PURIFICATION OF THREE PROTEINS FROM THE OUTER MEMBRANE OF THE ENVELOPE OF *ESCHERICHIA COLI*

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1. Introduction

Spheroplasts of *Escherichia coli* are bounded by two membranes [1]. Miura and Mizushima [2] have separated these membranes and have equated the 'inner membrane' to the plasma membrane of the cell since it contained ATPase and the components of the respiratory chain. The 'outer membrane' was shown by immunological techniques to be located on the outside surface of the cell [3]. Schnaitman [4] found that cell envelope preparations of *E. coli* obtained by disruption of the cells with a French press could be resolved on a sucrose gradient into plasma membrane-enriched and cell wall-enriched fractions. The latter fraction contained the murein layer in addition to the outer membrane of the spheroplast. Gel electrophoresis of the proteins extracted from the cell wall-enriched fraction suggested that the outer membrane was probably composed of only a limited number (ca. 6) of proteins.

In this paper we describe a method for the separation of three of the proteins of the outer membrane. Since spheroplasts do not represent a convenient source of material for large scale preparations we have used the whole cell envelope as a starting material, and have removed the inner (plasma) membrane components from it by treatment with EDTA at an alkaline pH, and detergents.

2. Experimental

2.1. *Extraction of outer membrane proteins*

E. coli (strain 482 of the culture collection of the National Research Council of Canada) was grown and harvested as previously described [5]. A suspension of 10 g bacteria in 30 ml 0.01 M tris-HCl (pH 7.5) containing 1 mM $MgCl_2$ and a few crystals of DNase was disrupted in a French Pressure Cell (Aminco) at 20,000 psi. Sodium deoxycholate (10%) was then added to give a final concentration of 0.2% and the mixture was centrifuged at 13,000 *g* for 5 min to remove unbroken cells. The cell free extract was centrifuged at 100,000 *g* for 30 min to sediment the cell envelopes. The envelopes (252 mg protein) were suspended to 30 ml in 0.2 M KCl containing 1 mM EDTA and 0.4 M NH_4OH, and sedimented as before. (This washing procedure removed considerable amounts of protein and was much superior to washing with 0.01 M tris-HCl, pH 7.5). The KCl–EDTA–NH_4OH-washed envelopes (99 mg protein) suspended in 30 ml 0.01 M tris-HCl (pH 7.5) were stirred with 3 ml 10% (v/v) aqueous Triton X-114 at 0° for 30 min and then sedimented as before. The triton-extracted envelopes (65 mg protein) were then extracted in sequence with 30 ml 0.5% (w/v) sodium dodecyl sulfate (SDS) at 37° for 1 hr, twice, and finally with 6 ml 2.5% (w/v) SDS at 100° for 3 min. After each treatment the extracted envelopes were sedimented by centrifuging at 100,000 *g* for 30 min. In this way three extracts ('first, second and third SDS extracts') were obtained which contained 19.4, 5.1 and 29.5 mg protein, respectively. The final residue of murein contained 2.3 mg protein.

2.2. *Chromatography of SDS extracts*

The third SDS extract from a large scale preparation was dialysed against water to remove deter-

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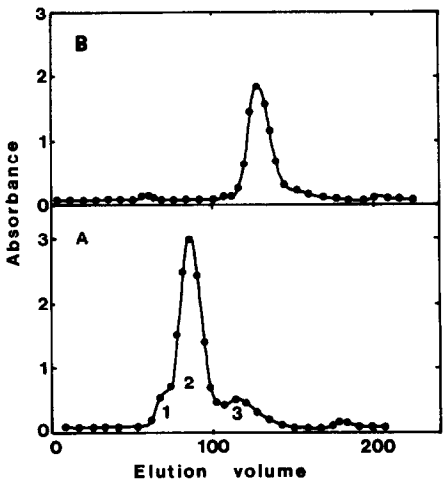

Fig. 1. Separation of the third SDS extract from *E. coli* envelopes on a column of Sephadex G-100 (A). In B peak 2 from Sephadex G-100 was separated on a column of Sepharose 6B. See Experimental for details of method.

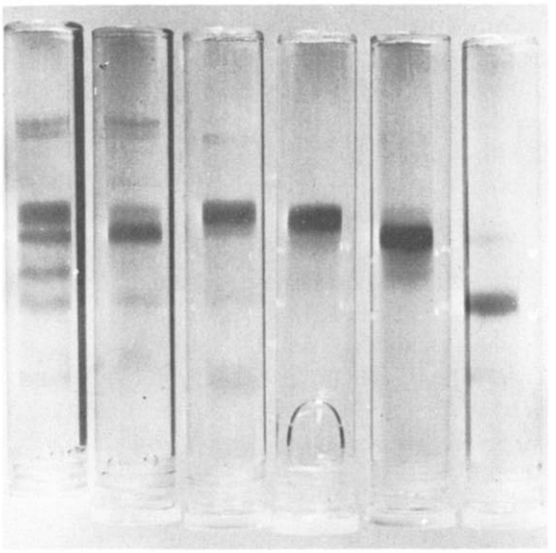

Fig. 2. Electrophoresis on 5% polyacrylamide gels of proteins extracted from the *E. coli* envelope. The gels from left to right are (a) 2.5% SDS extract of triton-extracted envelopes, (b) first SDS extract, (c) third SDS extract, (d) purified protein A, (e) purified protein B, and (f) purified protein D. See Experimental for details of method.

gents and then lyophilized. The dried material (66.8 mg protein) was dissolved in 8 ml 2.5% (w/v) SDS, heated at 100° for 3 min, and then applied to a column (2.5 X 39 cm) of Sephadex G-100 equilibrated with 0.1 M sodium phosphate buffer (pH 7.2) containing 1% (w/v) SDS. Elution was performed with the same buffer. Three peaks of material absorbing at 280 nm were observed (fig. 1A). Fractions eluting at 78 to 94 ml were dialysed and lyophilized. The dried material (27.5 mg protein) was dissolved in 4 ml w/v 2.5% SDS, heated at 100° for 3 min, and then chromatographed on a column (2.5 X 40 cm) of Sepharose 6B equilibrated with the same phosphate-SDS buffer. A single peak of material absorbing at 280 nm was obtained (fig. 1B). The fractions eluting at 120 to 140 ml were collected as protein A (26.1 mg).

2.3. *Miscellaneous techniques*

Protein was estimated by the Lowry method [6].

For routine analysis of protein components electrophoresis was performed on 5% polyacrylamide gels containing 0.1% SDS in 0.1 M sodium phosphate buffer (pH 7.2). The samples for electrophoresis were depolymerized by heating at 100° with 2.5% SDS for 3 min [7]. The gels were stained with Coomassie blue [8], and destained electrolytically.

For molecular weight determinations electrophoresis was performed on 7.5% and 10% polyacrylamide gels using the split gel technique of Dunker and Rueckert [9]. Samples were prepared as described by these authors except that the samples were heated at 100° for 3 min prior to electrophoresis.

3. Results and discussion

Direct extraction of the triton-extracted envelopes with 2.5% SDS at 100° for 3 min extracted the bulk of the protein present. Polyacrylamide electrophoresis of the extract showed that at least ten bands were present (fig. 2a). The five most prominent of these were named A_1, A_2, B, C and D. The bands A_1 and A_2 were of equal intensity, and often did not resolve into two bands on gel electrophoresis. For this reason, and because we could not separate the components, this material was treated as a unit (protein A). The other proteins did not under any

conditions of electrophoresis resolve into more than one band. Proteins A–D were shown by gel electrophoresis to be enriched in the outer membrane fraction isolated by the method of Miura and Mizushima [2].

Extraction of triton-extracted envelopes with 0.5% SDS at 37° resulted in the preferential solubilization of protein B together with smaller amounts of other proteins (fig. 2b). A second extraction with 0.5% SDS resulted in extraction of most of the remaining protein B but some protein A was also removed. The third SDS extract (2.5% SDS; 100°) contained protein A as the main component together with smaller amounts other proteins including proteins B, C and D (fig. 2c).

Purification of protein A was obtained by chromatography of the third SDS extract on a column of Sephadex G-100 (fig. 1A). Three peaks were obtained. Peak 2 consisted primarily of protein A. Peak 1 contained the proteins moving more slowly than protein A on gel electrophoresis. Peak 3 contained predominantly protein D with smaller amounts of protein B and two proteins with lower molecular weight (fig. 2f). Further purification of protein A from peak 2 was obtained on a column of Sepharose 6B (fig. 1B). The single absorbance peak contained mainly protein A (fig. 2d).

Chromatography of the first SDS extract on Sephadex G-100 also gave two peaks. The second peak contained predominantly protein D but was contaminated with variable amounts of triton. Rechromatography of the material from the first peak on Sepharose 6B yielded a single peak which contained predominantly protein B (fig. 2e).

The molecular weights of proteins A_1, A_2, B, C and D were determined by gel electrophoresis using bovine serum albumin (monomer and dimer), γ-globulin (heavy and light chains), pepsin, trypsin, myoglobin, and cytochrome *c* to calibrate the distance of migration. The molecular weights of the purified proteins were the same as those determined directly from extracts of the triton-extracted envelope, and were 44,000, 38,100, 33,400, 28,200 and 21,400 for proteins A_1, A_2, B, C and D respectively.

Acknowledgements

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