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Isolation of Outer Membrane Proteins from *Haemophilus Influenzae* by Preparative SDS and Native Gel Electrophoresis

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Abstract

Nontypable *Haemophilus influenzae* (NTHI) are gram-negative bacteria that are common causes of respiratory tract infections. The protein composition of the outer membrane of NTHI comprises approximately twenty proteins of which four to six account for most of the protein content and the remainder are relatively minor components.[1] The isolation of appropriate quantities of proteins from the outer membrane of NTHI that are conserved between strains is essential if they are to be considered as vaccine candidates. One such protein of interest to the researchers in this field is P6, a 16 kDa lipoprotein that contains an epitope that is a common determinant among all strains of *H. influenzae*.[2]

Due to the number of lipoproteins in the outer membrane, traditional column chromatography methods often result in poor resolution and/or purification of insufficient quantities of protein. The use of preparative electrophoresis overcame some of these problems and facilitated efficient production of suitable quantities of protein. The method also proved to be reproducible between protein extracts from different strains of NTHI. Here we report the methods that were used to isolate proteins from strains of NTHI by both native and SDS-preparative polyacrylamide gel electrophoresis. The proteins isolated by these methods have been tested in immunization studies.[3,4,5] The method was used to purify several proteins. Semi-purified preparations from an initial Model 491 Prep Cell run were applied to a second Model 491 Prep Cell gel to achieve the high levels of purification.

Method

NTHI was grown overnight at 37 °C in 5% CO_2 on 100 plates of supplemented brain heart infusion agar. The bacteria were harvested and washed twice by centrifugation. A crude outer membrane preparation was obtained using a previously described method.[6] Briefly, this involved resuspension of the washed pellet in 20 ml of 1 M sodium acetate and 0.01 M β-mercaptoethanol, pH 4, for 45 min before 80 ml of 5% (w/v) Zwittergent 3–14 in 0.5 M calcium chloride were added. The suspension was then stirred for a further 90 min. Ethanol was added to a final concentration of 20% (v/v) and the suspension left overnight at 4 °C. Following centrifugation at 17,000 x g for 10 min at 4 °C, the supernatant was collected and the ethanol concentration adjusted to 80% (v/v). This suspension was left overnight at 4 °C, centrifuged at 17,000 x g for 20 min at 4 °C, and the pellet resuspended in a buffer containing 0.05% (w/v) Zwittergent 3–14, 0.05 M Tris, and 0.01 M EDTA, pH 8, stirred at room temperature for 1 h and centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant was then dialyzed overnight against distilled H_2O at 4 °C, frozen to -70 °C and then lyophilized. This step produced an extract containing approximately 35–40 mg protein.

Electrophoresis Sample Preparation

The dried bacterial extract was resuspended in a minimal amount of distilled H_2O (0.5-1 ml) and further dissolved in 4 times the volume of either SDS-reducing buffer (as per the Model 491 Prep Cell manual) or the same buffer without SDS for the native separation. The SDS-preparation was incubated at 37 °C for at least 30 min prior to loading onto the stacking gel.

Running Conditions

Preparative Electrophoresis—SDS

An initial separation of the components of the outer membrane preparation was performed using an 80 ml 12% resolving gel and a 15 ml 4% stacking gel prepared in the 37 mm ID tube. The Model 491 Prep Cell was set up for electrophoresis using an electrode buffer containing 25 mM Tris, 0.2 M glycine, 1% (w/v) SDS, pH 8.3, in the cathode and anode reservoirs and an elution buffer of 25 mM Tris, pH 7.5. After application of the sample to the top of the stacking gel, the cell was run at maximum settings of 10 W power and 50 mA current. Elution of fractions commenced when the bromophenol blue indicator band reached the base of the separating gel*. Fractions of 6 ml volumes were collected using a peristaltic pump set at a flow rate of 1 ml/min. Eluted material was continuously monitored for protein content with a UV detector set at 280 nm.

*In monitoring the migration of the bromophenol blue band, migration should be between 1.5–2 cm per hour. If it is much faster or slower than this, the current is adjusted down or up respectively.

Fractions containing the 16 kDa protein, P6, contaminated with lipo-oligosaccharide (LOS) and other proteins, were pooled, lyophilized, and reconstituted in 1–2 ml of SDS sample buffer. This was loaded onto a 20 ml, 16% resolving gel with a 10 ml, 4% stacking gel in the 28 mm Model 491 Prep Cell tube. Electrophoresis of the sample was performed using the same electrode and elution buffers as before, and run at 10 W and 40 mA. Four ml fractions were collected at a flow rate of 1 ml/min. These conditions separated the P6 from the LOS and other contaminants.

Proteins in the MW range between 26–40 kDa were pooled, lyophilized, and reconstituted in 2–3 ml of SDS sample buffer. This sample was loaded on a 60 ml, 14% resolving gel with a 10 ml, 4% stacking gel polymerized in the 37 mm ID gel tube and run at 10 W and 45 mA, using the same electrode and elution buffers as in previous work. Four ml fractions were collected at a flow rate of 1 ml/min. Proteins of approximately 26, 28, 30, and 39 kDa were successfully separated.

Pooled fractions were concentrated and any SDS removed from the protein sample by a previously described method.[7] The purified protein preparations could then be desalted. Aliquots of the purified proteins were stored at concentrations between 2–5 µg/µl in 10% (v/v) glycerol at -70 °C.

Preparative Electrophoresis—Native

Native separation of the 16 kDa P6 protein from the outer membrane extract was performed using 30 ml, 16% resolving gel and 5 ml 4% stacking gels in the 28 mm ID gel tube prepared by the method used for SDS-electrophoresis. The chambers were set up for electrophoresis using 50 mM Tris, 0.4 M glycine, pH 8.3, in the cathode chamber (upper), 25 mM Tris, 0.14 M glycine, pH 6.8, in the anode chamber (lower), and an elution buffer of 50 mM Tris, pH 7.5. After the sample was loaded on the top of the stacking gel, electrophoresis was carried out at maximum setting of 10 W and 40 mA for the first 3 h and then the current setting was increased to 45 mA. Fractions were collected in 4 ml volumes at a flow rate of 1 ml/min. Those fractions containing P6 were pooled and dialyzed prior to protein determination. Aliquots of the purified proteins were stored at concentrations between 2–5 µg/µl in 10% (v/v) glycerol at -70 °C.

Fraction Analysis for SDS and Native Preparative PAGE

Fractions were concentrated to a volume of 200 µl by lyophilization. Initially every fifth fraction was analyzed for protein composition by analytical SDS-PAGE.[8] The protein concentrations of samples were determined after desalting using the BCA Protein Assay Reagent and albumin standard.

Conclusions

P6 was purified by both native and SDS-PAGE. Several proteins have been purified using SDS-PAGE in which the denaturation step was carried out at 37 °C rather than being boiled in the SDS loading buffer. The purified proteins included P6, a 16 kDa protein, and other proteins of 26 kDa, 28 kDa, 30 kDa, and 39 kDa. Figure 1 shows the protein profile of the pooled fractions eluted from the 12% column. The fractions containing P6 with LOS contamination, when applied to a 16% resolving gel resulted in the pattern shown in Figure 2 with a total recovery of approximately 1 mg of pure P6 from 35–40 mg of protein in the original extract. The remaining pooled fractions were further separated using a 14% resolving gel which resulted in the final purification of the other proteins (Figure 3). Yields of these proteins ranged from 0.5 to 5 mg depending on the relative amounts of the particular protein in the original extract.

The particular conditions used in the native PAGE were designed to elute only a 16 kDa protein known as P6, while the LOS and many other proteins in the extract were excluded by charge and molecular size from the gel (based on analysis of material that remained on the top of the gel). The same electrophoresis conditions isolated P6 in four different strains of

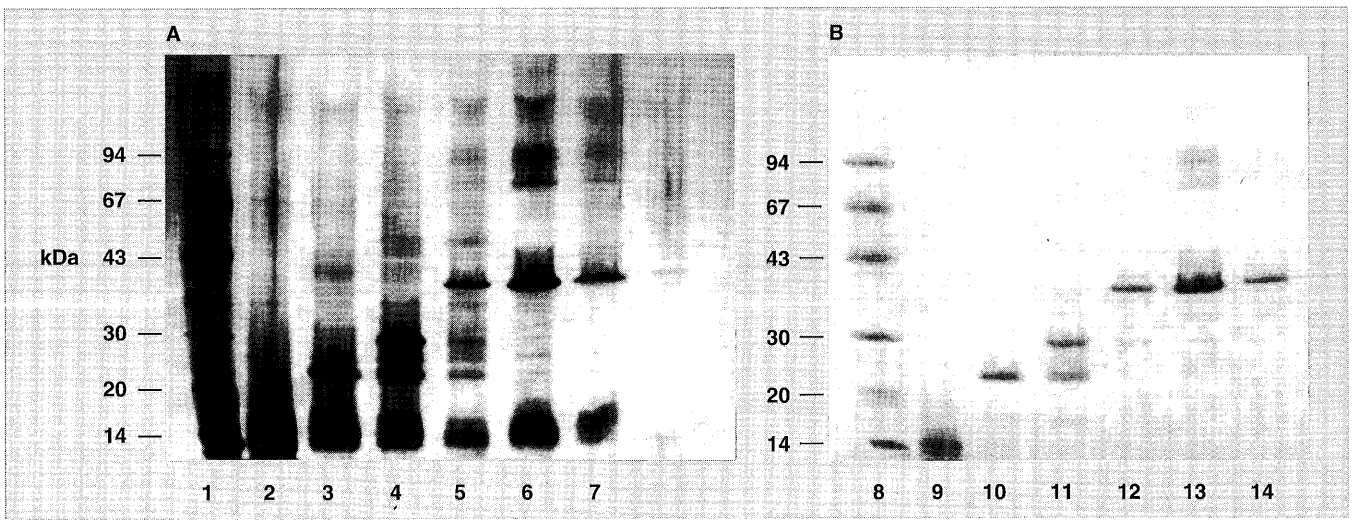

Fig. 1. SDS-PAGE analysis with (A) silver staining and (B) Coomassie® staining of pooled fractions following initial separation of an outer membrane extract with a 12% acrylamide/bis column. A. lane 1, low molecular mass standards; lanes 2 to 7, different pooled batches for the purification of P6 (lane 2), 26 to 30 kDa (lanes 3 and 4), and 39 kDa (lanes 5 to 7). **B.** Same as gel A but Coomassie stained. Coomassie stain does not stain lipo-oligosaccharide or protein bands of low concentration. Values of molecular mass standards are shown on the left.

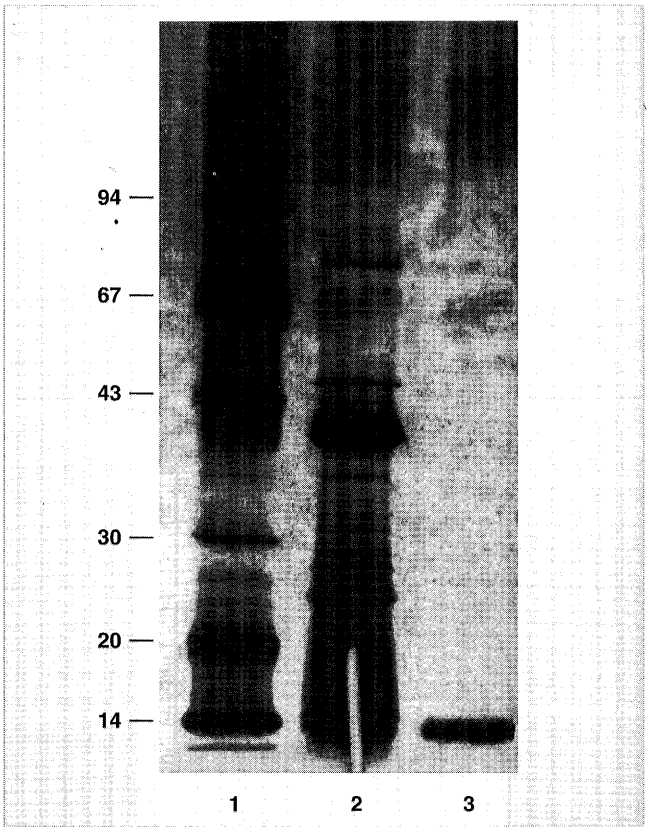

Fig. 2. SDS-PAGE analysis of P6, a 16 kDa lipoprotein. Sample was run on a gradient of 10 to 15% polyacrylamide gel and silver stained. Lanes: 1, molecular mass standards (values in kilodaltons shown on left side); 2, outer membrane extract of strain NTHI; 3, P6.

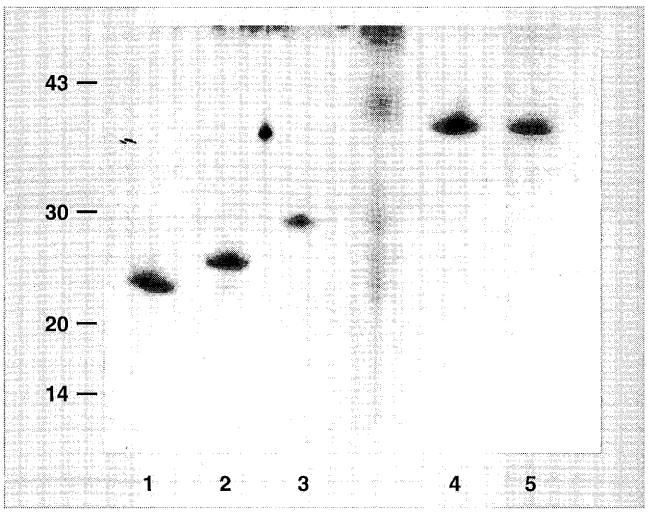

Fig. 3. SDS-PAGE analysis of isolated proteins. Samples of the proteins were run on a 10-15% gradient polyacrylamide gel and silver stained. Lanes: 1, 26 kDa; 2, 28 kDa; 3, 30 kDa; 4 and 5, 39 kDa. The values (in kilodaltons) for the molecular weight standards are shown on the left.

H. influenzae. A yield of approximately 1 mg of P6 was obtained from the 35–40 mg of protein in the outer membrane extract. This native method was designed to favor the migration of P6 and there was no recovery of other proteins in the extract.

Helpful Hints

We do not denature proteins by boiling the protein samples prior to application to the top of the column. Rather, we found it best to incubate them for at least 30 minutes at 37 °C.

We eliminated the SDS and glycine from the elution buffer to reduce the presence of these reagents in the concentrated fractions. The large amounts of these following lyophilization caused problems with analyzing the protein profile of the fractions.

Detailed discussions of this work can be found in references 3–5. Some of this work was presented at an Australian Electrophoresis Society Conference in 1994.

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