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Note

Analysis of membrane proteins by ion-exchange high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) has been used for the purification and the analysis of membrane proteins by reversed-phase[1,2], size exclusion[3–5] and ion-exchange[4] methods in the presence and absence of a surfactant(s). Although HPLC is a powerful technique for purifying and analysing membrane proteins, the presence of a surfactant often results in poor separations of proteins. The low resolution of membrane proteins in size exclusion HPLC in the presence of a surfactant is unavoidable, because surfactants and membrane proteins form comicells, increasing the total mass. The use of ionic surfactants with a small aggregation number, such as sodium dodecylsulphate (SDS) and sodium deoxycholate, reduces the problem, but there is no guarantee that the biological activity of the membrane protein is preserved.

In this study, we have examined a newly developed ion-exchange column (TSKgel DEAE-5PW) for the analysis of proteins from the outer membrane of *Escherichia coli* K-12 and the intrinsic membrane proteins obtained from human erythrocyte in the presence of a non-ionic surfactant. Satisfactory separations were obtained.

EXPERIMENTAL

Preparation of membrane fraction and extraction of membrane proteins

Membranes of human erythrocyte were prepared essentially according to the procedure described by Dodge *et al.*[6], the only modification being the use of an osmolysis buffer containing 10 m*M* Tris–HCl (pH 8) in the presence or absence of 5 m*M* EDTA. The membranes were subjected to sonic oscillation in a cup-type sonicator (Kubota, Tokyo, Japan) at 180 W for 3 min. The outer membranes of *E. coli* K-12 were prepared according to the procedure described by Smit and Nikaido[7]. Membranes were solubilized in a solution of 1–2% $C_{12}E_8$–10 m*M* EDTA–10 m*M* Tris–HCl (pH 8) (see next section for definition of C_mE_n) and an appropriate amount of the sample was injected into the HPLC column after centrifugation at 16,000 *g* for 5 min.

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Ion-exchange HPLC

HPLC was performed with a Model SP8700 high-speed liquid chromatography system (Spectra-Physics, San Jose, CA, U.S.A.) consisting of a pump, a gradient mixer, an injector and a UV-8 UV detector (Toyo Soda, Tokyo, Japan). The ion-exchange HPLC column used was TSKgel DEAE-5PW (Toyo Soda) of dimensions 7.5 × 0.75 and 15 × 2.15 cm I.D. for analytical and preparative purposes, respectively. The column was equilibrated with 10 m*M* Tris–HCl (pH 8) containing 1 m*M* polyoxyethylene glycol alkyl ether (C_mE_n) in the presence and absence of 5 m*M* EDTA. The column was eluted with a linear gradient of 0–0.5 *M* sodium chloride in 1 m*M* $C_{12}E_8$–10 m*M* Tris–HCl (pH 8) (buffer A).

Other methods

Protein was quantified by measuring the absorption at 280 nm. Polyacrylamide gel electrophoresis was performed in the presence of SDS according to the procedure described by Laemmli[8]. All chemicals used were the best grade commercially available.

RESULTS AND DISCUSSION

Before the membrane proteins were analysed, one should ensure that the surfactant to be used does not interfere with the performance of TSKgel DEAE-5PW

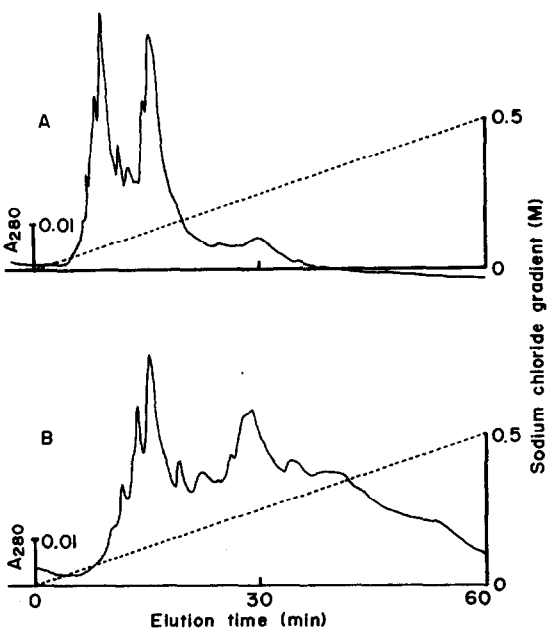

Fig. 1. Elution profiles of the partially purified hexokinase. The partially purified hexokinase (Sigma, type III) was dissolved (10 mg/ml) in 10 m*M* Tris-HCl buffer (pH 8) in the presence and absence of 1% $C_{12}E_8$ and 0.12 ml of the solution was injected into a TSKgel DEAE-5PW column (7.5 × 0.75 cm I.D.) equilibrated with the same buffer. The column was eluted with a linear gradient of 0–0.5 *M* sodium chloride in the above solution, at a rate of 1 ml/min. UV absorption was recorded with a flow-type UV detector. A, Chromatography run in 10 m*M* Tris–HCl (pH 8); B, similar to A, except that the buffer contained 1 m*M* $C_{12}E_8$.

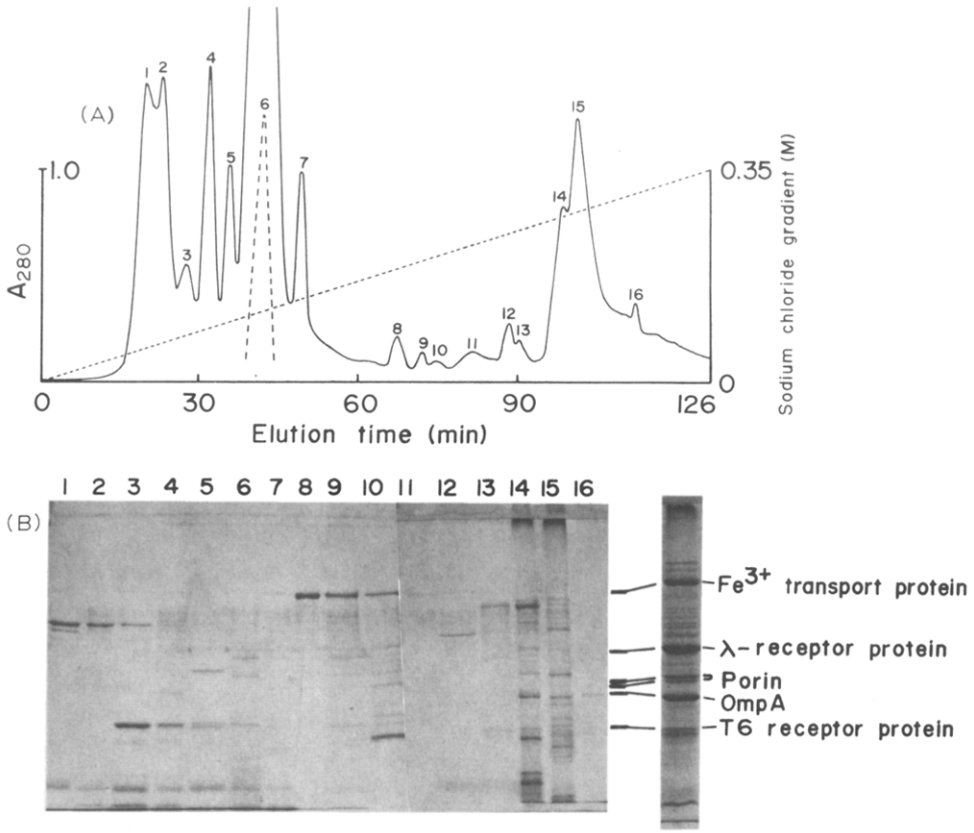

Fig. 2. Elution profile of the outer membrane proteins of *E. coli* K-12 and its analysis by polyacrylamide gel electrophoresis. A, The outer membranes prepared from *E. coli* K-12 (3.1 mg of proteins) were solubilized in 1.0 ml of a solution of 2% $C_{12}E_8$–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and applied to a TSKgel DEAE-5PW column (15 × 2.15 cm I.D.) equilibrated with buffer A. The column was eluted with a linear gradient of 0–0.5 *M* sodium chloride in buffer A at a flow-rate of 2 ml/min. The eluates were collected in 1 ml fractions. B, The samples in the fractions corresponding to the peaks were dialysed against dry Ficoll 400 and 50 m*M* Tris–HCl (pH 6.5) consecutively, and were subjected to SDS–polyacrylamide gel electrophoresis (10% gel) after heating at 100°C for 5 min in the solubilization buffer[8]. The lane on the far right shows the protein bands from the whole outer membrane of *E. coli* K-12.

for the resolution of soluble proteins. We tested this by running the ion-exchange chromatography of partially purified hexokinase (Sigma, type III) in the presence and absence of 1 m*M* $C_{12}E_8$, under otherwise identical conditions, and comparing the elution profiles. As shown in Fig. 1, the elution profiles in the presence and absence of the surfactant gave very similar results. The differences were (i) more protein peaks were resolved in the presence of $C_{12}E_8$ than in the absence of the surfactant and (ii) the retention times of the individual peaks in the presence of the surfactant were slightly longer than in the absence of the surfactant.

As an example of membrane proteins, we chose the outer membrane proteins from *E. coli* K-12, which were solubilized in a solution containing 2% $C_{12}E_8$–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and the soluble fraction was applied to a TSKgel DEAE-5PW column after removing the insoluble materials by centrifugation. The

column was eluted with a linear gradient of 0–0.5 *M* sodium chloride in buffer A (Fig. 2A). The results showed a clear separation of about sixteen UV-detectable peaks. The eluates were fractionated and the samples were subjected to SDS–polyacrylamide gel electrophoresis after heating in SDS. The electropherogram shown in Fig. 2B revealed the following: (i) peaks 1–3 contained predominantly a protein species with an apparent molecular weight of about 60,000; (ii) peaks 3 and 4 contained the proteins with a molecular weight of about 26,000, which might correspond to T6 receptor protein; (iii) the protein species found in a large amount in peaks 8–10 corresponded to the mobility of one of the Fe^{3+} transport proteins; (iv) peaks 14 and 15 contained a number of protein species and many of those in peak 14 showed different electrophoretic mobilities from those in peak 15; (v) most of the UV-detectable materials in peak 6 seemed to be non-proteinaceous, as the electropherogram in Fig. 2B showed only a few minor protein bands.

As mentioned above, the peptidoglycan-associated outer membrane proteins were solubilized little by the surfactant used. The insoluble residues were re-treated with a solution containing 2% Triton X-100–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and the soluble materials were analysed on a TSKgel DEAE-5PW column as described above (see Fig. 3). The material eluted as the arrowed peak was identified as a homogeneous λ-receptor protein as shown in the inset electropherogram.

Membranes of human erythrocyte prepared in the presence of EDTA were solubilized in a solution of 1% $C_{12}E_8$–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and applied to a TSKgel DEAE-5PW column, then the column was eluted with a linear gradient of 0–0.5 *M* sodium chloride in buffer A. The elution profile (Fig. 4A) showed about seventeen UV-detectable peaks. In contrast, the erythrocyte membranes prepared in the absence of EDTA were poorly resolved (Fig. 4B). Although the reason for this difference is not clear, we assume that the membranes prepared in the absence of EDTA contained divalent cations and possibly were not solubilized well under the conditions used. In order to ascertain the effect of EDTA, the erythrocyte membranes prepared in the absence of EDTA were solubilized in a solution of 1% $C_{12}E_8$–10 m*M* Tris–HCl (pH 8) plus 10^{-5}–10^{-2} *M* EDTA and analysed using a TSKgel DEAE-5PW column with buffer A. The results showed that an increasing concentration of EDTA resolved some peaks slightly better than for the samples prepared

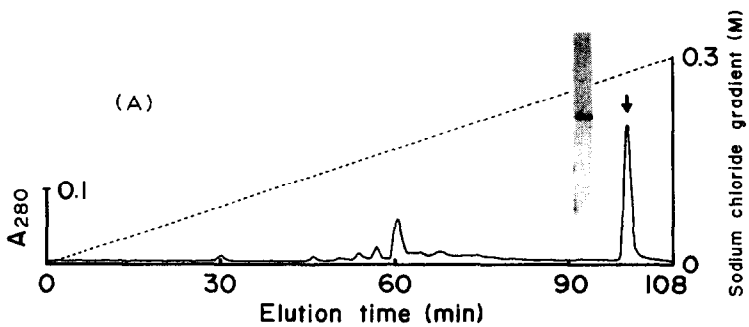

Fig. 3. Elution profile of Triton X-100–EDTA extracts. The $C_{12}E_8$-insoluble outer membrane materials (a leftover from the experiment in Fig. 2) was treated with 10 ml of a solution of 2% Triton X-100–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and the solubilized materials were analysed as described in Fig. 2. The inset shows the electropherogram of material from the arrowed peak.

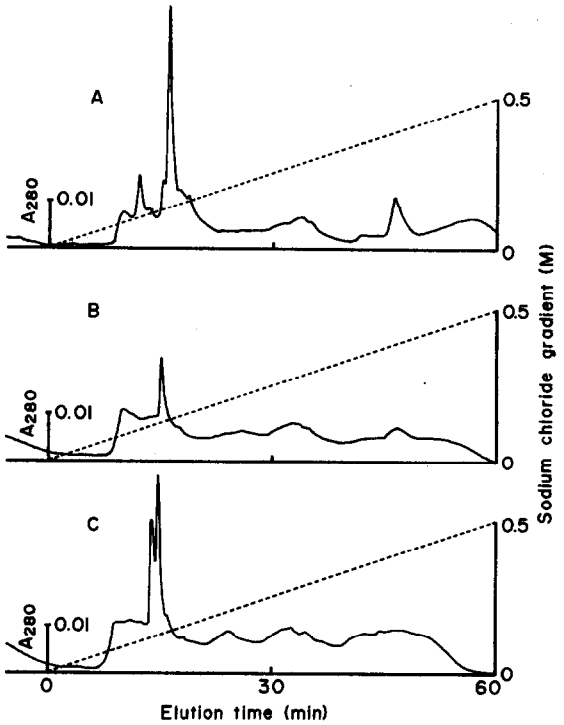

Fig. 4. Elution profiles of the membrane proteins of human erythrocyte. The membranes of human erythrocyte (8 mg of proteins) were solubilized with 1 ml of a solution containing 1% $C_{12}E_8$–10 m*M* Tris–HCl (pH 8) in the presence and absence of 5 m*M* EDTA. The sample (1.2 mg of proteins per 0.15 ml) was applied to a TSKgel DEAE-5PW column (7.5 × 0.75 cm I.D.) and the column was eluted as described in Fig. 1B. A, The erythrocyte membranes prepared in the presence of EDTA were solubilized in an EDTA-containing solution and analysed in buffer A. B, The erythrocyte membranes prepared in the absence of EDTA were run in buffer A. C, The erythrocyte membranes prepared in the absence of EDTA were solubilized in a solution containing 1 m*M* EDTA and run in buffer A.

in the absence of EDTA (Fig. 4C; only one example shown), but never resolved the peaks as well as in Fig. 4A. The results suggested that the poor separation of erythrocyte membrane proteins shown in Fig. 4B was not due simply to the elution buffer used, but depended largely on the conditions used for the membrane preparation.

As the presence of a surfactant is essential for the chromatography of membrane proteins, the effect of the concentration of $C_{12}E_8$ in the buffer was examined. Erythrocyte membranes were solubilized in a solution of 1% $C_{12}E_8$–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and were analysed in the same buffer containing $7 \cdot 10^{-5}$ *M* [the same concentration as the critical micellar concentration (CMC)], 1 m*M* and 5 m*M* $C_{12}E_8$ (the chromatogram obtained in the presence of 5 m*M* $C_{12}E_8$ is not shown, as the elution profile was the same as that obtained in the presence of 1 m*M* $C_{12}E_8$). The results shown in Fig. 5 indicated that a surfactant concentration above the CMC is essential for a good separation. Therefore, care should be taken to avoid using surfactant concentrations below the CMC. This poor resolution is probably due to the aggregation of proteins in the presence of an insufficient concentration of the surfactant.

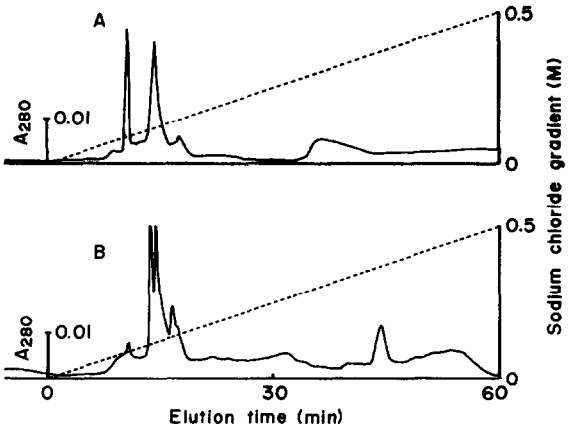

Fig. 5. Effect of surfactant concentration on the elution profile of the membrane proteins of erythrocyte. The erythrocyte membranes prepared in the presence of EDTA were solubilized in a solution containing EDTA as in Fig. 4A, and run in a solution containing $7 \cdot 10^{-5}$ *M* $C_{12}E_8$ (A) or $1 \cdot 10^{-3}$ *M* $C_{12}E_8$ (B). All other conditions as in Fig. 4A.

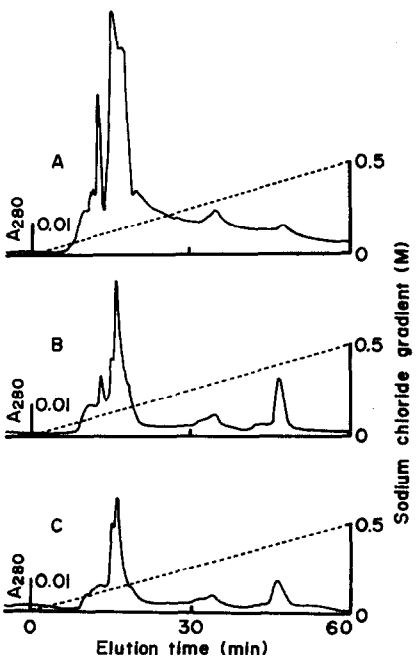

Fig. 6. Effect of chain length of ethylene glycol in $C_{12}E_n$ on the elution profile of the erythrocyte membrane proteins. The erythrocyte membranes prepared in the presence of EDTA were solubilized in a solution containing 1% $C_{12}E_{5,6 \text{ or } 7}$. The conditions for the chromatography were the same as in Fig. 4A, except that 1 m*M* of (A) $C_{12}E_5$, (B) $C_{12}E_6$ and (C) $C_{12}E_7$ were used instead of $C_{12}E_8$. Compare the elution profile with Fig. 4A.

The effect of a homologous series of surfactants, with different chain lengths of ethylene glycol or of the alkene of polyoxyethylene glycol alkyl ether, was examined. The results in Fig. 6 show that an increasing chain length of ethylene glycol in polyoxyethylene glycol dodecyl ether ($C_{12}E_n$) gave a better separation of membrane proteins (see Figs. 4A, 5B and 6) than the $C_{12}E_n$ of shorter ethylene glycol chains. These results suggest that the solubilizing activity of the surfactant directly influenced the resolution of ion-exchange chromatography. A large peak at a retention time of about 20 min seemed to be an aggregate or a mixture of a number of proteins, as

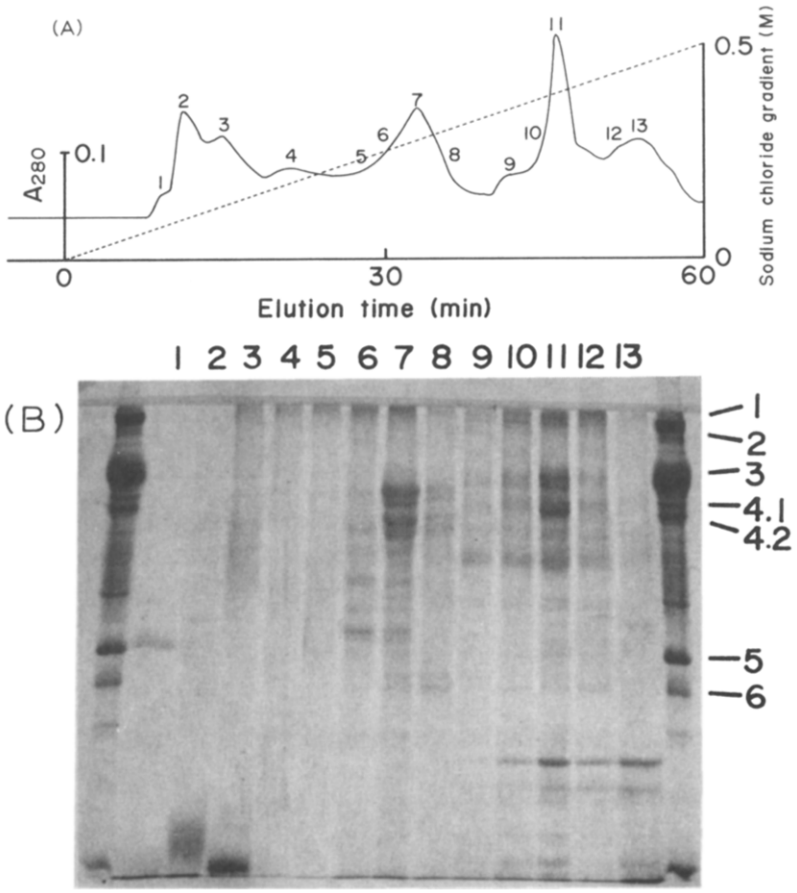

Fig. 7. Large-scale preparation of the membrane proteins of human erythrocyte. The erythrocyte membranes prepared in the presence of EDTA (12 mg of proteins) were solubilized in 1 ml of a solution containing 1% $C_{12}E_8$–5 m*M* EDTA–10 m*M* Tris–HCl (pH 8) and were applied to a TSKgel DEAE-5PW column (7.5 × 0.75 cm I.D.). The column was eluted as described in Fig. 4A. The eluates were collected in 1-ml fractions. The samples were dialysed against a large excess of 50 m*M* Tris–HCl (pH 6.8) overnight and concentrated to appropriate volumes by dialysis against dry Ficoll 400. The samples were subjected to electrophoresis in the presence of SDS using 10% polyacrylamide gel after heating at 100°C for 5 min in the solubilization buffer[8]. A, Absorption at 280 nm was recorded manually. B, Electropherogram. The numbers in A correspond to the numbers in the lanes in B. The lane on the far right shows the protein bands from the whole erythrocyte membrane. The numbers on the right in B indicate the location of known membrane proteins from erythrocyte membrane according to ref. 10.

analysed by polyacrylamide gel electrophoresis (Fig. 7B). Therefore, the appearance of this peak may be a sign of poor resolution. The use of octa-, hexa- and pentaethylene glycol decyl ether ($C_{10}E_n$) resulted in essentially the same results as in Fig. 6B and C (data not shown). However, the minor peaks tends to be lost with the use of $C_{10}E_n$. Although the reason for this poor resolution of membrane proteins in the solution containing $C_{10}E_n$ is not clear, the most likely possibility is the poor solubilizing activity of the surfactant. This interpretation is consistent with a report by Lichtenberg *et al.*[9], who stated that an increasing chain length of the hydrocarbon increased the solubilizing ability of the surfactant. Ion-exchange HPLC of membrane proteins in the presence of a non-ionic surfactant gave satisfactory separations, as monitored with the UV detector and by SDS–polyacrylamide gel electrophoresis. In order to determine the protein composition of the peak fractions, a relatively large amount of the erythrocyte membrane proteins was applied to a TSKgel DEAE-5PW column (7.5 × 0.75 cm I.D.). The eluates were collected in fractions as shown in Fig. 7A and subjected to SDS–polyacrylamide gel electrophoresis after heating in SDS. The electropherogram showed a reasonably good separation of the membrane proteins, although fractions 7 and 11 contained either a mixture of several proteins or a complex of proteins (Fig. 7B).

It can be concluded that the ion-exchange HPLC of membrane proteins could be made just like the chromatography of soluble proteins in the presence of a non-ionic surfactant, without losing the high performance. The advantages of using an ion-exchange HPLC column are (i) the resolution of membrane proteins is fairly good, if the proteins are solubilized in the appropriate surfactant, and (ii) a single chromatographic run takes only 90–120 min. Therefore, the system can be used to isolate labile membrane proteins.

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