroacetic acid treatment, the usual method used for the extraction of teichoic acids.

Teichoic acids are a class of carbohydrate associated with the cell walls and cytoplasmic membranes of several gram-positive species of bacteria. These polymers of ribitol phosphate or glycerol phosphate, to which sugars and

ester-linked alanine may be attached, were designated as teichoic acids by Armstrong et al (1958). Their chemical nature has been investigated by Baddiley et al (1961) and has been confirmed by studies on their enzymatic synthesis (Burger and Glaser, 1964; Glaser, 1964; Glaser and Burger, 1964).

Studies on ribitol teichoic acids by Armstrong et al (1958, 1959) indicate a basic structure consisting of ribitol units joined by phosphodiester linkages. The presence of ester-linked D-alanine was also characteristic. The glucosyl residues of the polymer were either glucose as in B. subtilis (Baddiley and Davison, 1961) or N-acetyl amino sugars as in S. aureus (Sanderson et al, 1961).

Glycerol teichoic acids yield glycerol, alanine, glycerophosphate and usually a sugar upon acid hydrolysis (Baddiley and Davison, 1961) and are found in the cytoplasmic membranes as well as in the cell wall of bacteria. They show a similar structure to that of ribitol teichoic acids (Kelemen and Baddiley, 1961). The presence of a glycerol teichoic acid in the cell wall of B. stearothermophilus has been reported by Wicken (1966). The release of teichoic acid occurred very slowly when cells were treated with

dilute trichloroacetic acid, similar to results reported for B. megaterium (Ghuysen, 1961). D-alanine, attached to the polymer through the glucose hydroxyl group was reported to be a major component of the polysaccharide.

Several studies have shown that teichoic acids constitute an important antigenic component of several genera of gram-positive bacteria as these polymers are known to confer species-specificity (McCarty, 1959; Juergens et al, 1960; Haukenes, 1962). The group D antigen of streptococci has been identified as an intracellular teichoic acid present in the cytoplasmic membrane (McCarty, 1959). The addition of teichoic acid to high titer sera prior to agglutination tests inhibits agglutination of staphylococcal cell walls (Juergens et al, 1960, 1963; Sanderson et al, 1961). The antigenic determinant of the teichoic acid isolated from S. aureus 80/81 strain was shown to be β-linked N-acetyl glucosamine (McCarty and Morse, 1964). Also, the passive hemagglutination of tanned red blood cells sensitized with teichoic acid from the same strain was inhibited by the addition of β-N-acetyl glucosamine derivatives to antisera prior to testing (Nathenson and Strominger, 1962).

From the work previously discussed, it can be seen that a variety of sophisticated biochemical techniques are currently being used in the field of immunochemistry for the isolation, purification and characterization of microbial antigens. Both Haukenes (1962) and Hofstad (1965) have used anion exchange chromatography to isolate and purify antigenic teichoic acid complexes from buffered salts extracts of crushed S. aureus cells. Keeler and Pier (1965) used DEAE cellulose columns to purify extracellular antigens of Nocardia asteroides. Gel-filtration techniques have also been used to purify precipitinogenic material found in culture filtrates of Mycobacterium tuberculosis (Lind, 1965). Chromatography on Sephadex G-50, G-100 and G-200 resulted in a partial separation of six antigen factors, three of which were stable to 100° C for 15 min. In all the above systems, agar gel diffusion was used to assay for serological activity of the isolated fractions.

MATERIALS AND METHODS

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Cultures

The strain Bacillus megaterium ATCC 14581 was used throughout the studies. Bacillus megaterium strain "M" (received from Dr. Tomcsik), Bacillus subtilis ATCC 9945A, Bacillus cereus ATCC 10206, Bacillus anthracis ATCC 14578, and laboratory strains of Bacillus pumilis, Bacillus mycoides, Bacillus fusiformis and Bacillus polymyxa were used to test cross reactivity. The cultures were maintained in brain heart infusion, BHI, (B.B.L.) media and subcultured at frequent intervals.

Mass Cultures

B. megaterium ATCC 14581 was grown in 600 ml BHI broth in 2-liter Fernbach flasks on a rotary shaker at 30° C for 12 hours. The cells were harvested at 0° C in a Servall RC_2 centrifuge at 7,000 x g for 20 minutes, washed twice with 0.1 M phosphate buffer pH 7.0 and stored at -15°C.

Whole Cell Antigens

B. megaterium, *Bacillus* "M", *B. anthracis* and *B. cereus* were grown on BHI agar in Roux bottles at 28° C for 20 hours. The cells were suspended in 10 ml of 0.85% saline, washed twice in saline and resuspended in 0.2% formal saline to give a density of 38% light transmission on the Beckman model C colorimeter, corresponding to MacFarland tube no. 3. The cells were inactivated by storage at 4° C for 4 to 7 days with the exception of *B. anthracis* which was heated at 100° C for 45 min. The vaccines were tested for sterility and stored at 5° C.

Cell Wall Antigen

Purified cell walls, obtained by the French pressure cell technique, were suspended in 0.2% formal saline and prepared as described for whole cell antigens.

Antisera Production

Male albino rabbits were used for the production of high titer antisera. Sera were collected by cardiac puncture and stored at -15° C.

A whole cell suspension was injected according to the method of Kabat and Mayer (1961). Each animal received a total of 140 to 200 mg antigen over a period of 5 to 7 weeks.

A cell wall suspension was injected according to the method of Slavin (1950) using calcium alginate as adjuvant. A total of 30 mg cell wall suspension was given over a period of 9 weeks.

Buffered Sonic Extract

A cell mass of 30 grams wet weight B. megaterium ATCC 14581 was suspended in 15 ml of 0.1 M phosphate buffer pH 7.0. The slurry of 45 ml was sonicated at maximum current for 1 hour and centrifuged at 10,000 x g at 0° C for 30 min. The supernatant was labeled "buffered sonic extract" and stored at -15° C.

Hot HCl Extract

A crushed cell mass, as used for the buffered extract, was treated with 4 volumes of 0.1 N HCl at $90\text{-}95^{\circ}$ C for 25 min, cooled on ice to room temperature, adjusted to pH 7.0-7.2 with 1 N NaOH and centrifuged at 12,000 x g in the Servall RC_2 for 20 min. The clear yellowish supernatant

was concentrated to approximately 1/10 volume by dialysis against moving air at room temperature, dialysed for 12 hours against 6 liters of 0.02 M phosphate buffer pH 7.0 at 4° C and stored at -15° C. This was labeled "hot acid extract".

Alcohol Precipitate of HCl Extract

Twenty ml of hot acid extract were adjusted to pH 5.0 with 1/10 the volume of a 20% sodium acetate-HAc solution, allowed to stand at room temperature for 5 to 10 min and centrifuged at 20,000 x g at 0° C for 20 min. The supernatant was treated with 2.5 times the volume of 95% ethanol at -15° C and stored at -15° C overnight. The white flocculant alcohol precipitate was collected by centrifugation at 12,000 x g for 20 min, suspended in 10 ml 0.02 M phosphate buffer pH 7.0 and recentrifuged at 10,000 x g for 10 min. The supernatant was labeled "ethanol precipitate" and, together with the insoluble residue, was stored at -15° C.

Column Chromatography

(A) Sephadex G-100

The ethanol precipitate was eluted by gel-filtration on a Sephadex G-100 column. A column 2.5 cm x 35 cm was packed and washed with 0.02 M phosphate buffer pH 7.0. Two ml of ethanol precipitate were applied to the column and eluted with the equilibrating buffer at room temperature at a flow rate of 20 ml per hour. Fifty 4-ml fractions were collected and assayed for absorption at 260 mμ and 280 mμ on the Unicam SP 700 or the Gilford spectrophotometer. Fractions were assayed for the presence of carbohydrate by a modification of the anthrone test of Scott and Melvin (1953). Appropriate fractions of the absorption peaks were tested for serological activity by immunodiffusion and passive hemagglutination.

(B) Dowex-1-Cl

The alcohol precipitate was eluted by ion exchange chromatography on Dowex-1-Cl, according to the method of Haukenes (1962). Dowex-1-Cl resin, in the formate form, was packed into a 2.5 cm x 18 cm column and equilibrated with

0.005 M ammonium formate pH 5.0 at 4° C. Two ml of the ethanol precipitate, previously dialysed against the starting buffer, were applied to the column and eluted with 200 ml of 0.005 M ammonium formate into 9.5 ml fractions. A linear gradient from 0.005 M ammonium formate to 2.0 M ammonium formate was then run and 100 fractions collected. Fractions were analysed for absorption at 280 mμ on the Gilford spectrophotometer. The fractions comprising the main absorption peaks were pooled, dialysed against 0.02 M phosphate buffer pH 7.0 for 12 hours and tested for serological activity by immunodiffusion.

Cell Walls

(A) French Pressure Cell

Approximately 30 grams wet weight of cell mass were suspended in distilled water to a final volume of 40 ml. This slurry was passed three times through a French pressure cell (Aminco) at 4° C, 20,000 lbs/sq. inch pressure. The crushed cell suspension was centrifuged at 1,000 x g for 20 min; the cell walls were recovered from the supernatant, washed once in 0.1 M phosphate buffer, twice in

1.0 M KCl and resuspended in 10 ml 1.0 M KCl. The crude walls were further purified by a modification of the sucrose density gradient technique of Yoshida et al (1961). Five ml of the suspension were layered on a 40 ml sucrose-KCl linear gradient, and spun at 460 x g at 4° C for 1 hour on a no. 215 swinging head in the International model H centrifuge. The cell walls were recovered from the cloudy layer of the gradient, washed three times with distilled water and stored at -15° C. Purity of cell wall preparations was confirmed by scanning on the Beckman DB spectrophotometer and on the phase contrast microscope.

(B) Sonication

Thirty grams of wet cell mass were suspended in 0.1 M phosphate buffer pH 7.0 to a total volume of 45 ml. The cells were disintegrated by sonication in the Raytheon sonic oscillator (10 KC) at maximum current for 15-20 min. The disrupted cells were centrifuged at 12,000 x g for 30 min, resuspended in 1.0 M KCl, homogenized and centrifuged at 1,000 x g for 20 min. The cell walls were recovered from the supernatant, washed 8-10 times in distilled water, lyophilized and stored at -15° C. Purity was assessed by

paper chromatography of the acid hydrolysates and by phase contrast microscopy.

Trichloroacetic Acid Extract

A 10 ml aqueous suspension of cell walls, prepared by the French pressure cell method, was adjusted to pH 3 with 50% trichloroacetic acid (TCA) and placed at 4° C for 3 hours. The precipitate was removed by centrifugation at 10,000 x g at room temperature for 20 min; the supernatant was dialysed against saline at 4° C for 12 hours and stored at -15° C.

Hot HCl Extract

One hundred mg (dry weight) of cell walls, prepared by sonication, were extracted in HCl at 95° C for 25 min as described above. The extract was concentrated against moving air, dialysed against 0.02 M phosphate buffer pH 7.0 overnight and stored at -15° C.

Serological Activity

(A) Agglutination Tests

The standard tube agglutination test was used to determine the serological activity of antisera. Halving dilutions of antisera in 0.4 ml volumes were added to an equal volume of antigen suspension, incubated at 37° C for 2 hours and at 4° C for 18 hours. Agglutination was read macroscopically with the aid of a Fisher-Kahn viewer and the titer recorded as the reciprocal of the highest dilution of serum giving a visible aggregate.

(B) Immunodiffusion

The double diffusion plate technique of Ouchterlony (1949) was followed, except that a horizontal trough was used as the antiserum reservoir. Special Noble agar (Difco) was layered over the bottom of a 9-cm plastic petri plate and covered with a second layer of 20 ml 0.8% agar in saline with 1% NaN_3. A horizontal trough (2 mm x 75 mm) was cut 7 mm from the antigen wells and filled with 0.30 ml undiluted antiserum. The wells were cut with a No. 1 cork borer and filled with 0.03 ml of antigen. The plates were

placed in a moist chamber at 25° C for 4 days and photographed by direct contact printing on Ilford single weight glossy paper.

(C) Immunoelectrophoresis

The method of Baillie and Norris (1964) was used, a modification of the technique introduced by Graber and Williams (1953).

The supporting glass plate (12 cm x 12 cm) was coated with a thin layer of 1% Agarose (N.B.C.), then layered with 20 ml 1% Agarose in 0.02 M barbital buffer pH 7.5 with 1% NaN_3. Horizontal troughs (o.2 cm x 9 cm) were cut 1.5 cm apart. Wells, 3.5 mm in diameter, were cut approximately 7 mm from the troughs and filled with test antigen. The gel was connected to 0.02 M barbital buffer pH 7.5 by Agarose wicks. The Shandon electrophoresis tank was connected to a Heathkit regulated power supply and the gel subjected to a potential gradient of 10 to 12 volts/cm for 3 hours at 4° C. Following the electrophoresis, the trough was filled with homologous whole cell antisera and the reaction allowed to develop in a moist chamber at 25° C for 1 to 3 days.

The agar gels were washed, dried and stained with