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ISOLATION OF OUTER MEMBRANE PROTEINS OF *ESCHERICHIA COLI* AND THEIR CHARACTERIZATION ON POLYACRYLAMIDE GEL

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SUMMARY

Proteins from the outer membrane of *Escherichia coli* were studied on a urea-dodecyl sulfate polyacrylamide gel by electrophoresis. A polyacrylamide gel containing sodium dodecyl sulfate and urea gave an excellent resolution of outer membrane proteins. Seventeen protein bands were reproducibly observed on a gel. By use of Sephadex G-200, DEAE-cellulose and polyacrylamide gel, eight proteins were purified to near homogeneity. Five of them were found to be heat-modifiable proteins. The behavior of these purified proteins was studied on a polyacrylamide gel under three different electrophoretic conditions, which had been used for the analysis of cell envelope proteins. Thus correspondence was made between these purified proteins and envelope proteins reported by other investigators.

INTRODUCTION

The outer membrane of *Escherichia coli* has a simple protein composition in comparison with the cytoplasmic membrane [1, 2]. However, except for a lipoprotein studied by Braun and other workers [3–6], these proteins have not been well characterized. This was due, in part, to their anomalous behavior on polyacrylamide gel electophoresis, depending on solubilization conditions, and in part to the lack of a suitable gel system for their separation. This made it difficult to compare results reported from various laboratories. Recently several envelope proteins of *E. coli* have been purified from Triton X-100-treated cell envelope [7, 8], ghosts [9] and dodecyl sulfate-treated peptidoglycan layer [10], and these proteins are thought to be constituents of the outer membrane.

We have used the purified outer membrane as a starting material and purified eight proteins to near homogeneity. The behavior of these purified proteins was studied on dodecyl sulfate-polyacrylamide gel by three different methods. These are described in this paper.

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MATERIALS AND METHODS

Bacteria E. coli YA21 used was a leucine auxotroph mutant derived from *E. coli* K-12 (*met*$^-$, F$^-$, λ^-) [1]. The cultivation of bacteria and the preparation of outer membrane were carried out as described previously [1]. The outer membrane I was used in the present work.

Analytical procedure. Protein concentration was measured by the method of Lowry et al. [11], using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of membrane proteins was carried out by three different methods.

Method I: the gel, containing urea as well as dodecyl sulfate, was essentially the same as that described previously [1]. In the present work, samples dissolved in 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution were incubated either at 37 °C for 30 min or at 100 °C for 5 min. The concentration of *N,N'*-methylenebisacrylamide was 0.1 % and the electrophoresis was carried out for 5–6 h until bromophenol blue used as a tracking dye reached the bottom of tube. Gels were fixed and stained according to the method of Maizel [12] or, alternatively, gels were fixed for 19 h with 20 % trichloroacetic acid instead of sulfosalycylic acid.

Method II: this procedure was described by Fairbanks et al. [13]. The concentration of acrylamide was 8 % in the present study.

Method III: this procedure was described by Bragg and Hou [14]. The concentration of acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were the same as those in Method I. Gels were fixed and stained according to the method of Fairbanks et al. [13].

Stained gels were scanned with Chromoscan (Joyce Loebl, England) with a filter 5-042.

Sephadex G-200 chromatography. The outer membrane was solubilized in 5 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution. The final concentration of protein was 7–20 mg/ml. The solubilized outer membrane was applied to a Sephadex G-200 column (2 × 110 cm) previously equilibrated with 0.5 % sodium dodecyl sulfate/0.1 M sodium phosphate buffer (pH 7.2). Sodium azide (0.02 %) was added to the buffer to prevent microbial growth. The column was eluted at 25 °C with the same buffer at a flow rate of 6 ml/h and fractions of 3 ml were collected. A Sephadex G-200 column (1.6 × 90 cm) equilibrated with 7 M urea/0.5 % sodium dodecyl sulfate/0.1 M sodium phosphate buffer (pH 7.2) was also used.

DEAE-cellulose chromatography. A sample (4.0 mg of protein in 2.4 ml) in 1 % sodium dodecyl sulfate was dialyzed for 72 h at room temperature against 500 ml of 0.5 % Triton X-100/6 M urea/25 mM Tris/acetate (pH 8.0 at 20 °C) with three changes of dialysis solution and applied to a DEAE-cellulose column (2 × 10 cm) previously equilibrated with the same buffer. The column was washed with 25 ml of the same buffer and eluted with 200 ml of a linear gradient of 0–0.3 M NaCl at a flow rate of 15 ml/h at room temperature.

Preparative gel electrophoresis. Proteins to be separated were dissolved in 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution and heated at 100 °C for 5 min. Then solid urea was added to a final concentration of 8 M, and electrophoresis carried out by Method I. Usually twelve gels were run at a time, applying 100 μg protein in 100 μl solution to each gel. After the electrophoresis, two gels were rapidly

stained with 20 % trichloroacetic acid/0.05 % Coomassie Brilliant Blue solution to locate the protein bands. The unstained gels were sliced at the position corresponding to individual protein bands, and the slices homogenized in water by the use of a Teflon-glass homogenizer, mixed with an equal volume of 1 % sodium dodecyl sulfate and left overnight at room temperature. Proteins extracted were separated from gel by centrifugation.

Protein nomenclature. The nomenclature of proteins from the outer membrane preparation used in the present study is given in Fig. 2.

RESULTS

Resolution of outer membrane proteins on polyacrylamide gel

The purified outer membrane was dissolved in sodium dodecyl sulfate solution and electrophoretic profiles of proteins were analyzed by three different methods (Fig. 1). Fig. 1A shows gel profiles obtained by Method II. When the outer membrane was dissolved in sodium dodecyl sulfate solution and heated at 100 °C for 5 min, a strong band was observed in the middle of the gel. The profile was different when the sample had not been heated before the electrophoresis. The strong band was not observed, but many slower moving bands and a faster moving band appeared, indicating that the strong band was composed of several proteins. The result was in agreement with those obtained by other investigators [15, 16]. Fig. 1B shows gel electrophoretic profiles obtained by Method I. The profile also varied depending on the temperature of solubilization, as described previously [1, 17]. However, by this method the separation of major proteins on a gel was better with heating at 100 °C than without heating. Fig. 1C shows gel electrophoretic profiles obtained by Method

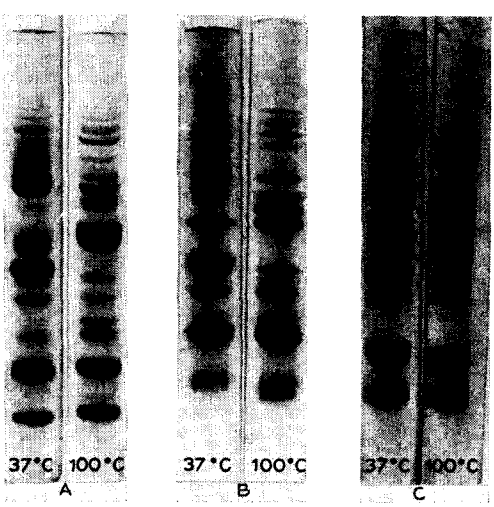


Fig. 1. Polyacrylamide gel electrophoresis of outer membrane proteins by three different methods. Electrophoresis was carried out by Method II (A), Method I (B) and Method III (C). The amount of protein in 50 μl of sample applied in (A), (B) and (C) was 25, 50 and 25 μg, respectively. Figures under each gel show the temperature at which the outer membrane had been heated in sodium dodecyl sulfate solution before gel electrophoresis.

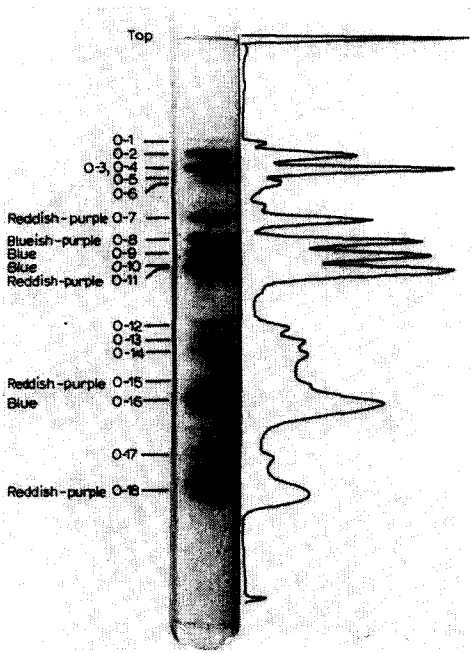


Fig. 2. Nomenclature of outer membrane proteins and their colors on gel. The sample had been heated at 100 °C for 5 min in sodium dodecyl sulfate solution. Method I was employed for gel electrophoresis under the same conditions as in Fig. 1B. The densitometric tracing of the gel with Chromoscan is also shown.

III. The migration of major proteins was similar to that obtained by Method I. However, resolution was not always as good as that obtained by Method I in our laboratory.

Since we intended to take advantage of an anomalous behavior of outer membrane proteins (heat modifiability) for their separation, a method which gives good results even after the heating of sample was preferable. Therefore we used Method I for the routine analysis of proteins. As will be described later, the colors of the protein bands differed from each other under Method I (Fig. 2), which is another advantage of the method.

Properties of individual protein bands on polyacrylamide gel

For convenience of description, we gave numbers O-1 to O-18 to bands which appeared reproducibly on gels when the outer membrane I was used (Fig. 2). Two bands were thought to consist of at least two proteins. Evidence which supported the idea is as follows. Protein O-4 was eluted in middle fractions from Sephadex G-200 columns when proteins had not been heated (Fig. 5), while O-3 was eluted near void volume (data not shown). A band which contained proteins O-10 and O-11 had two colors, blue in the upper part and reddish purple in the lower part. Longer migration sometimes, but not reproducibly, resulted in the resolution of the blue and reddish-purple bands. Furthermore, the repeated freezing and thawing reduced the rate of migration of the blue band, protein O-10, resulting in the resolution of two bands.

It was also noted that proteins O-10 and O-11 were separable on DEAE-cellulose chromatography in 2 % Triton X-100/6 M urea solution (Ichihara, S. and Mizushima, S., unpublished). The difference in color between proteins O-10 and O-11 was more distinct when gels were fixed with 20 % trichoroacetic acid instead of sulfosalycylic acid. Besides proteins O-10 and O-11, other bands also showed various colors. It should be noticed, however, that the tone of color is relative and may change slightly depending on protein concentration and on the electrophoresis run. The colors of some major bands are indicated in Fig. 2.

Sometimes proteins O-2 and O-3 appeared strongly on a gel and sometimes they almost disappeared. Recently, the appearance of these proteins in the outer membrane was found to be repressed by the presence of iron in a cultivation medium (Ichihara, S. and Mizushima, S., unpublished). Since the outer membrane used in the present paper was prepared mainly from cells grown in Bacto-casamino acids (technical), which contained a considerable amount of iron, we could not obtain any information on proteins O-2 and O-3 in the present paper. Some outer membrane preparations were slightly contaminated by flagella. Flagellin, which is also heat-modifiable [18], migrated to a position near protein O-5 (data not shown).

Specific extraction of membrane proteins

First we tested various reagents for the selective solubilization of proteins. Reagents tested were detergents (Brij 58, Tween 40, 60 and 80, Triton X-100, X-405, sodium cholate and sodium deoxycholate), salts (NaCl, NH_4Cl, KCl and LiCl), chaotropic agents (urea, guanidine · HCl and KSCN) and organic solvents (pyridine,

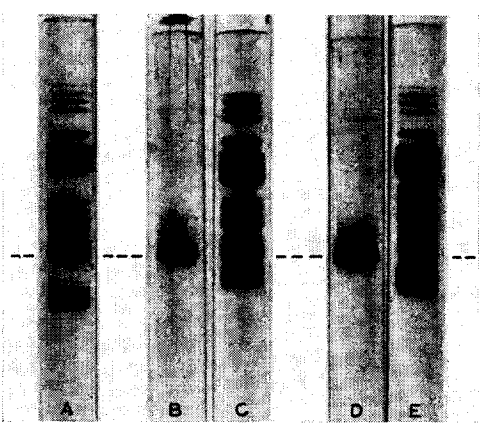


Fig. 3. Extraction of protein 0-16 from outer membrane. Outer membrane (1 mg protein) was treated with 1 ml of 1 M NaCl at room temperature with or without sonication for 2 min. Sonicator OT-5202 (Ohtake, Tokyo) was used. Then it was centrifuged at 100 000 × *g* for 30 min to separate clear supernatant solution from membrane pellet. The membrane pellet was suspended in 1 ml of water and both preparations were dialyzed against 200 ml of 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol overnight at 25 °C and analyzed on polycarylamide gel by Method I. Samples had been heated in sodium dodecyl sulfate solution at 100 °C before gel electrophoresis. (A) Original outer membrane (50 μg protein); (B) supernatant solution without sonication (200 μl); (C) membrane pellet without sonication (100 μl); (D) supernatant solution after sonication (200 μl); (E) membrane pellet after sonication (100 μl). The position of protein 0-16 on gel is indicated by a dotted line.

2-chloroethanol, acetone, ethanol, *n*-butanol and *n*-pentanol). However, these reagents did not selectively solubilize any proteins except proteins O-16 and O-18. When the outer membrane was incubated with 1 M NaCl at 30 °C for 30 min and centrifuged at 100 000 × *g* for 30 min, about half of protein O-16 was solubilized (Figs. 3B and 3C). The same effect was observed with 1 M NH_4Cl and 1 M LiCl. Although the remaining half of protein O-16 could not be solubilized by repeated washing with salts, it was almost completely solubilized when the sample was sonicated in 1 M NaCl (Figs 3D and 3E). Protein O-16 thus solubilized migrated to the same position as egg-white lysozyme on electrophoresis at acrylamide concentrations of 5, 8 and 12.5 %. Co-electrophoresis experiments under these conditions also confirmed the results. Furthermore, the sonicated outer membrane which was devoid of protein O-16 did not hydrolyze peptidoglycan, while the untreated outer membrane did (Yamada, H. and Mizushima, S., unpublished). Thus protein O-16 is certainly lysozyme that had been incorporated during the preparation of the outer membrane, and a half of the enzyme was seemingly enclosed inside the membrane vesicle.

It has been reported that only the Braun's lipoprotein remained soluble when *E. coli* envelope was solubilized in 1 % sodium dodecyl sulfate at 70 °C followed by the addition of trichloroacetic acid to a final concentration of 10 % [19]. We followed this method and found that protein O-18 remained soluble even in 15 % trichloroacetic acid/1 % sodium dodecyl sulfate solution. Judging from this result and its apparent molecular weight on a polyacrylamide gel, protein O-18 is certainly Braun's lipoprotein. It has been reported that the lipoprotein was localized in the outer membrane [20, 21].

Purification of outer membrane proteins

When the outer membrane was dissolved in 5 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution at 30 °C and applied to a Sephadex G-200 column, proteins were eluted as shown in Fig. 4. Three peaks were observed. Fig. 5 shows protein compositions of the fractions that are indicated by arrows in Fig. 4. The first peak contained proteins O-8 and O-9, and proteins of higher molecular weight except protein O-4. Proteins O-4 and O-7 were eluted between the first and the second peaks. Proteins O-10 and O-11 eluted in the second peak, and the third peak contained mainly proteins O-16 and O-18. On the other hand, when the dissolved outer mem-

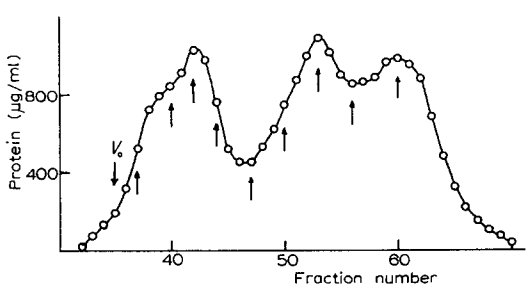


Fig. 4. Sephadex G-200 column chromatography of non-heated outer membrane proteins. Outer membrane (60 mg protein) was dissolved in 3 ml of 5 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution at 30 °C and chromatographed on Sephadex G-200 column (3 ml per fraction). Electrophoretic profiles of the fractions indicated by arrows are shown in Fig. 5.

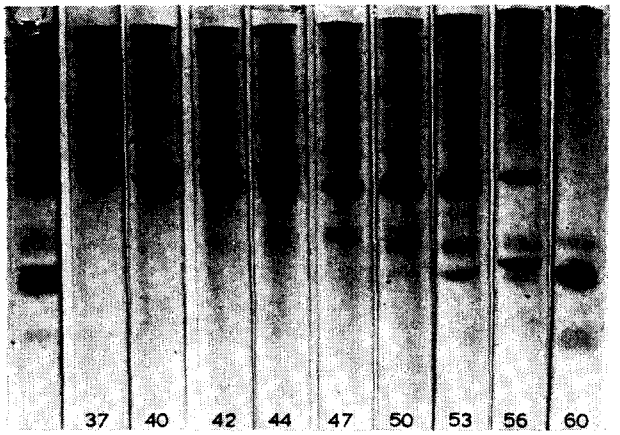
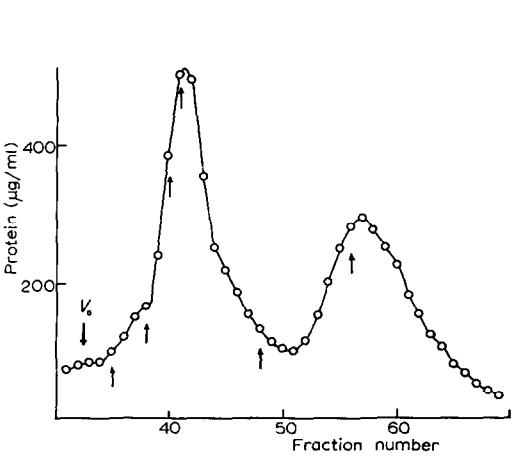


Fig. 5. Polyacrylamide gel electrophoretic profiles of fractions chromatographed on Sephadex G-200 without heat treatment. Fractions indicated by arrows in Fig. 4 were dialyzed overnight at 25 °C against 100-fold volume of 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution and heat treated at 100 °C. Method I was employed for gel electrophoresis. Figures under each gel show fraction number in Fig. 4. The gel on the left contained original outer membrane (about 50 μg protein).



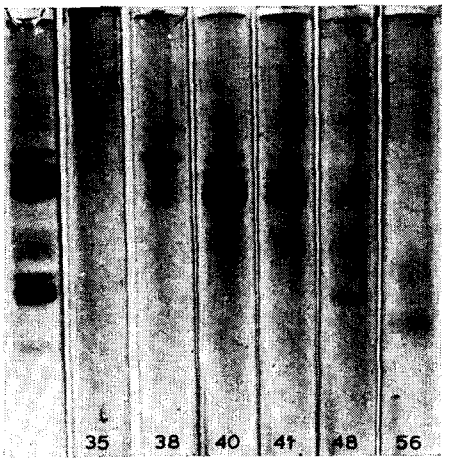


Fig. 6. Sephadex G-200 column chromatography of heated outer membrane proteins. Outer membrane (15 mg protein) was dissolved in 2 ml of 5 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution, heated at 100 °C for 5 min and chromatographed on Sephadex G-200 column (3 ml per fraction). Electrophoretic profiles of fractions indicated by arrows are shown in Fig. 7.

Fig. 7. Polyacrylamide gel electrophoretic profiles of fractions chromatographed on Sephadex G-200 after heat-treatment. Fractions indicated by arrows in Fig. 6 were dialyzed overnight at 25 °C against 100-fold volume of 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution and heated at 100 °C. Method I was employed for gel electrophoresis. Figures under each gel show the fraction number in Fig. 6. The gel on the left contained original outer membrane (about 50 μg protein).

brane was heated at 100 °C for 5 min, the elution pattern differed significantly, as shown in Fig. 6. Two peaks appeared. Fig. 7 shows the protein compositions of each fraction that are indicated by arrows in Fig. 6. It is clear that the elution position of some proteins was changed by the heat treatment in sodium dodecyl sulfate solution. Protein O-7 appeared first, followed by proteins O-8, O-9, O-10 and O-11 in the first peak. The second peak consisted of lower molecular weight proteins. Fraction 56 in Fig. 7 was not stained densely. This may be due to the weak staining property of protein in this fraction.

Taking advantage of this behavior, we fractionated outer membrane proteins by successive gel filtrations without and with heating of protein samples. Fig. 8 illustrates the results of the successive chromatography on Sephadex G-200 columns. First the outer membrane was dissolved in sodium dodecyl sulfate solution at 30 °C and fractionated on Sephadex G-200 (Fig. 8A). Fraction I in Fig. 8A, composed mainly of proteins O-8 and O-9, was concentrated by means of dried Sephadex and rechromatographed after heating. As shown in Fig. 8B, the elution positions of

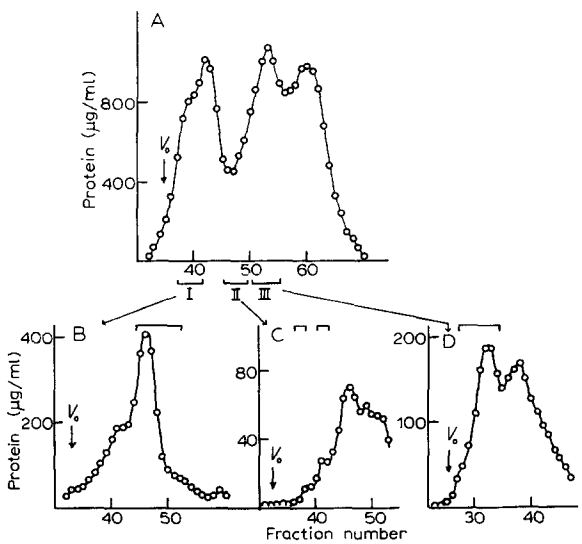


Fig. 8. Fractionation of outer membrane proteins by successive chromatography on Sephadex G-200 column with and without heat-treatment. (A) Outer membrane (60 mg protein) was dissolved in 3 ml of 5 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution at 30 °C and chromatographed on Sephadex G-200 column. Proteins in fractions 38–41, 46–49 and 51–55 were pooled as Fractions I, II and III, respectively. (B) Fraction I in A was concentrated to 4 ml by means of dried Sephadex, heated at 100 °C for 5 min and rechromatographed on the same column under the same conditions. Fractions 45–52 were combined and used for further purification of proteins 0-8 and 0-9. (C) Fraction II in A was concentrated to 4 ml by means of Collodion bag SM 13200 (Sartorius Membranefilter, GmbH, Gottingen), heated at 100 °C for 5 min and rechromatographed on the same column under the same conditions. Fractions 37–38 and 41–42 were pooled separately, concentrated by means of dried Sephadex and used as purified proteins 0-4 and 0-7, respectively. (D) Fraction III in A was concentrated to 3 ml by means of dried Sephadex and heated at 100 °C for 5 min. Then solid urea was added to a final concentration of 7 M and chromatography was carried out on Sephadex G-200 column in urea/dodecyl sulfate solution. Elution conditions were the same as those in A, except 7 M urea was also added in elution buffer. Fractions 28–34 were combined and used for further purification of proteins 0-10 and 0-11.

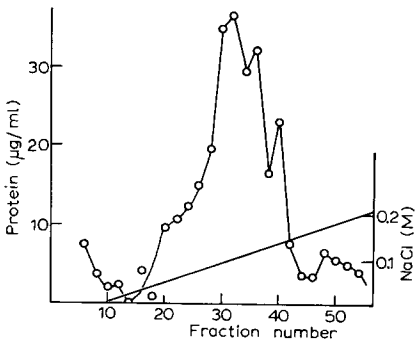


Fig. 9. Separation of proteins 0-10 and 0-11 on a DEAE-cellulose column. A mixture of proteins 0-10 and 0-11 (4.0 mg in 2.6 ml) from Fig. 8 D was dialyzed against 0.5 % Triton X-100/6 M urea/25 mM Tris/acetate (pH 8.0) and chromatographed on a DEAE-cellulose column. Fractions of 2.5 ml each were collected and the amount of protein was determined. Fractions 24–32 and 39–45 were pooled as Fractions I and II, respectively.

proteins O-8 and O-9 have shifted to a lower molecular weight region. Thus proteins O-8 and O-9 were separated from other proteins.

Main constituents of Fraction II in Fig. 8A were proteins O-4, O-7, O-10 and O-11. The fraction was also concentrated, heated and rechromatographed (Fig. 8C). Proteins O-4, O-7 and O-10 moved to a higher molecular weight region, to different extents. Protein O-4 was eluted near the void volume and protein O-7 followed. Thus pure proteins O-4 and O-7 were obtained.

Fraction III, which contained proteins O-10, O-11 and those of lower molecular weight, was rechromatographed in urea/sodium dodecyl sulfate solution after heating. As shown in Fig. 8D, proteins O-10 and O-11 were separated from others by this rechromatography.

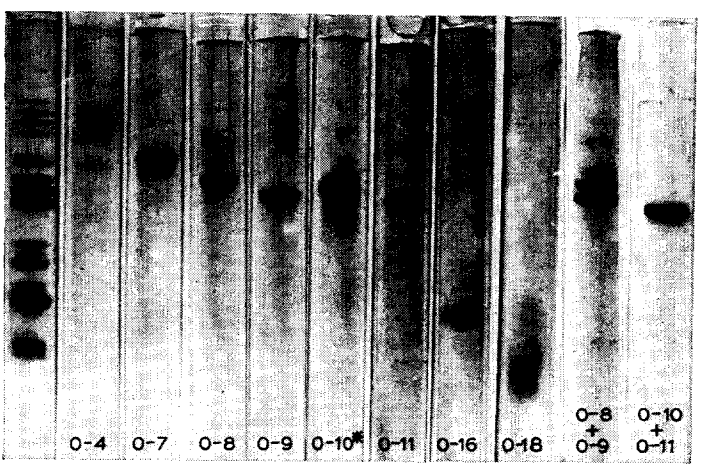


Fig. 10. Polyacrylamide gel electrophoresis of isolated proteins by Method I. Purified proteins in 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol solution (50 μl) were used. Amount of protein in each gel was about 5–10 μg. The gel on the left contained original outer membrane, and the gels on the right mixtures of proteins 0-8 and 0-9 from Fig. 8B and of proteins 0-10 and 0-11 from Fig 8D, as indicated. All preparations had been heated at 100 °C for 5 min before gel electrophoresis.

Since proteins O-8 and O-9 were not separable from each other on Sephadex G-200, about 100 μg of the mixture of these proteins was applied to a polyacrylamide gel, electrophoresis was carried out by Method I and proteins O-8 and O-9 were then recovered separately, as described in Materials and Methods. Proteins O-10 and O-11 were separated by an ion-exchange chromatography. Recently, a mixture of urea and Triton X-100 was found to be an effective solubilizing medium for the outer membrane (Ichihara, S. and Mizushima, S., unpublished). Therefore we separated proteins O-10 and O-11 by DEAE-cellulose chromatography using this solubilizing medium. To remove sodium dodecyl sulfate, the mixture of proteins O-10 and O-11 was dialyzed against 0.5 % Triton X-100/6 M urea/25 mM Tris/acetate (pH 8.0 at 20 °C). Then the mixture was chromatographed as shown in Fig. 9. The protein in Fraction I in Fig. 9 gave a broad blue band on polyacrylamide gel and did not migrate to the position for protein O-10 in the orignial outer membrane (Fig. 10). Therefore, we tentatively designated the protein as protein O-10*. The protein in Fraction II in Fig. 9 gave a sharp reddish-purple band on a polyacrylamide gel and the position was the same as that for protein O-11. A peak between Fractions I and II contained both proteins.

Examination of purified proteins on polyacrylamide gel

Fig. 10 shows the electrophoretic profiles of purified proteins as examined by Method I. The position of individual proteins was also examined, using the mixture of the outer membrane and individual proteins (data not shown). All purified proteins migrated to the same positions as those of corresponding proteins in the original outer membrane except proteins O-8 and O-10*. Purified protein O-8 migrated slightly slower than that in the original outer membrane. However, the preparation used for the final purification of proteins O-8 and O-9 contained only these two proteins (Fig. 10). Furthermore, repeated freezing and thawing of the outer membrane often resulted in a slight shift of the position to that of purified protein O-8. We

Fig. 11. Polyacrylamide gel electrophoresis of isolated proteins by Method II. Purified proteins in 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol (50 μl) were used. The amount of protein in each gel was about 5–10 μg except 0-16 (about 20 μg). The gel on the left contained original outer membrane. All preparations had been heated at 100 °C for 5 min before gel electrophoresis.

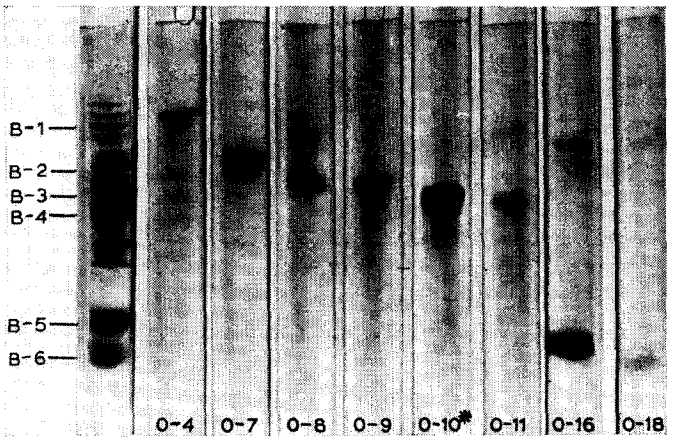


Fig. 12. Polyacrylamide gel electrophoresis of isolated proteins by Method III. Purified proteins in 1 % sodium dodecyl sulfate/1 % 2-mercaptoethanol (50 μl) were used. The amount of protein in each gel was about 5–10 μg except 0-16 (about 20 μg). The gel on the left contained original outer membrane. All preparations had been heated at 100 °C for 5 min before gel electrophoresis.

concluded, therefore, that purified protein O-8 was a modified form of that in the original outer membrane.

Purified protein O-10* migrated more slowly than protein O-10 of the original outer membrane, although the sample used for the purification of proteins O-10 and O-11 contained only these two proteins which migrated normally (Fig. 10). As already described, the repeated freezing and thawing reduced the rate of migration of a blue band, protein O-10, and made the band broader. Therefore we concluded that the purified protein O-10* was also a modified form of protein O-10 in the original outer membrane. The nature of the modification of proteins is unknown. The purified protein O-18 also migrated as a broad band, while the protein gave a sharp band by Method II.

Fig. 11 shows the electrophoretic profiles of purified proteins as examined by Method II. The gel electrophoresis of individual purified proteins mixed with the outer membrane was also carried out (data not shown). Proteins O-4, O-7, O-16 and O-18 corresponded to bands F-1, F-2, F-4 and F-5, respectively. Proteins O-8, O-9, O-10 and O-11 corresponded to F-3. Thus band F-3 consisted of at least four proteins.

Fig. 12 shows the electrophoretic profiles of the purified proteins as examined by Method III. The position of individual proteins was also examined using the mixture of the outer membrane and individual proteins (data not shown). A major band, B-3, seemed to consist of two bands. Proteins O-4, O-7, O-11, O-16 and O-18 corresponded to B-1, B-2, B-4, B-5 and B-6, respectively. Both proteins O-8 and O-9 corresponded to B-3. Protein O-10* positioned between B-3 and B-4. Since purified protein O-10* migrated more slowly than protein O-10 in the original outer membrane by Method I, protein O-10 is probaly another component of band B-4 of Method III.

DISCUSSION

Many investigators repeatedly showed that the outer membrane proteins of

Gram negative enteric bacteria exhibited an anomalous behavior on sodium dodecyl sulfate-polyacrylamide gel, depending on solubilization conditions [1, 2, 8, 22]. Upon heating in sodium dodecyl sulfate solution, a major band which has an apparent molecular weight of about 42 000 appeared on the polyacrylamide gel. This band was shown to consist of several proteins [7, 16]. To study the outer membrane proteins, therefore, a method which can resolve this band into several bands must be developed.

Bragg and Hou described a method which employed an alkaline cathode buffer [14]. By this method, the major band was resolved into two to three bands. In the present paper, we showed that the gel electrophoresis in the presence of both urea and sodium dodecyl sulfate gave an excellent resolution of heated proteins from the outer membrane. By this method, proteins in the outer membrane preparation were resolved into at least 17 bands. The major band, mentioned above, was resolved into four bands (O-8, O-9, O-10 and O-11). The method had another advantage, since each band was stained with different color. When the staining procedure of Fairbanks et al. [13] was employed on the urea-dodecyl sulfate gel, bands stained more distinctly, but the color was the same throughout a gel, except for protein O-18.

We purified eight proteins. Each protein gave essentially a single band on a polyacrylamide gel under three different conditions. However, still we can not say much about the purity of these protein preparations. Large scale isolation and further studies on the nature of proteins must be carried out. Also, at present, we have no information on whether these are simple or complexed proteins. Judging from the elution pattern from Sephadex G-200 before and after heat treatment of proteins, five of them were thought to be heat modifiable (proteins O-4, O-7, O-8, O-9 and O-10). We are uncertain whether O-11 is heat modifiable or not. This characteristic property is thought to be related to the conformation of proteins. Nakamura et al. [23] found that outer membrane proteins were unusually rich in β-structured polypeptide and Mizushima [17] found that this conformation was not destroyed in sodium dodecyl sulfate solution at room temperature and was destroyed upon heating at 100 °C in the solution.

It is well known that the ratio of sodium dodecyl sulfate/protein and the solubilizing temperature are important for the complete solubilization of membrane proteins. In the standard conditions for gel electrophoresis, the amount of sodium dodecyl sulfate in samples was ten times as much in weight as that of membrane protein and the samples had been heated at 100 °C for 5 min. Furthermore, the gel pattern was the same even when a small amount of membrane (5 μg of protein) was treated with 500 μg of the detergent in 50 μl solution. These facts supported the idea that the heterogeneity of protein bands on the urea-dodecyl sulfate gel after the heat treatment is not due to the incomplete dissociation of membrane proteins.

On the other hand the solubilization was found to be incomplete to some extent when the membrane was not heated in the detergent solution, because preliminary experiment showed that the dense background color in the upper part of a gel in Fig. 1 (Fig. 1B, 37 °C) was due to the incomplete dissociation of a few outer membrane proteins (data not shown.) The broad elution pattern of individual proteins from Sephadex G-200 (Fig. 4) may also be due to the heterogeneity in the state of protein or some interactions between proteins.

One of the aim of the present study was to determine the protein composition of outer membrane. For this purpose we have used purified outer membrane as a

starting material, purified eight proteins and compared their behavior by three different methods of gel electrophoresis (Methods I–III) which had been used for the study of outer membrane proteins. The method of gel electrophoresis described by Weber and Osborn [24] and Inouye and Pardee [25] are thought to be essentially the same as Method II. Through the present work it became easier to compare proteins studied by several investigators. However, there is still a problem in comparison, because many investigators purified envelope proteins which were not from the isolated outer membrane. Also it is difficult to claim that all proteins found in our purified outer membrane preparation were originally localized in the outer membrane itself. Some proteins might bind secondarily to the outer membrane during treatment, such as by lysozyme or EDTA. The following discussion will be carried out on the assumption that these envelope proteins are also components of our outer membrane preparation.

Proteins O-8, O-9, O-10 and O-11 are probably identical with proteins 1, 2, 3a and 3b, respectively, purified from cell envelope by Schnaitman [7]. Evidence which supports this idea is as follows. The behavior of proteins O-8 and O-9, and proteins 1 and 2 obtained on a polyacrylamide gel by Method III was essentially the same. These proteins were also similar in their elution profiles from Sephadex G-200. In *E. coli* YA21, the amount of protein O-9 was significantly reduced when glucose was used as a carbon source for cultivation (data not shown). Schnaitman also indicated that the amount of protein 2 in a cell envelope preparation was reduced significantly when the cultivation was carried out in glucose [26]. Proteins O-10 and O-11, and proteins 3a and 3b seemed to migrate to the same position on a polyacrylamide gel with Method III. The order of elution from a DEAE-cellulose column was proteins O-10 and O-11 in the present paper (although the eluting solution contained 6 M urea) and then proteins 3a and 3b by Schnaitman [7].

Judging from the position on a polyacrylamide gel, protein A_1 of Bragg and Hou [8, 14], ghost protein I of Garten and Henning [9] and an envelope protein of Rosenbusch [10] are probably identical with protein O-8. However, there is a possibility that these preparation contained protein O-9 too, because it seems to be difficult to separate proteins O-8 and O-9 on a polyacrylamide gel under conditions used for the analysis of these protein preparations. Furthermore, protein A_1 and ghost protein I have been reported to be possibly composed of two similar polypeptides.

Protein O-7, another major protein, was perhaps specific to the strain used, because we could not find the protein in a few other strains of *E. coli* examined.

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