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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF MEMBRANE PROTEINS ISOLATED FROM ERYTHROCYTE GHOSTS

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SUMMARY

Membrane proteins extracted from erythrocyte ghosts with sodium dodecyl sulphate (SDS), 3-(3-cholamidopropyl)-dimethylamminopropane sulfonate (CHAPS) or octylglucoside have been analyzed in various reversed-phase high-performance liquid chromatographic systems. Only SDS was able to solubilize considerable amounts of membrane proteins with mol.wt. $>15\,000$ daltons, but these membrane proteins were recovered in poor yield from a silica-based C_4 column eluted with an acetonitrile gradient in trifluoroacetic acid (TFA). A resin-based phenyl column eluted with a similar TFA–acetonitrile gradient was found to be a better choice with respect to the recovery of membrane proteins with mol.wt. $>15\,000$ daltons, and when this column was eluted with an acetic acid gradient with increasing amounts of acetonitrile, erythrocyte ghost membrane proteins solubilized in SDS (mol.wt. 10 000–200 000 daltons) were separated in six major and several minor components with satisfactory recovery.

INTRODUCTION

Due to their lipophilic character, the solubilization and separation of membrane proteins normally require the use of detergents and classical protein purification strategies —designed for water-soluble proteins— are of limited value. Consequently the number of reports dealing with high-performance liquid chromatographic (HPLC) separation of membrane proteins is limited. The development of stationary phases for protein separation has been directed towards the

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water-soluble proteins with molecular weight (MW) $<25\,000$ daltons, and reversed-phase separation has been the most popular separation principle. Only a few new stationary phases for high-performance ion-exchange chromatography (HPIEC), hydrophobic interaction chromatography (HIC) or size-exclusion chromatography (SEC) have been commercially available in the last few years.

The separation principle in reversed-phase (RP)-HPLC is hydrophobic interaction between the stationary phase and hydrophobic areas in the sample molecules, but due to the extremely high hydrophobicity of most membrane proteins, the binding forces between membrane proteins and most RP stationary phases are too strong. In order to elute the membrane proteins, extremely rigorous mobile phases have to be used, and even then many membrane proteins are recovered in very poor yield.

Moreover, the detergents normally used for solubilizing many membrane proteins are not compatible with RP stationary phases for protein separation. Consequently the most common HPLC techniques used for the purification of membrane proteins have been HPSEC (which may be used in the presence of ionic as well as non-ionic detergents) and —to a lesser extent— HPIEC and HIC (compatible with non-ionic detergents).

We have recently evaluated a number of commercially available HIC stationary phases for their potential use for the separation of membrane proteins from erythrocyte ghosts using mobile phases containing 3-(3-cholamidopropyl)-dimethylamminopropane sulfonate (CHAPS), octylglucoside or sulfobetaine[1]. In this report we present the separation of erythrocyte membrane proteins extracted with sodium dodecyl sulphate (SDS), CHAPS or octylglucoside using a silica-based as well as a resin-based reversed-phase column eluted with trifluoroacetic acid (TFA)–acetonitrile. A novel mobile phase additive (acetic acid) was evaluated in the resin-based RP column, and the resulting separations of the extracted membrane proteins have been evaluated with SDS polyacrylamide gel electrophoresis (PAGE) followed by silver staining.

MATERIALS AND METHODS

HPLC equipment

Pumps: Waters M6000A or M510, Gynkotek 300C or Spectra Physics SP 8700. Sample injectors: U6K, WISP 710B or 712 (Waters), Rheodyne 7125. Gradient controllers: Waters 760, 721 or 840 chromatography control station, Gynkotek 250 B. UV-Photometers: Linear UVIS 200, Waters M440 and M481 or Hitachi L4200. Columns: TSK Phenyl 5 PW RP (75 mm × 4.6 mm I.D.) (Toyo Soda), Nucleosil C_4 (300 Å) (250 mm × 4.0 mm I.D.) (Macherey Nagel).

The mobile phases are given in the figure legends.

Chemicals

TFA was sequential grade (Applied Biosystems). All other chemicals were of HPLC quality or similar purity. Water was drawn from a Milli-Q plant, and the buffers were Millipore-filtered (0.45 μm) and degassed (ultrasound/vacuum) before use.

The columns were operated at room temperature (*ca.* 22°C) at 1.0 or 0.5 ml/min. The UV absorption of the column eluate was measured continuously (210 or 280 nm) and fractions were collected manually.

Samples

Erythrocyte ghosts were prepared as described[2] with the exception that trasylol was added to the washing buffer (0.15 mg/ml) in order to reduce the enzymatic digestion during the washing procedure. The erythrocyte ghosts were extracted with 1% SDS, CHAPS or octylglucoside in 10 m*M* Tris · HCl–5 m*M* EDTA pH 8.0 and remaining solid material was removed by centrifugation before analysis.

The protein concentration in the SDS extract was 2.2–5.1 mg/ml (Bio-Rad Coomassie Blue protein assay, serum albumin as a standard).

SDS-PAGE was performed in a Pharmacia Phast-Gel apparatus (8–25% gradient gels with separation range 8000–300 000 daltons). The electrophoresis and the silver staining were performed as described by the manufacturer[3].

RESULTS

TSK Phenyl 5 PW RP

The separation of SDS-solubilized membrane proteins from erythrocyte ghosts using the TSK Phenyl 5 PW RP column eluted with 0.1% TFA–acetonitrile is shown in Fig. 1. Although the acetonitrile gradient was extended up to 90%, virtually no material was eluted from the column in the first half of the chromatogram. Injection of 25 μl membrane protein solution resulted in a separation of 10–15 components with fairly good peak shape (Fig. 1, upper panel) and when the extraction buffer was injected the column had virtually no "memory" from the previous injection of membrane proteins. Injections of 100 and 200 μl membrane protein solution resulted in a gradually reduced separation efficiency (Fig. 1, middle and lower panels).

The fractions marked A–D were diluted in one volume of distilled water and lyophilized. The residue was dissolved in Tris–SDS–mercaptoethanol, placed in a bath of boiling water for 5 min and analyzed by SDS–PAGE (Fig. 2). The membrane proteins are separated into fractions primarily according to their molecular weight: in the first part of the chromatogram two major components with MW slightly lower and higher than the lowest molecular weight marker (14 400 daltons) are seen, and one of them probably represents the globin chains from residual haemoglobin (MW 15 000 daltons). In the last fraction (D), membrane proteins with MW > 80 000 daltons are found, clearly separated from components with lower MW.

Membrane proteins extracted with 1% CHAPS or 1% octylglucoside were analyzed under similar conditions, and the column separations are shown in Fig. 3. When 200 μl membrane protein solution were injected the resulting chromatograms showed that the amount of material eluted from the TSK Phenyl 5 PW RP column was much less for CHAPS and octylglucoside-solubilized membrane proteins than for SDS-solubilized membrane proteins (judged by the area under the UV curves in Fig. 1, lower panel and Fig. 3, upper and lower panels). SDS-PAGE of collected fractions corresponding to the major peaks obtained after fractionation of CHAPS and octylglucoside-solubilized membrane proteins showed no material with MW higher than *ca.* 15 000 daltons (data not shown) in accordance with SDS-PAGE of the three extracts of erythrocyte ghosts (Fig. 4): none or extremely small amounts of material with MW > 15 000 daltons was found in the octylglucoside and the CHAPS extracts, whereas several components with MW from 15 000 to 200 000 daltons were present in the SDS extract.

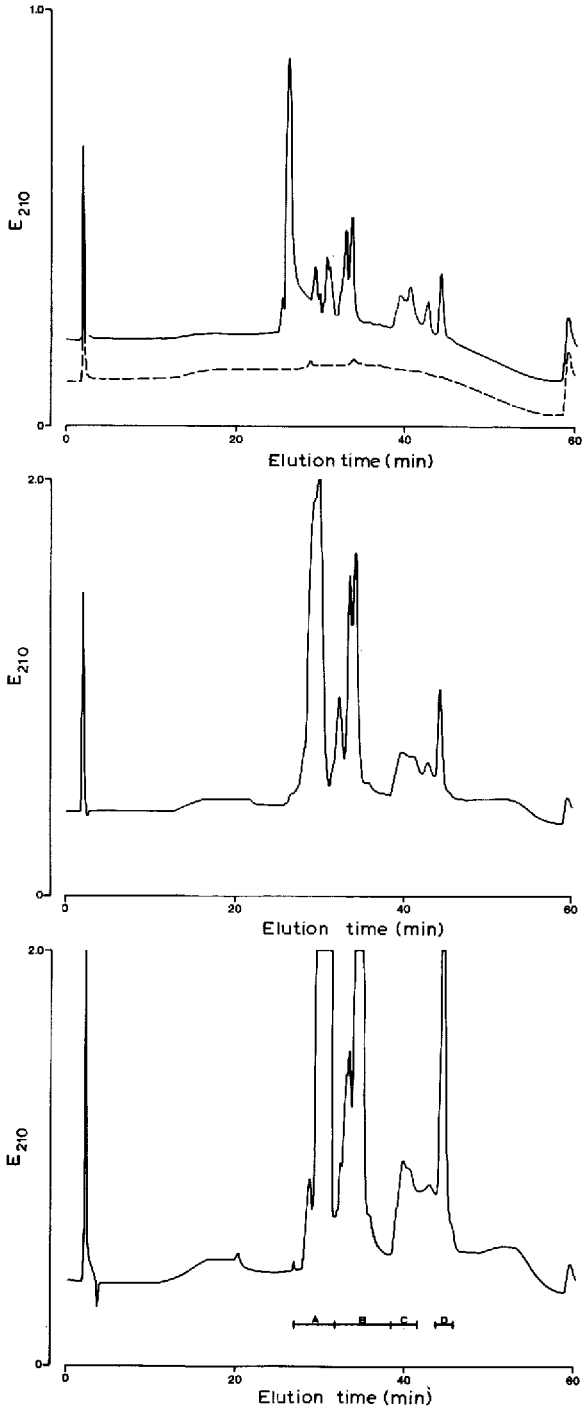

Fig. 1. Separation of 25 (upper panel), 100 (middle panel) and 200 μl (lower panel) SDS-solubilized erythrocyte membrane proteins using a TSK Phenyl 5 PW RP column (75 mm × 4.6 mm I.D.) eluted at 0.5 ml/min with an acetonitrile gradient (0–90%) in 0.1% TFA during 45 min. In the upper panel the broken line indicates the injection of 25 μl of the SDS extraction buffer. E_{210} = Absorbance at 210 nm.

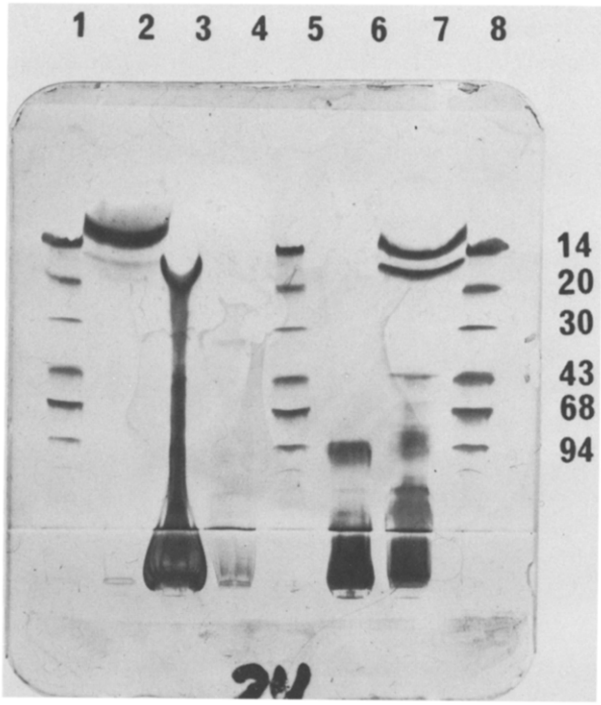

Fig. 2. SDS-PAGE of fractions A–D in Fig. 1 (lower panel). Lanes 1–8 show the separation in 8–25% gradient. SDS-PAGE gels (separation range 8000–200 000 daltons) of molecular weight markers (lanes 1, 5 and 8), fractions A (lane 2), B (lane 3), C (lane 4), D (lane 6) and the total eluate collected from 0 to 60 min (lane 7).

In order to obtain a better distribution between the peptide-like material (with MW $\leqslant$ 15 000 daltons) and membrane proteins with MW 25 000–200 000 daltons the TSK Phenyl 5 PW RP column was eluted with an acetic acid gradient in combination with increasing amounts of acetonitrile, and the resulting separation of SDS-solubilized erythrocyte ghost membrane proteins is shown in Fig. 5. Four major fractions as well as several minor components were separated when 100 μl membrane protein solution were applied to the column. Injection of 250 and 500 μl membrane protein solution resulted in a compressed chromatogram with reduced resolution of the first fractions.

The fractions indicated in Fig. 5, right panel, were analyzed by SDS-PAGE (Fig. 6), and it was demonstrated, that the elution order was correlated to MW in SDS, *i.e.*, the components eluted in the last part of the gradient were those with the highest MW. The last half of the chromatogram corresponded to membrane proteins with MW $>$ 60 000 daltons, and components with MW higher than the upper fractionation range for the SDS-PAGE gel (200 000 daltons) were demonstrated in the last fraction.

It was further demonstrated that the amount of membrane protein which could be extracted with CHAPS was considerably smaller than that obtained using SDS for the extraction, and that the CHAPS-solubilized membrane components were eluted

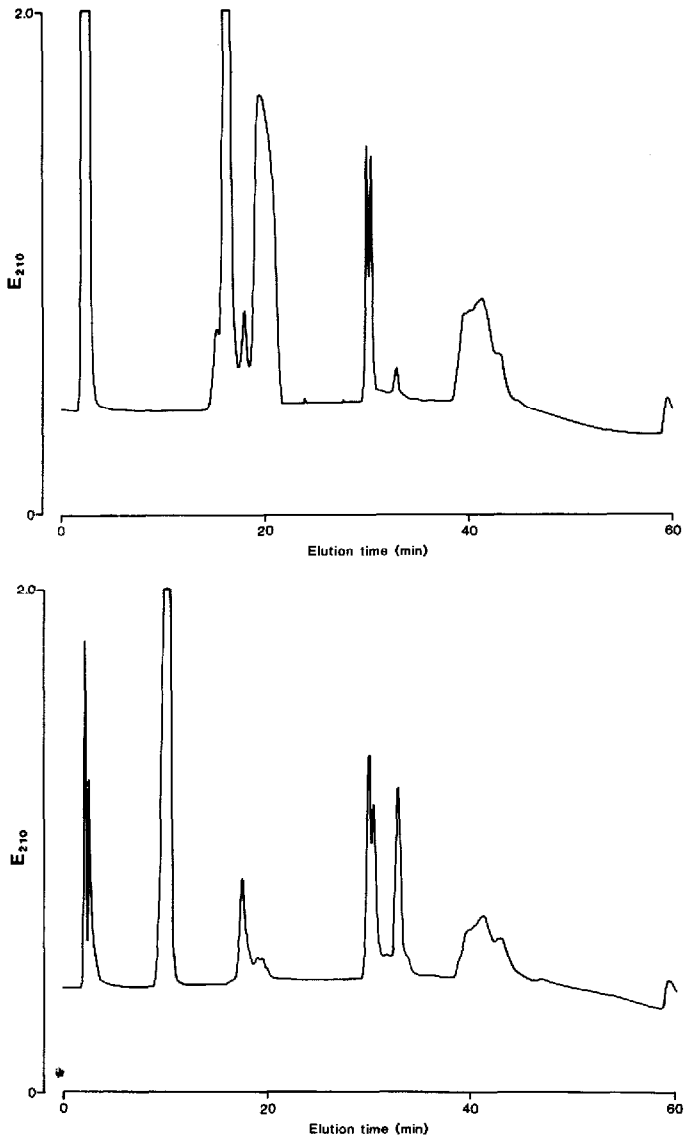

Fig. 3. Separation of 200 μl erythrocyte membrane proteins extracted in 1% CHAPS (upper panel) or in 1% octylglycoside (lower panel). Chromatographic conditions as in Fig. 1.

in the part of the chromatogram which corresponds to membrane components with MW $<$ 15 000 daltons (Fig. 5, lower panel left). The recovery of the membrane proteins in this acetic acid–acetonitrile mobile phase was estimated by comparing the area under the UV curve after gradient elution of membrane protein solution to that obtained after sample injection directly in the UV-photometer. A recovery of 95–100% was measured for the SDS-solubilized membrane proteins.

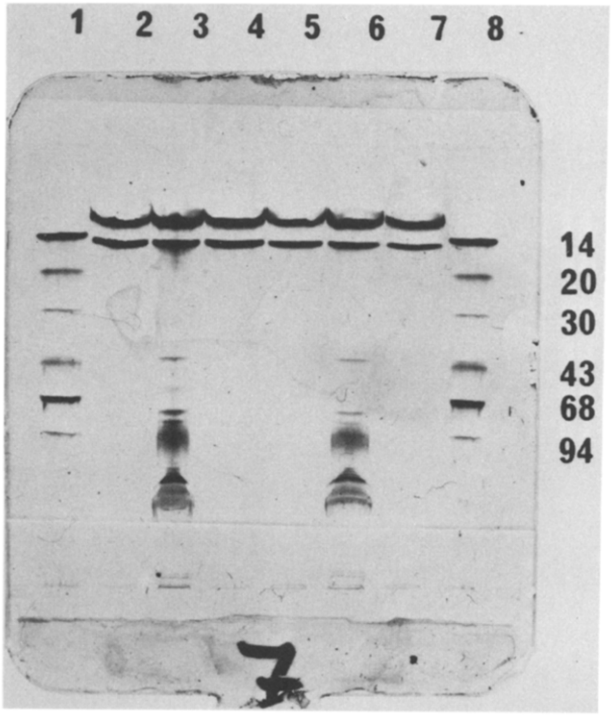

Fig. 4. SDS-PAGE of erythrocyte membrane proteins extracted in 1% SDS (lane 3 and 6), CHAPS (lanes 2 and 5) or octylglucoside (lanes 4 and 7). Molecular weight markers (are in lanes 1 and 8).

If the gradient was changed from the "simple" linear 0–100% to a gradient composed of two linear segments in order to obtain an higher resolution especially in the first part of the chromatogram, an improved separation of erythrocyte membrane proteins was obtained (Fig. 7, upper panel). SDS-PAGE of the individual fractions clearly demonstrated that the resolution was the best obtained so far (Fig. 8) and that isolation of individual membrane proteins may be possible with this technique.

The mobile phases used for eluting the TSK Phenyl 5 PW RP column are slightly more acidic than recommended by the manufacturer, and during continuous use for several months an increase in back pressure and reduced column performance were noticed. Whether this was caused by statioary phase degradation or was the result of several injections of rather crude, unfiltered biological samples is not clear at present, but the column performance as well as the back pressure could be normalized after eluting the column with 0.2 *M* sodium hydroxide and cleaning the dismounted inlet and outlet filters in 50% nitric acid in an ultrasonic bath for 30 min.

Nucleosil C_4

In order to compare a silica-based reversed-phase column to the resin-based TSK Phenyl column, a Nucleosil C_4 (300 Å) column was eluted with an acetonitrile gradient in 0.1% TFA. The membrane proteins extracted in SDS were separated into a major and several minor components (Fig. 7, lower panel), but only the minor

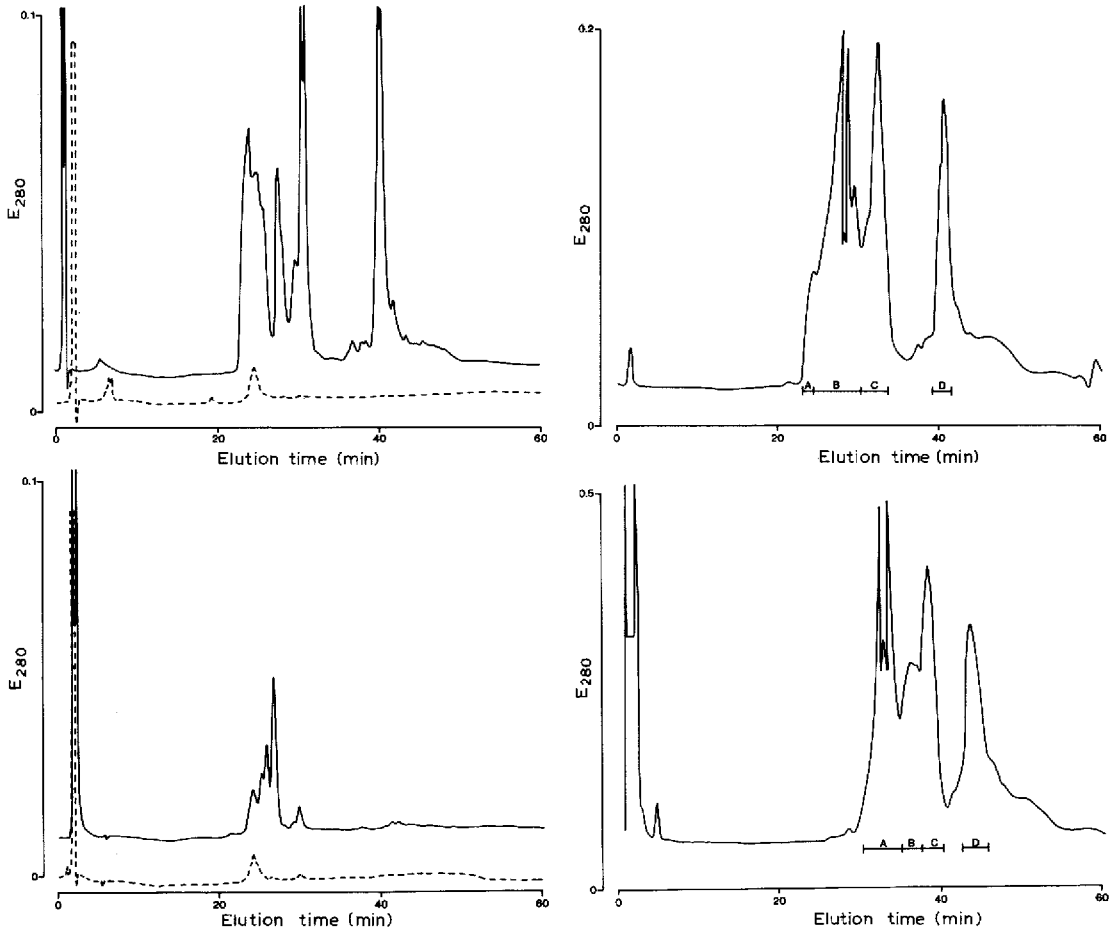

Fig. 5. Separation of 100 (upper panel left), 250 (upper panel right) or 500 μl (lower panel right) SDS-solubilized erythrocyte membrane proteins and 100 μl CHAPS-solubilized erythrocyte membrane proteins (lower panel left). Column: TSK Phenyl 5 PW RP (75 mm × 4.6 mm I.D.). Buffers: A, 20% acetic acid; B, acetic acid–acetonitrile (40:60). A linear gradient from 100% A to 100% B during 45 min was used at 0.5 ml/min. The dashed lines indicate the injection of 100 μl of the buffer used for extraction of the membrane proteins.

peaks in the last part of the chromatogram were found to contain membrane proteins with MW > 15 000 daltons, and a considerably lower amount than was found after elution of the TSK Phenyl column with the similar mobile phase (Fig. 9).

DISCUSSION

The erythrocyte membrane contains *ca.* 52% protein, 40% lipid and 8% carbohydrate[4] and the membrane proteins extracted with detergents have been analyzed primarily using SDS-PAGE[2,4]. It has been demonstrated that SDS-PAGE will separate six major and several minor components with MW from 15 000 (globin chains

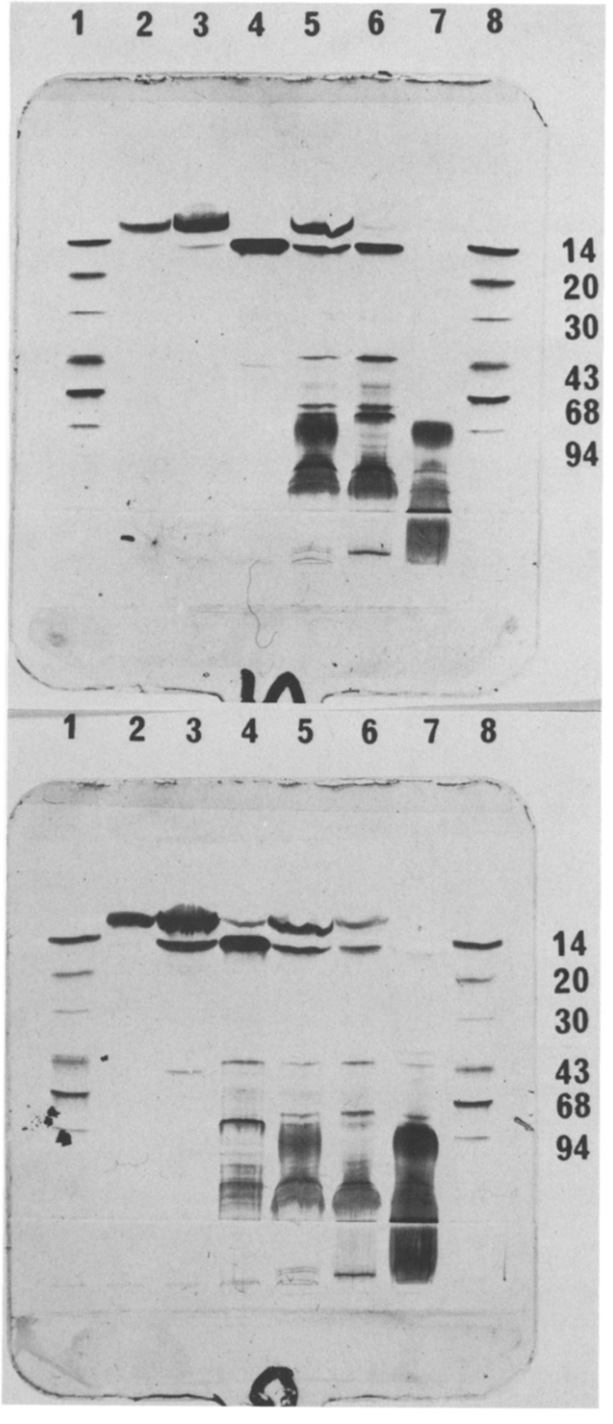

Fig. 6. Upper gel: SDS-PAGE of the fractions indicated in Fig. 5, upper panel right; fraction A (lane 2), the major peak in fraction B (lane 3), the minor peak in fraction B (lane 4), total membrane extract in SDS (lane 5), fraction C (lane 6), fraction D (lane 7) and molecular weight markers (lanes 1 and 8). Lower gel: SDS-PAGE of the fractions indicated in Fig. 5, lower panel right; fraction A (lane 2), fraction B, initial half (lane 3), fraction B, terminal half (lane 4), total membrane extract in SDS (lane 5), fraction C (lane 6), fraction D (lane 7) and molecular weight markers (lane 1 and 8).

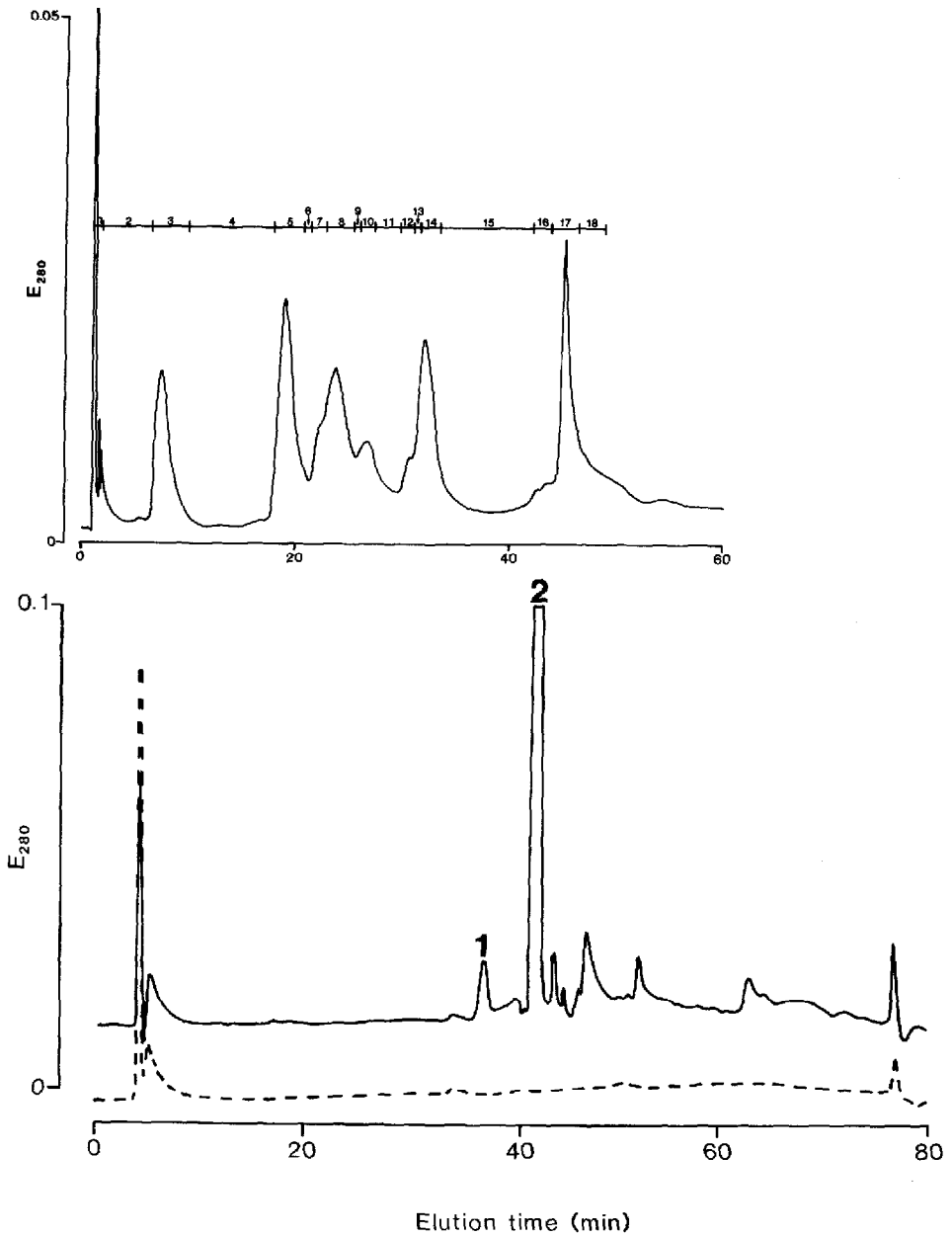

Fig. 7. Upper panel: separation of 100 μl SDS-solubilized erythrocyte membrane proteins using the stationary and mobile phase decribed in Fig. 5. Gradient 80% A (10 min), 80 to 50% A (35 min), 50 to 0% A (15 min), 0% A (15 min). Flow-rate: 0.5 ml/min. Lower panel: separation of 200 μl SDS-solubilized erythrocyte membrane proteins using a 250 mm × 4.0 mm I.D. Nucleosil C_4 (300 Å) column eluted at 1.0 ml/min with an acetonitrile gradient (0 to 90%) in 0.1% TFA over 60 min. The dashed line indicates injection of 100 μl SDS extraction buffer.

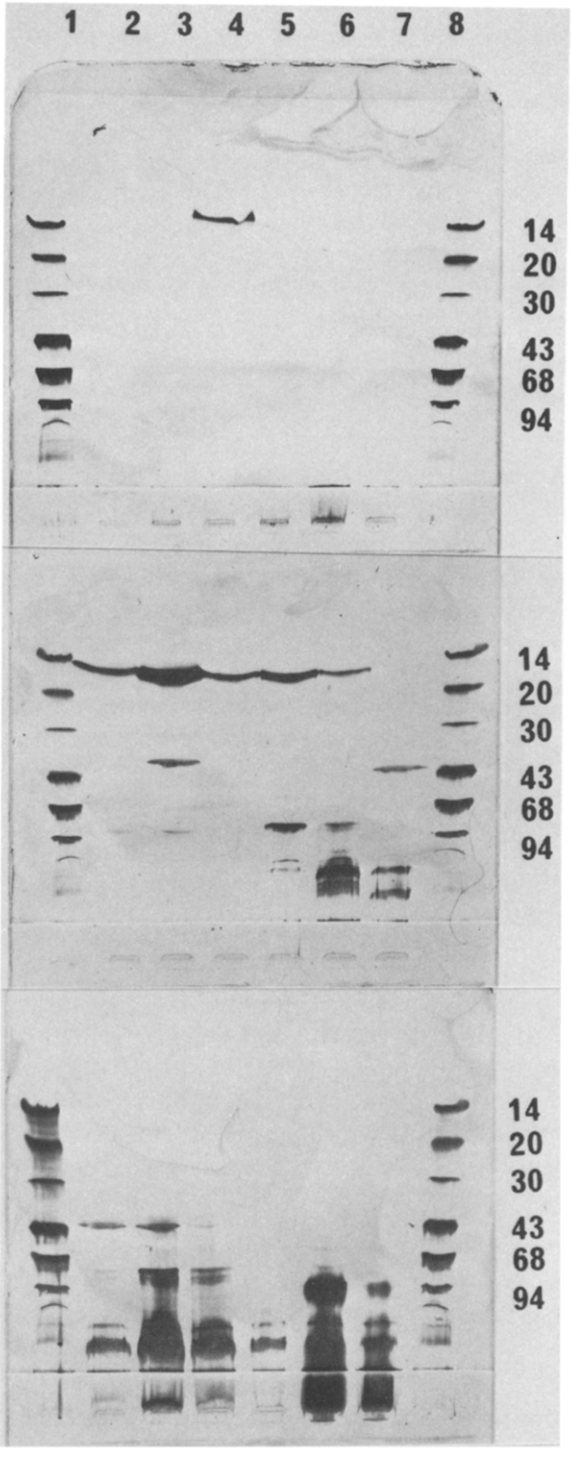

Fig. 8. SDS-PAGE of fractions 1–18 indicated in Fig. 7. Upper gel: lanes 2–7 correspond to fractions 1–6 respectively. Middle gel: lanes 2–7 c orrespond to fractions 7–12 respectively. Lower gel: lanes 2–7 correspond to fractions 13–18 respectively. In all three gels the molecular weight markers are shown in lanes 1 and 8.

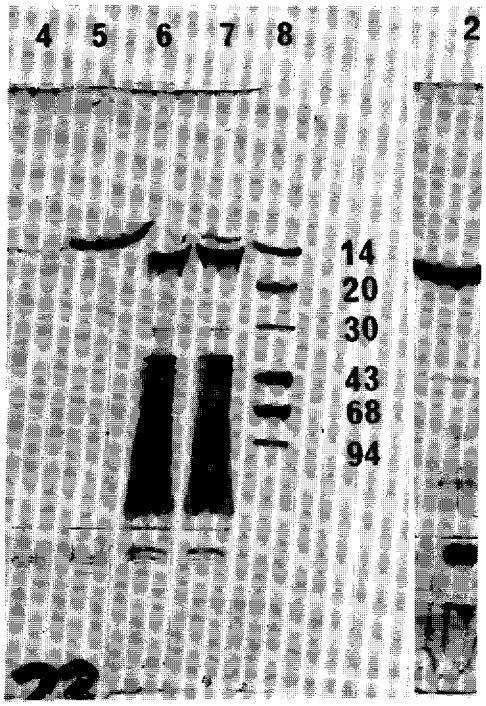

Fig. 9. SDS-PAGE of the peaks indicated in Fig. 7: peak 1 (lane 4), 2 (lane 5), total membrane proteins extracted in SDS (lane 6 and 7), molecular weight markers (lane 8) and the pooled column eluate collected from immediately after peak 2 to the end of the chromatogram (lane 2).

from haemoglobin) to $>150\,000$ daltons. Due to easy accessibility and a variety of proteins with well known MW, this source of membrane protein seems attractive for initial investigations concerning the potential use of HPLC for the separation and isolation of membrane proteins.

Erythrocyte ghost membrane proteins extracted with polyoxyethylene glycol alkyl ether ($C_{12}E_8$) have been analyzed using HPIEC with the same detergent in the mobile phase. The separation was reasonably good judged by SDS-PAGE, but only the non-ionic detergent was used for membrane protein extraction. No recovery figures were given[5]. The influence of the type of detergent used for solubilizing the erythrocyte ghost has been examined using HPSEC with the proper detergent in the mobile phase. The elution strength was found to be SDS > CHAPS > Triton X-100, and it was concluded that HPSEC is a useful technique for monitoring the solubilizing process[6].

TSK Phenyl 5 PW RP as well as TSK Phenyl 5 PW columns have been evaluated for the potential separation of SDS-solubilized erythrocyte ghost membrane protein or whole ghosts injected directly. An acetonitrile gradient (0 to 95%) in 0.05% TFA was used. Compared to other RP columns with smaller pore sizes, the resolution of the large-pore TSK Phenyl columns was found to be advantageous. The column eluate was not characterized in any respect (except for the UV absorption)[7].

The recovery of erythrocyte membrane proteins from the two TSK Phenyl

columns has been estimated using the TFA–acetonitrile gradient described above. It was concluded that it was advantageous to solubilize the membrane proteins in SDS before analysis, but even then recoveries of up to 40–50% are predictable[8].

In the present study we have eluted a TSK Phenyl 5 PW RP column with TFA–acetonitrile and with a new mobile phase containing increasing amounts of acetic acid (20–40%) in an acetonitrile gradient (0–60%). In both cases it could be demonstrated, that CHAPS and octylglucoside primarily solubilized membrane components with MW < 15 000 daltons, whereas the SDS-solubilized membrane proteins in addition to the 15 000 daltons material included several proteins with MW 25 000–200 000 daltons. Although such membrane proteins can be eluted from the TSK Phenyl column with TFA–acetonitrile, the chromatogram in Fig. 1 clearly shows that the column eluted under these conditions favours the separation of lower MW polypeptides, *i.e.*, from 3000 to 15 000 daltons. These components may be separated completely, whereas the membrane proteins with higher MW are eluted in one or two groups virtually without separation.

However, the elution of hydrophobic proteins with MW around 100 000 daltons from a RP column is quite remarkable. In order to expand the high-molecular-weight separation range, the TSK Phenyl column was eluted with an acetic acid gradient in acetonitrile. It was clearly demonstrated that the column now operated in another fashion to that obtained with TFA–acetonitrile, since the CHAPS membrane extracts (which contained only membrane components with MW < 15 000 daltons) were eluted in the initial quarter of the chromatogram (Fig. 5), whereas the membrane proteins solubilized with SDS and containing components with MW 15 000–200 000 daltons were resolved in several fractions in the major part of the chromatogram (Fig. 7, upper panel). Isolation of membrane proteins with MW > 50 000 daltons, sufficiently pure for sequencing, should be possible with minor developmental work.

HPLC separation of a membrane protein mixture is an enormous challenge due to the complexity of the sample and (partly) the inapplicability of several HPLC principles which have produced brilliant results in the separation and characterization of water-soluble proteins. Due to the well known principle for estimating the molecular weight of polypeptides in SDS, HPSEC with mobile phases containing SDS has traditionally been the initial step in an HPLC characterization of membrane proteins, but HPSEC offers probably the least satisfactory separation capacity of the most common HPLC methods, and for several reasons, primarily economic, preparative HPSEC of polypeptides has been reported in very few cases.

RP-HPLC of water-soluble polypeptides is normally optimized by varying the stationary as well as the mobile phase, and as indicated in the present report, successful RP-based separation of membrane proteins depends upon similar developmental work: a silica-based C_4 column eluted with TFA–acetonitrile (a perfect combination for several polypeptide separations) was considerably less suited for membrane protein separation than was the resin-based phenyl column (with a different chemistry) eluted with the same mobile phase.

Further, a considerable expansion of the separation range for the resin-based RP column resulted from exchanging TFA with acetic acid as the mobile phase additive. The use of high amounts of acetic acid seems a promising alternative to the classical RP mobile phase additives. Good resolution and high recovery of erythrocyte membrane proteins was obtained, and the degree of solubilization using various detergents can be rapidly estimated.

Similar separations to those reported here were obtained if the TSK Phenyl column was eluted with an acetic acid gradient, *i.e.*, 20–90% acetic acid without addition of any organic modifier[9], indicating the possibility to perform RP-HPLC analyses of proteins without incorporation of acetonitrile, propanol or other organic solvents.

However, only few commercially available RP columns will tolerate continuous use of high concentrations of acetic acid, and this mobile phase is far from any physiological level. A successful separation of many membrane proteins will therefore require new concepts in column design as well as a critical revision of the choice of mobile phases. Investigations concerning the last point are in progress in this laboratory.

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