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Purification of *Pseudomonas aeruginosa* Endotoxin by Membrane Partition Chromatography

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A procedure is described for obtaining large quantities of purified endotoxin of *Pseudomonas aeruginosa* by using Diaflo ultrafiltration. This method allowed us to isolate from the protein-lipopolysaccharide complex two low-molecular-weight substances which do not play any antigenic role. It provides a useful tool for immunological purposes.

Membrane partition chromatography has been successfully used for both concentration and purification purposes (2, 4, 6). We previously reported a procedure for the purification of the protein-lipopolysaccharide complex (Pr-LPS) isolated from the cell envelope of *Pseudomonas aeruginosa* by use of gel filtration of Sepharose 4B (8). The present paper deals with the purification of such a complex by partition chromatography on a membrane.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* strain 115 from a pathogenic source was grown on Penassay medium (Difco) at 37 C. The cells were harvested at the end of the log phase.

Isolation of Pr-LPS complex. Cell walls were isolated by the method of Cox and Eagon (1) except that the enzymatic treatment was omitted. Pr-LPS complex was prepared by incubation with ethylenediaminetetraacetic acid (7).

Diafiltration procedure. A sample of a 50-ml solution of Pr-LPS in 0.03 M tris(hydroxymethyl) aminomethane-hydrochloride buffer (*p*H 8) was placed in a Diaflo ultrafiltration stirred cell (model 50; Amicon Corp, Lexington, Mass.) equipped with the highest cut-off XM-100 membrane. Operating pressure was kept at 25 psi, and the above buffer was used during diafiltration as a wash solution. The per cent transmittance of the eluate was ultraviolet-monitored until a nonabsorbing final ultrafiltrate was obtained.

Ultracentrifugal analysis. Sedimentation coefficient analysis was carried out in a Spinco model E analytical ultracentrifuge. Runs were made at 20,410 rev/min by using a 2° single-sector cell with a 12-mm filled Epon centerpiece in an An-D rotor at 20 C. Sedimentation patterns were recorded with schlieren optics. Corrections to obtain $S_{20,w}$ values were made by the method of Schachman (9).

Antisera. Rabbit anti-Pr-LPS serum was obtained by an immunization scheme of administering infections for a total period of 7 weeks. Four intramuscular injections of 0.1, 0.25, 0.50, and 0.75 ml of a sterile saline stock solution (2.5 mg/ml) of nonpurified Pr-LPS complex emulsified with equal volumes of Freund's incomplete adjuvant (Difco) were given weekly during the first 4 weeks. Then three intravenous injections of 0.1, 0.2, and 0.4 ml were administered weekly for the last 3 weeks. The animals were bled by cardiac puncture on the 14th day after the final inoculation (N. Rubio and A. Portolés, Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. II, *in press*).

Immunoelectrophoresis. Immunoelectrophoresis was performed on microscope slides containing 3 ml of 2% Difco Noble agar in 0.0375 ionic strength Veronal buffer (*p*H 8.6) as the support medium. Slides were processed at room temperature for about 90 min in an Elphor electrophoresis chamber with the use of a 0.035 M Veronal buffer (*p*H 8.6) and a voltage gradient of 6 v/cm.

Gel filtration chromatography. The material retained by the XM-100 membrane was chromatographed on Sepharose 4B in columns (K 25/45 model from Pharmacia, Uppsala, Sweden) as previously described (8). Column effluent was monitored at 260 nm in a Beckman DB-G spectrophotometer equipped with a liquid microaperture flow cell (model 97460).

RESULTS

A single peak was noted (Fig. 1B) when the diafiltered retentate was gel-filtered, in contrast to the profile obtained with the unpurified material (Fig. 1A). Moreover, a more clear-cut peak was registered from the ultrafiltered purified material.

The ultraviolet spectra of the diafiltration eluates show an intense absorption band at 280

nm in the first diafiltration; in addition, a second peak was detected at 260 nm during the second and third ones (Fig. 2). Both maxima became negligible after several dilutions. Two fractions were found when the second peak obtained from Sepharose 4B chromatography of the unpurified material (Fig. 1A) was concentrated and rechromatographed on Sephadex G-100 columns (Fig. 3).

The ultracentrifugal analysis of the Diaflo retentate material depicts a single peak with an $S_{20,w}$ of 13.4 (Fig. 4).

When unpurified and membrane partition-purified materials were subjected to immunological evaluation, identical precipitating bands showing two humps were found for both types of materials (Fig. 5).

DISCUSSION

The experimental data presented above demonstrate that diafiltration provided us with a tool for rapidly obtaining large quantities of Pr-LPS from *P. aeruginosa* which can be easily separated from lower-molecular-weight impurities.

The fact that this Pr-LPS gave a single schlieren peak upon sedimentation in an ultracentrifuge and a fairly symmetrical peak when

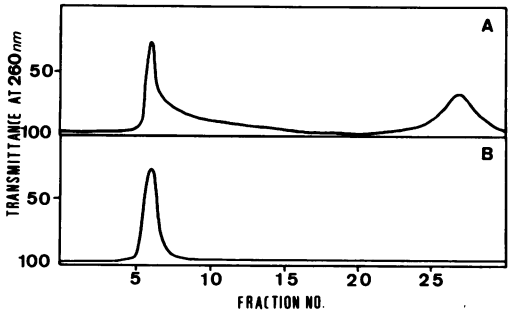

FIG. 1. *Sepharose 4B elution patterns of nonpurified (A) and membrane partition chromatography-purified (B) protein-lipopolysaccharide complex from Pseudomonas aeruginosa eluted with 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride plus 0.1 M NaCl (pH 7.6) buffer.*

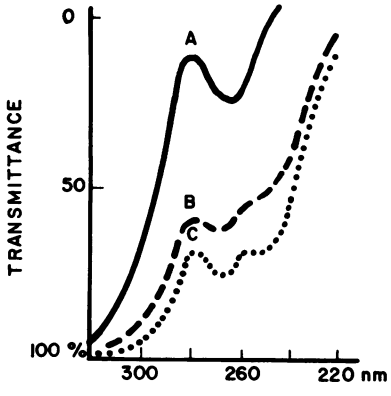

FIG. 2. *Absorption spectra of the diafiltration eluate obtained by use of the Diaflo XM-100 membrane. Eluates were collected during the first (A), second (B), and third (C) diafiltrations.*

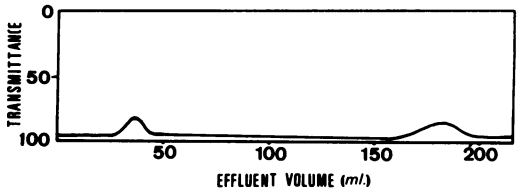

FIG. 3. *Sephadex G-100 chromatographic elution of the protein-lipopolysaccharide contaminant fraction on a column (2.5 by 40 cm) equilibrated with 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride plus 0.1 M NaCl (pH 7.6) buffer. Absorbance was monitored at 260 nm.*

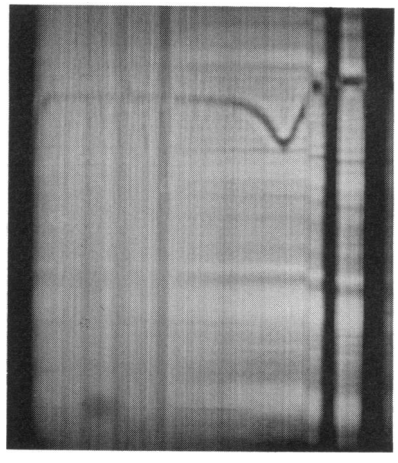

FIG. 4. *Schlieren pattern of partition chromatography-purified protein-lipopolysaccharide complex. The photograph was taken 66 min after 20,410 rev/min was reached. The sample concentration was 5 mg/ml in 0.15 M NaCl.*

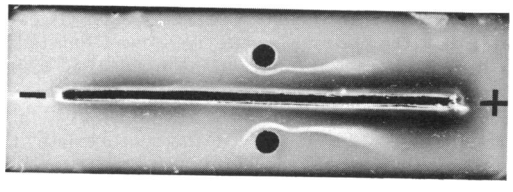

FIG. 5. *Immunoelectrophoretic comparison of the reaction of unpurified (upper well) and partition chromatography-purified endotoxin (lower well) against anti-endotoxin serum.*

it was gel-filtered on Sepharose 4B offers reasonable evidence for the purity of this diafiltrate material. On the other hand, the immunological pattern shows two humps in the precipitation arc which could indicate that the electric field breaks this substance down into subunits. Furthermore, we have recently found (*unpublished data*) that, when the ultrafiltered material was treated with sodium deoxycholate, two shlieren peaks were obtained; this indicates that this material might be degraded into subunits as has been reported elsewhere (3).

All of these experimental findings seem to demonstrate that a pure endotoxin was obtained by membrane partition chromatography.

Our results also suggested that the second fraction obtained by Sepharose 4B chromatography of the unpurified material (Fig. 3) contained two different substances that have absorption maxima at 260 and 280 nm (Fig. 2). Since the same precipitation bands were found when pure and unpurified antigen were diffused against the anti-unpurified Pr-LPS serum (Fig. 5), we can say that the above fraction does not play any antigenic role. These materials could not be covalently bound to the Pr-LPS molecule but are merely contaminants as has been previously suggested (5).

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ERRATA

Procedure for Isolation and Enumeration of *Vibrio parahaemolyticus*

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Volume 23, no. 1, p. 26, column 2, line 3 under Materials and Methods, add: Cultures A4280, A4281, A6540, A7606, and B1650 were obtained from R. Weaver (CDC) as "*Vibrio parahaemolyticus*-like" organisms, with some question as to the exact taxonomic position. They are listed here as *V. parahaemolyticus* because of the similarity of tissue infection isolates with Japanese strains of *V. parahaemolyticus* (J. Bacteriol. **98**:511–518).

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Volume 23, no. 2, p. 212, column 2: Figure 4 is printed upside down.