

IMMUNOCHEMICAL AND ELECTROPHORETIC CHARACTERIZATION OF PHENOL-SOLUBLE COMPONENTS FROM THE CELL WALL OF *SERRATIA MARCESCENS* BIZIONO

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ABSTRACT.—Phenol-extraction method of Westphal was applied to the whole cells of *Serratia marcescens*, Bizio. An ethanol-precipitable material (fraction WP) was isolated from the phenolic phase. The lipid-free fraction (fraction LFWP) was found to consist mainly of protein (80.8%) and carbohydrate (12%). The heterogeneity of the proteinous material was shown by polyacrylamide gel electrophoresis, and the presence of a glycoprotein was manifested by the periodic acid-Schiff staining. Attempts to isolate the glycoprotein in pure form were unsuccessful. Immunochemically, fraction LFWP possessed a common antigenic determinant with the sodium dodecyl sulfate extracted cell wall glycoprotein and the phenol-soluble components from the endotoxin of the same bacterial strain.

Aqueous phenol extraction method (Westphal *et al.*, 1952) has long been shown to be of high efficiency in fractionation of bacterial cell wall materials. The treatment yields phenol and aqueous soluble fractions. Lipopolysaccharides (LPS) isolated from the aqueous phase have been studied in detail and shown to constitute mainly the cell wall endotoxin complex (LPS-protein complex). However, the components in the phenolic phase have not yet been studied extensively.

When the cell walls of *pseudomonas aeruginosa* were treated with aqueous phenol (Clarke *et al.*, 1967) a methanol-precipitable material was isolated from the phenol layer whose composition was described as almost entirely protein. When this technique was applied to the endotoxin from *Serratia marcescens* 08 and *Escherichia coli* 0 141:K 85 (B) (Wober and

Alaupovic, 1971), the ethanolprecipitable material was found to consist of the protein moiety of the LPS-protein complex and a small segment of lipid A. Similar extraction with phenol of *Escherichia coli* B led to the isolation of a material from the phenol-soluble fraction, which was shown to be glycoprotein in nature (Okuda and Weinbaum, 1968).

This report represents the study of the immunochemical and electrophoretic behaviors of the materials isolated from the phenol layer after the whole cell or isolated cell wall of *Serratia marcescens*, Bizio was extracted with aqueous phenol.

MATERIALS AND METHODS

Serratia marcescens, Bizio grown on an inorganic medium was supplied by General Biochemicals, Chagrin Falls, Ohio. The cells were harvested at the late log phase. Wet cells were treated with 45% aqueous phenol for 30 minutes at 65-70 C (Westphal *et al.*, 1952). To the water-washed (three times) phenolic phase 95% ethanol was added in a ratio of 9.5:1 (ethanol:phenol) (Wober and Alaupovic, 1971; Tsang, 1971). The mixture was allowed to stand overnight. The precipitate (fraction WP) was isolated by centrifugation at 10,000 rpm for 15 minutes. After washing with ethanol and acetone fraction WP was submitted to chloroform-methanol 2:1, v/v extraction in a Soxhlet apparatus. The lipid-free residue was dried and designated fraction LFWP. Carbohydrate content of

the fraction LFWP was estimated by use of the anthrone method (Koehler, 1952) with beta-D-glucose as standard. Protein was measured with bovine serum albumin as standard (Lowry *et al.*, 1951). Hexosamine estimation was performed using beta-D-glucosamine as standard (Rondle and Morgan, 1955). Thin-layer chromatographic analysis (TLC) for neutral sugars was performed on Cellulose plates (Raadsveld and Klomp, 1971) with the following solvent system: freshly distilled ethyl acetate-pyridine-distilled water (100: 35:25, v/v/v). The spots were detected with anilinium phosphate in 70% ethanol. Amino acid components were separated on Silicia gel H plates using the following solvent systems: chloroform-methanol-17% ammonium hydroxide (2:2:1, v/v/v) for first dimension and phenol-distilled water (75:25, w/w) for second dimension (Kellerman *et al.*, 1970). Spots were made visible by ninhydrin (0.2%) in acetone spray. TLC for extracted free lipids was also run on Silica gel H plates. The following solvent system was employed: chloroform-methanol-concentrated ammonium hydroxide (75:25: 4, v/v/v). Spray used was molybdenum blue stain. Paper chromatographic analysis was carried out on Whatman No. 1 paper by the descending method. The developing solvent system used was ethyl acetate-pyridine-distilled water (3:6:1.0:1.5, v/v/v). Neutral sugars were detected with ammoniacal silver nitrate spray and amino sugars with ninhydrin spray.

Polyacrylamide gel electrophoresis was performed on 7.5% acrylamide in 8M urea, pH 9.5 at 5 mA per tube for 1 hour according to the modified procedure of Davis (Davis, 1964). The gel columns were stained separately with 0.25% Coomassie blue for protein and with periodic acid-

Schiff carbohydrate stain (Zacharius *et al.*, 1969).

Double diffusion immunological studies were performed on microslides (Ouchterlony, 1962), and a solution of 1% agar in 0.05M Veronal buffer, pH 8.6, was used as the supporting medium. For immunoelectrophoresis the same supporting medium was used. Electrophoresis was run for 45 minutes at 10 mA per agar-covered rack in a Gelman Deluxe electrophoresis chamber and gel diffusion precipitation was performed (Ouchterlony, 1962). The slides were stained with amido black for better visualization.

Fraction LFWP was fractionated by column chromatography on Sephadex G-200 gel and Sepharose 6B. Sample was solubilized by 0.05M tris-HCl buffer, pH 7.6, containing 0.5% sodium dodecyl sulfate (SDS). Columns were eluted by the same buffer and fractions of 3 ml were collected. Aliquots of 0.2 ml were taken for protein analysis (Lowry *et al.*, 1951).

RESULTS AND DISCUSSION

When the cell walls of *Pseudomonas aeruginosa* (Clarke *et al.*, 1967) were extracted with aqueous phenol, a menthanol-precipitable protein was obtained from the phenol layer with a yield of 24.5% of the cell walls. However, when the intact cells or isolated cell wall of *Serratia marcescens*, Bizio were submitted to the same treatment, the yields of the ethanol-precipitable material (fraction WP) from the phenolic phase were 7.3% (based on the dry whole cells) and 15% (based on cell wall), respectively. The amount of extractable lipids was 3.2% of fraction WP, in which alpha-lecithin and phosphatidyl ethanolamine were detected by TLC. The lipid-free fraction (fraction LFWP) was found to consist mainly of protein (80.8%) and carbohydrate

(12%). Tsang and co-workers (Tsang *et al.*, 1971) reported a sodium dodecyl sulfate (SDS)-extracted glycoprotein from the same bacteria, which contained less protein (58%) and higher lipid (33%). This may indicate that SDS is milder, less effective, and also less selective in protein extraction from the outer layer than phenol (Pusztai, 1966).

A total of 13 common amino acids and 3 unidentified spots were detected by TLC in fraction LFWP. They were similar to those obtained from the cell wall protein of *Brucella abortus* (Kellerman *et al.*, 1970). The carbohydrate components of the extract (fraction LFWP) were identified as mainly glucose and glucosamine. Surprisingly, trace amount of mannose, galactose, and even rhamnose were also observed. The existence of these sugars may suggest the possible contamination by any phenol-soluble lipopolysaccharide (Raff *et al.*, 1968; Tsang, 1971).

Fraction LFWP was purified by gel filtration on a Sephadex G-200 column. Although three peaks were obtained, the resolution was poor.

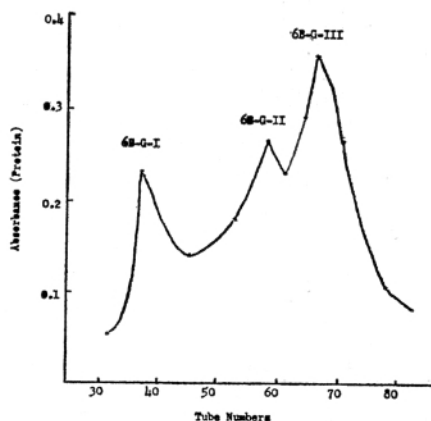


FIGURE 1—Fractionation of Phenol-soluble Proteins from Cell Walls of *Serratia marcescens*, Bizio on Sepharose 6B.

When Sephrose 6B was used, the separation was improved but far from satisfactory (Figure 1). The three peaks were designated 6B-G-I, 6B-G-II, and 6B-G-III, respectively. It is hoped that ion-exchange chromatographic technique will give better separation.

On polyacrylamide gel electrophoresis fraction LFWP gave three discrete protein bands with Coomassie blue, which were also positive with the periodic acid-Schiff staining procedure (Zacharius *et al.*, 1969). However, after fraction LFWP was washed exhaustively with water, only one carbohydrate band was observable while all three protein bands were retained. It is tempting to suggest that the ethanol-precipitable material may contain at least one component which is glyco-protein in nature. Upon gel electrophoresis fraction 6B-G-I migrated as a single diffused protein band, while both of fractions 6B-G-II and 6B-G-III gave four bands. All these three subfractions showed positive reaction with periodic acid-Schiff stain and gave the same carbohydrate band as observed in the washed fraction of LFWP. This electrophoretic study confirms the presence of at least one glycoprotein in the cell wall which had been suggested previously (Tsang *et al.*, 1971).

The antigenicity of the phenol extracted proteinaceous material (fraction LFWP) and its subfractions from Sepharose 6B (fractions 6B-G-I, 6B-G-II, and 6B-G-III) were characterized by means of immunodiffusion and immunoelectrophoresis. Fraction LFWP formed three precipitation lines with anti-whole cell serum in both types of analysis (Figure 2, pattern 1 and Figure 3, pattern 1). The same fraction showed one line in cross-reaction with the SDS-soluble glycoprotein (fraction S-SB) using the anti-serum (anti-S-SBC) to the purified

fraction S-SBC (Tsang *et al.*, 1971) (Figure 2, pattern 2). On agar immunoelectrophoresis, fraction LFWP and fraction S-SB demonstrated similar mobility (Figure 3, pattern 2). It is possible that these two different preparations from the same bacteria may share a common antigenic component. From a previous study (Tsang, 1971), the phenol-soluble components from endotoxin of *Serratia marcescens*, Bizio were shown

to be a mixture of phenol-soluble lipopolysaccharide (fraction LPS-GP-I) and lipoglycoprotein (fraction LPS-GP-II). When fraction LFWP was allowed to react with antibodies prepared against fraction LPS-GP-I (anti-GP) two lines were detected (Figure 2, pattern 3). A similar result was obtained on immunoelectrophoresis (Figure 2, pattern 3). The antigenic specificity of fraction LFWP and its cross-reactivity with both fractions

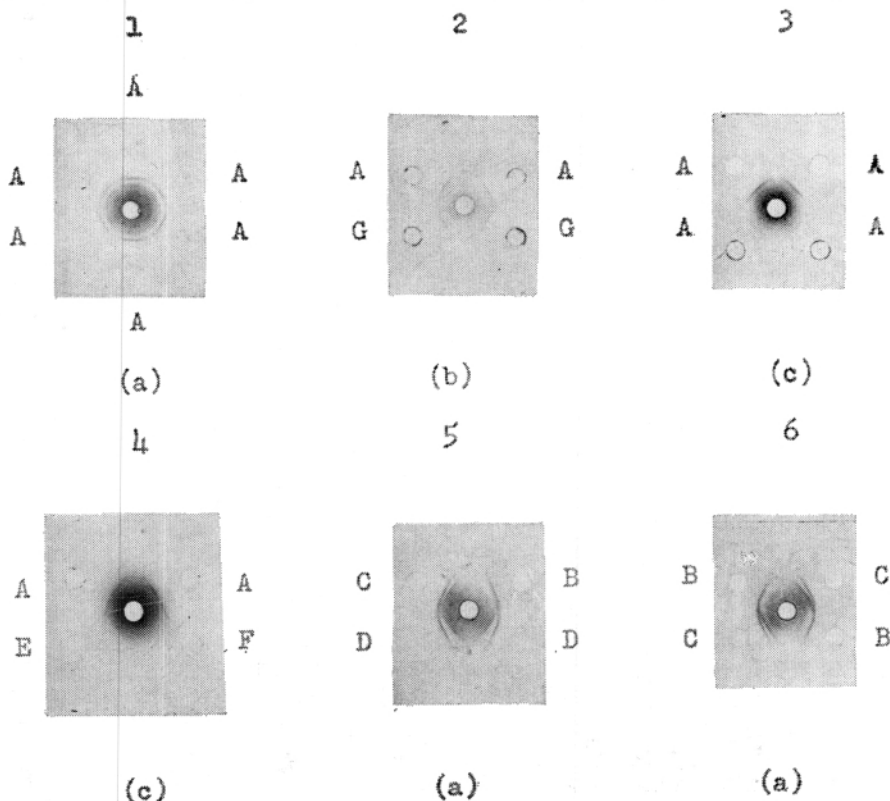


FIGURE 2.—Immunodiffusion Patterns of Fraction LFWP and Its Subfractions from Sepharose 6B.

Antibodies contained in the central wells:

- (a) against whole cells, Bizio
- (b) against fraction S-SBC
- (c) against fraction LPS-GP-I

Antigens applied:

- A: fraction LFWP
- C: fraction 6B-G-II
- E: fraction LPS-GP-I
- G: fraction S-SB

- B: fraction 6B-G-I
- D: fraction 6B-G-III
- F: fraction LPS-GP-II

LPS-GP-I and LPS-GP-II are shown in Figure 2, pattern 4. It is interesting to note that the antigenic determinant common to the two phenol-soluble fractions from the endotoxin may also be shared by the phenol-soluble protein from the cell walls. Indeed, a similar cell wall protein from *Pseudomonas aeruginosa* was

also shown to cross-react with the protein moiety of endotoxin (Homma and Suzuki, 1964).

On immunodiffusion, subfractions 6B-G-II and 6B-G-III gave three precipitin lines against anti-whole cell serum while subfraction 6B-G-I gave only two. However, a common antigenic component is present in all

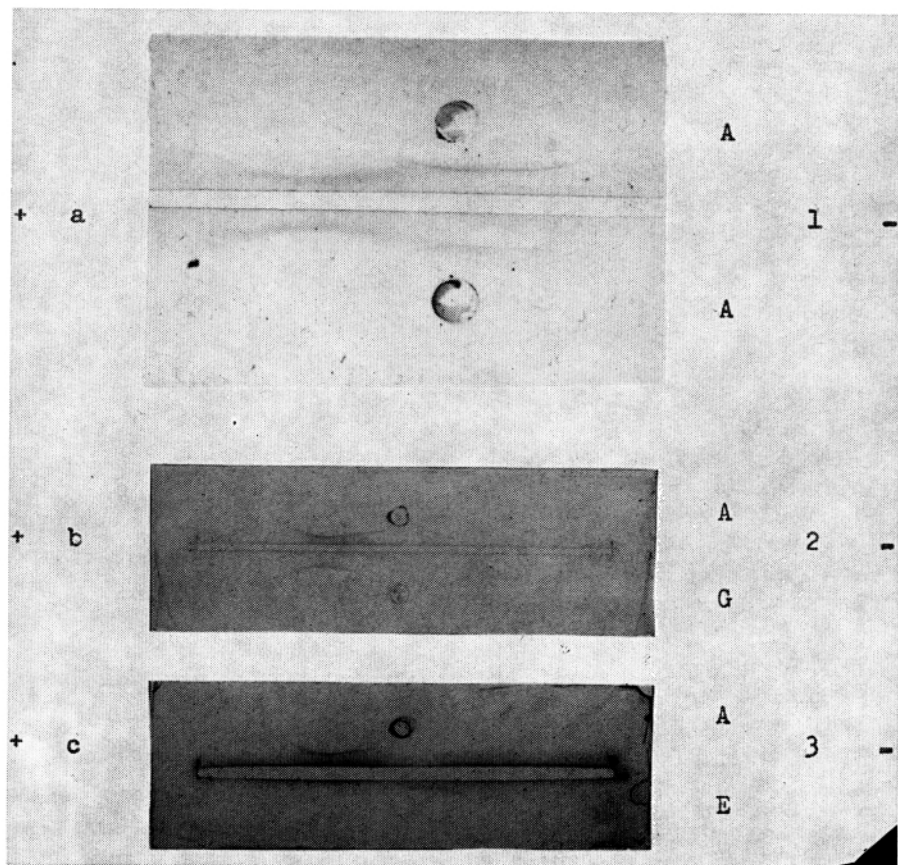


FIGURE 3.—Immunoelectrophoresis Patterns of Fraction LFWP.

Antibodies contained in the three central troughs:

- a: against whole cells, Bizio
- b: against fraction S-SBC
- c: against fraction LPS-GP-I

Antigens applied:

- A: fraction LFWP
- G: fraction S-SB
- E: fraction LPS-GP-I

three fractions (Figure 2, patterns 5 and 6). On immunoelectrophoresis against anti-GP, two identical components with varied intensities were present in all three fractions (Figure 4, patterns 1, 2, and 3). These results demonstrate the common antigenic property and the immunochemical heterogeneity of the subfractions.

Hot aqueous phenol extraction has revealed valuable information about the outer surface structure of Gram-negative bacteria; but when the intact cells were used, the source of

fractions cannot be certain. Therefore, in separate experiments, isolated cell walls instead of the whole cells were used for extraction. The ethanol-precipitable material was shown to be similar to fraction LFWP chemically (79% protein, 10.3% carbohydrate), electrophoretically (3 components), and immunochemically (3 components against anti-whole serum, 2 components against anti-phenol-soluble LPS, and 1 component against anti-SDS extractable glycoprotein).

From the present electrophoretic

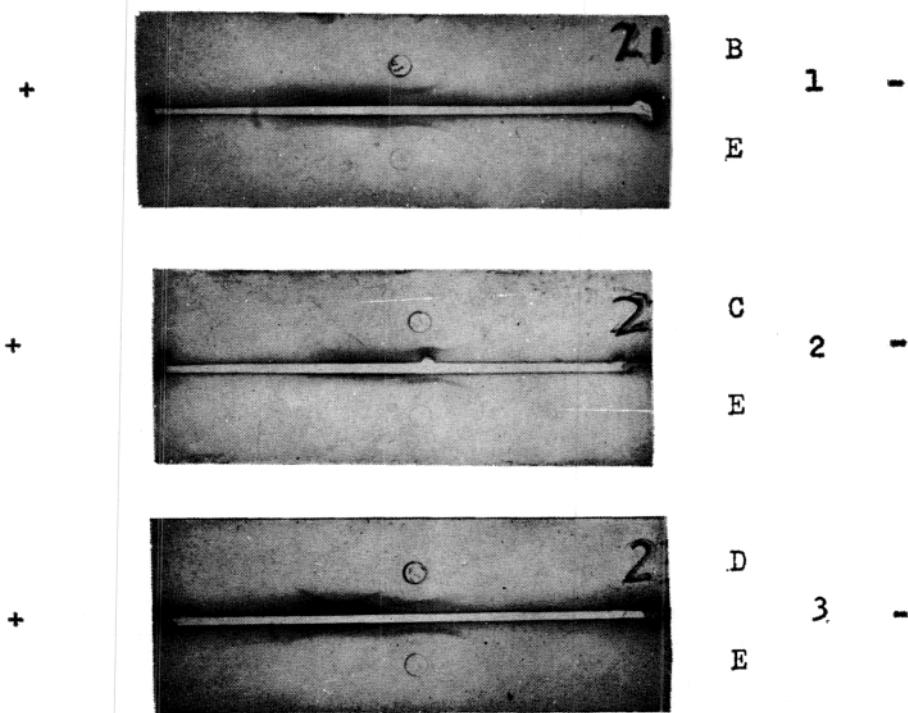


FIGURE 4.—Immunoelectrophoresis Patterns of Subfractions from Sepharose 6B Column Chromatography.

All troughs contain antiserum against fraction LPS-GP-I
Antigens in the wells:

- B: fraction 6B-G-I
- C: fraction 6B-G-II
- D: fraction 6B-G-III
- E: fraction LPS-GP-I

and immunochemical studies two conclusions may be drawn. First, the presence of an antigenic glycoprotein in the phenol-soluble components from the cell wall was confirmed. Secondly, the possible contamination by phenol-soluble lipopolysaccharide in the phenol-phase may present a purification problem for the study of phenol-soluble cell wall glycoprotein.

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