Journal of Chromatography, 515 (1990) 399–406
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1811

Separation of membrane protein–sodium dodecyl sulphate complexes by high-performance liquid chromatography on hydroxyapatite

TAKAAKI HIRANUMA*, TSUNEYOSHI HORIGOME and HIROSHI SUGANO
Department of Biochemistry, Faculty of Science, Niigata University, 2-Igarashi, Niigata 950-21 (Japan)

ABSTRACT

The analytical conditions for the high-performance liquid chromatography of protein–sodium dodecyl sulphate complexes on ceramic hydroxyapatite were optimized for the purification of membrane proteins and the microanalysis of membrane protein mixtures. About 12 mg of erythrocyte membrane protein could be separated by ceramic hydroxyapatite chromatography at one time and about 0.8 mg of purified anion carrier protein was obtained on one-step purification. Erythrocyte membrane proteins could be analysed with buffers not containing dithiothreitol and detection at 230 nm. A two-step method, *i.e.*, combined diethylaminoethyl and hydroxyapatite chromatography, made it possible to separate many membrane proteins.

INTRODUCTION

High-performance liquid chromatography (HPLC) is one of the most useful methods for the separation and analysis of proteins. Recently, many soluble proteins have been purified by HPLC. However, with membrane proteins, solubilization of the samples is necessary prior to separation or analysis by HPLC. Various detergents have been used for membrane solubilization, depending on the purpose of the experiment.[1] However, most of the detergents used could not solubilize whole membrane proteins, *e.g.*, some intrinsic and high-molecular-weight proteins could not be solubilized by detergents other than sodium dodecyl sulphate (SDS). Therefore, the development of a chromatographic method for the separation of membrane proteins dissolved in SDS is very important for the analysis of whole membrane proteins.

Moss and Rosenblum[2] studied the separation of protein–SDS complexes by conventional hydroxyapatite chromatography. We have improved the resolution of their method by using a ceramic hydroxyapatite HPLC column and applied the modified method to membrane protein analysis[3]. The retention times of protein–SDS complexes increased with increasing hydrophobicity of the proteins. Whole membrane proteins were well separated with this method.

In this study, we examined the conditions for ceramic hydroxyapatite HPLC

0021-9673/90/$03.50

for the microanalysis and preparative separation of membrane protein–SDS complexes. The two-step separation of whole membrane proteins was also examined through the combination of this method with ion-exchange chromatography.

EXPERIMENTAL

Materials

All reagents except 3-(tetradecyldimethylammonio)-1-propanesulphonate (Zwittergent 14) were purchased from Wako (Osaka, Japan). Zwittergent 14 was synthesized by the method of Gonenne and Ernst[4]. Biochemical-grade SDS was used.

Membrane preparations

Rat erythrocyte membranes and rat liver rough microsomal membranes were prepared according to the methods reported previously[3]. The rat brain myelin fraction was prepared by the method of Norton and Poduslo[5].

Buffers for HPLC

Buffer A was 0.01 *M* sodium phosphate buffer (pH 6.5) containing 1.0% SDS and 0.1 m*M* calcium chloride. Buffer B was 0.5 *M* sodium phosphate buffer (pH 6.5) containing 1.0% SDS and 7.5 μM calcium chloride. Buffers A′ and B′ were buffers A and B containing 0.5 m*M* dithiothreitol, respectively. Buffer C was 20 m*M* Tris–HCl buffer (pH 8.0) containing 0.4% Zwittergent 14. Buffer D was 20 m*M* Tris–HCl buffer (pH 8.0) containing 0.4% Zwittergent 14 and 1.0 *M* sodium chloride.

Columns used for HPLC

The columns used for hydroxyapatite HPLC were Hibar RT 100-8 hydroxyapatite-MP (100 mm × 8.0 mm I.D.), (Cica-Merck, Tokyo, Japan) and TSKgel HA-1000 (75 mm × 7.5 mm I.D.) (Tosoh, Tokyo, Japan). A TSKgel DEAE-5 PW column (75 mm × 7.5 mm I.D.) (Tosoh) was used for anion-exchange chromatography.

HPLC apparatus

Chromatographic equipment consisting of a model L-5000 LC controller, Model 655A-11 and 655A-12 pumps, a Model 655A variable-wavelength UV monitor, a Model D-2500 chromato-integrator (Hitachi, Tokyo, Japan), an injector with a 1-ml loop (Rheodyne, Cotati, CA, U.S.A.) and a fraction collector (LKB, Bromma, Sweden) was used.

Solubilization of membrane proteins

To a 140-μl aliquot of a protein solution (5.5 mg protein/ml buffer A) were added 50 μl of 10% SDS and 10 μl of 2-mercaptoethanol, followed by incubation at 37°C for 1 h. The solution was diluted with an equal volume of buffer A or A′ and then centrifuged at 8500 *g* for 10 min. A 250-μl aliquot of the supernatant was applied to a ceramic hydroxyapatite column. In exchange chromatography, Zwittergent 14 and 40 m*M* Tris–HCl (pH 8.0) were used instead of SDS and buffer A.

Standard conditions for ceramic hydroxyapatite HPLC

Rat erythrocyte membrane protein–SDS complexes (0.48 mg of protein) were applied to a TSKgel HA-1000 column equilibrated with buffer A′, followed by elution with a 69-min linear gradient from 55 to 100% of buffer B′ at 30°C. The flow-rate was 0.6 ml/min.

Two-step separation of membrane proteins

First step. A TSKgel-5PW column was equilibrated with buffer C. Rat erythrocyte membrane protein (4.5 mg) solubilized as above was applied to the column, followed by elution with a 120-min linear gradient, from 0 to 100%, of buffer D at a flow-rate of 1.0 ml/min. The column temperature was maintained at 30°C and the effluent was monitored by measuring the absorbance at 280 nm. The eluted sample was separated into ten fractions, as shown in Fig. 6A.

Second step. Each fraction was separated by hydroxyapatite HPLC in the presence of SDS. In the case of fraction 1, a 1.8-ml aliquot (1/5 volume) of the fraction was added to 1.35 ml of 10% SDS, 0.27 ml of 2-mercaptoethanol and 1.98 ml of buffer A, followed by incubation at 37°C for 1 h. After centrifugation at 8500 *g* for 10 min, a 5-ml aliquot of the supernatant was applied to a hydroxyapatite column. After washing with buffer A for 5 min the sample was eluted under the standard conditions except that a linear gradient from 50 to 95% of buffer B was used.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

To 20-μl of each fraction was added concentrated dithiothreitol solution (final concentration 24 m*M*), followed by incubation at 37°C for 1 h. Prior to application of the sample to a gel, sulphydryl groups in the sample were blocked with iodoacetamide[6]. An aliquot of each fraction of the column eluent was analysed by SDS-PAGE according to the method of Laemmli[7]. Linear gradient acrylamide gels (4–15 or 6–15%) were used. After electrophoresis, the gels were stained with silver according to a modification of the method of Morrissey, as reported previously[8]. M, P, B, A and C denote the positions of molecular weight marker proteins: M = myosin (MW 205 000); P = phosphorylase *b* (MW 97 400); B = bovine serum albumin (MW 66 000); A = actin (MW 42 000); and C = carbonic anhydrase (MW 29 000).

RESULTS AND DISCUSSION

Preparative hydroxyapatite chromatography

When the usual amount of rat erythrocyte membrane protein–SDS complexes was applied to a ceramic hydroxyapatite column and eluted with buffers containing 1% SDS, good resolution was obtained, as can be seen in Fig. 1. Most proteins were bound on the column, and then not only low but also high-molecular-weight proteins such as the α- and β-chains of spectrin were eluted as sharp peaks with a phosphate buffer concentration gradient. These results show that this method is useful for separating and analysing membrane proteins which cannot be separated by ion-exchange or reversed-phase chromatography.

As a basis for the preparative separation of membrane proteins, the amount of membrane protein that can be applied to a column was examined using rat erythrocyte membranes. As can be seen in Fig. 2, with an increase in the amount of applied

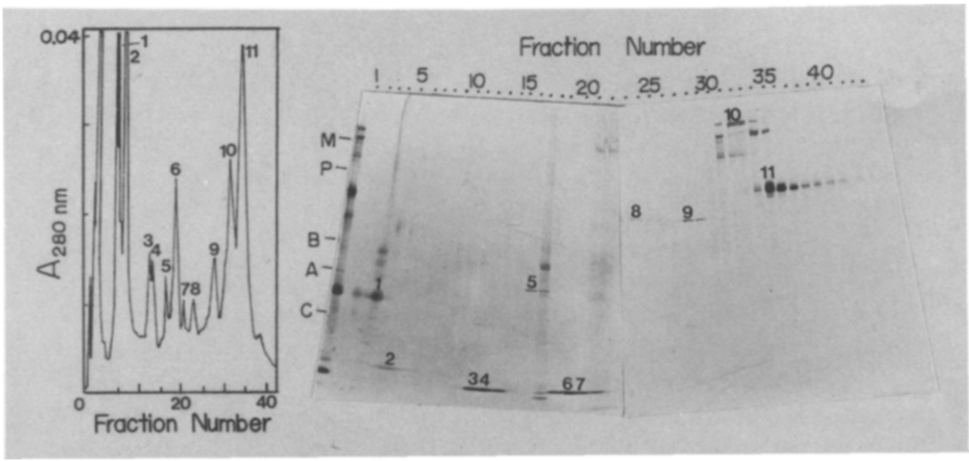

Fig. 1. Separation of erythrocyte membrane protein–SDS complexes by ceramic hydroxyapatite HPLC. Left: rat erythrocyte membrane protein–SDS complexes (0.48 mg of protein) were separated under the standard conditions. The eluate was collected in 1.8-ml (Nos. 1–4) or 0.9-ml fractions (Nos. 5–44). Right: the fractions were analysed by SDS-PAGE under the standard conditions. Proteins numbered in the gel are as follows: 1,2 = unknown; 3,4 = haemoglobin α_1- and α_2-chains; 5 = actin; 6,7 = haemoglobin β_1- and β_2-chains; 8 = band 4.1; 9 = band 4.2; 10 = spectrin α-chain; 11 = anion carrier protein.

protein, the separation of each membrane protein became poorer, the protein–SDS complexes were eluted faster and the tailing of peaks increased. When 24 mg of protein were applied, part of the sample protein could not be adsorbed on the column, being eluted in the flow-through fraction (data not shown). These results suggested that the amount of sample should be kept at less than 12 mg of protein.

Generally, a decrease in the flow-rate increases the resolution in the chromatography of macromolecules such as proteins. A protein–SDS complex has a Stoke's radius larger than that of the respective native globular protein. Therefore, we chose a lower flow-rate of 0.3 ml/min for the preparative separation of rat erythrocyte mem-

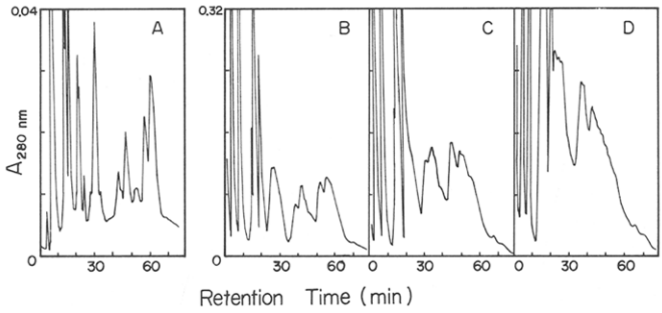

Fig. 2. Loadability of the hydroxyapatite column. Various amounts of rat erythrocyte membrane proteins were separated with a Hibar RT 100-8 hydroxyapatite-MP column under the standard conditions except that a 47–92% buffer B′ gradient was used. Amounts of rat erythrocyte membrane proteins: A, 0.48; B, 3.0; C, 6.0; D, 12 mg.

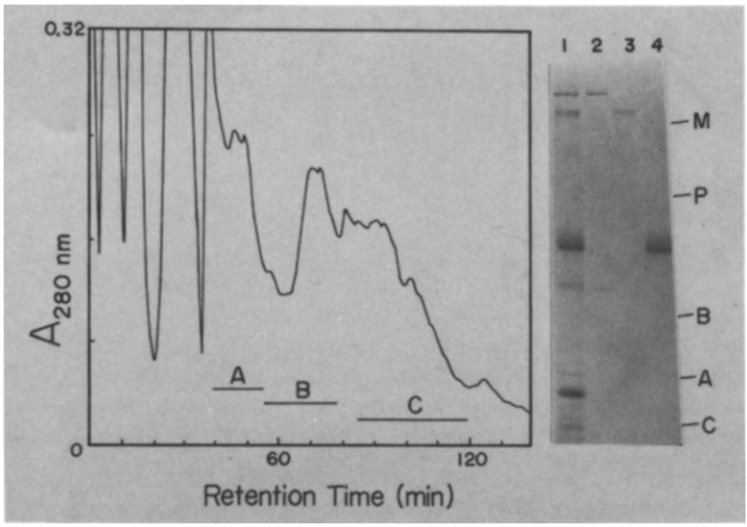

Fig. 3. Purification of spectrin α-chain, spectrin β-chain and anion carrier protein from erythrocyte membranes by hydroxyapatite HPLC. Left: rat erythrocyte membrane (12 mg of protein) was separated with a 138-min linear gradient and a flow-rate of 0.3 ml/min. Other conditions as in Fig. 2. The eluate was collected in 0.9-ml fractions. Right: SDS-PAGE of the isolated spectrin α-chain, spectrin β-chain and anion carrier protein fractions. Aliquots of the A, B and C fractions (left) which contained spectrin α-chain, spectrin β-chain and anion carrier protein, respectively, were subjected to SDS-PAGE and then the gel was stained with silver. Lanes: 1 = rat erythrocyte membrane protein; 2 = fraction A; 3 = fraction B; 4 = fraction C. Marker proteins as in Fig. 1.

brane proteins (Fig. 3). The eluate was analysed by SDS-PAGE and it was shown that all the peaks that appeared at 80–110 min were anion carrier protein. Therefore, fractions were collected as shown in Fig. 3. Fraction A, B and C contained spectrin α-chain, spectrin β-chain and anion carrier protein as the main components, the contents being 84, 71 and 95%, respectively. About 0.7, 0.6 and 0.8 mg of protein were obtained from these fractions through the one-step purification. This method could be applied to the purification of other membrane proteins with little changes in the gradient.

Microanalysis of membrane proteins

Under the standard conditions, samples were analysed using buffers containing dithiothreitol to prevent the oxidation of sulphydryl groups during the analysis. Therefore, the absorbance at 280 nm had to be used for protein detection because buffers containing dithiothreitol absorb light of wavelength shorter than 240 nm. However, the use of a shorter wavelength was necessary to increase the sensitivity. We therefore attempted to separate erythrocyte membrane proteins with buffers not containing dithiothreitol and with detection at 230 nm (Fig. 4). It was found that dithiothreitol in the elution buffer could be omitted without any change in the elution pattern (compare Fig. 4A and C). Pretreatment of a sample with iodoacetamide to block sulphydryl groups in the sample also had no effect on the pattern. The use of the absorbance at 230 nm made it possible to reduce the sample amount to 100 μg of

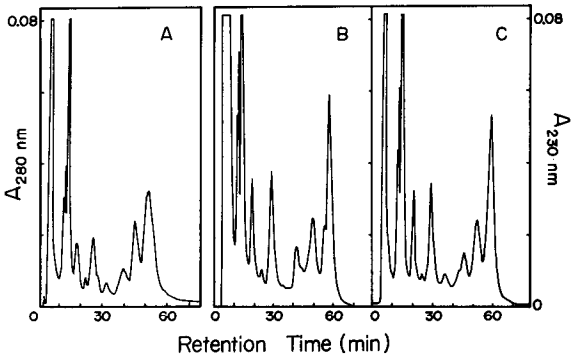

Fig. 4. Influence of omission of dithiothreitol from the elution buffer on the elution pattern. Rat erythrocyte membrane protein–SDS complexes were separated under the standard conditions except that buffers A and B were used instead of buffers A′ and B′, and chromatography was performed at room temperature. Buffer A not containing dithiothreitol but with 24.4 μM of sodium azide added was used in B and C. The amounts of samples were (A) 480, (B) 96 and (C) 96 μg. The sample in B was treated with iodoacetamide to block sulphydryl groups prior to analysis[6].

protein (Fig. 4). Correction of the baseline was also attempted because some lots of the second buffer showed a slightly higher absorbance than that of the first, and the baseline increased during analysis. Cytidine shows some absorbance in the ultraviolet region and does not adsorb to hydroxyapatite. Therefore, the baseline was corrected by the addition of a suitable amount of cytidine to the first buffer when necessary. The addition of an adequate amount of cytidine reduced the change in the baseline during analysis and as little as 24 μg of protein could be analysed constantly (Fig. 5). This sample amount is comparable to that necessary for SDS-PAGE analysis with Coomassie blue staining. This method was applied to the analysis of proteins of erythrocyte membrane, a myelin fraction and a rough microsomal membrane fraction, good

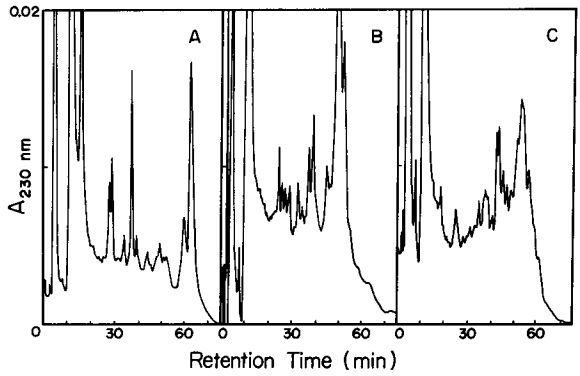

Fig. 5. Microanalysis of membrane proteins. Samples were separated by ceramic hydroxyapatite chromatography under the standard conditions except that a 50–95% buffer B gradient was used. Samples containing 24 μg of protein of (A) rat erythrocyte membrane, (B) rat brain myelin fraction and (C) rat liver rough microsomal membrane were analysed.

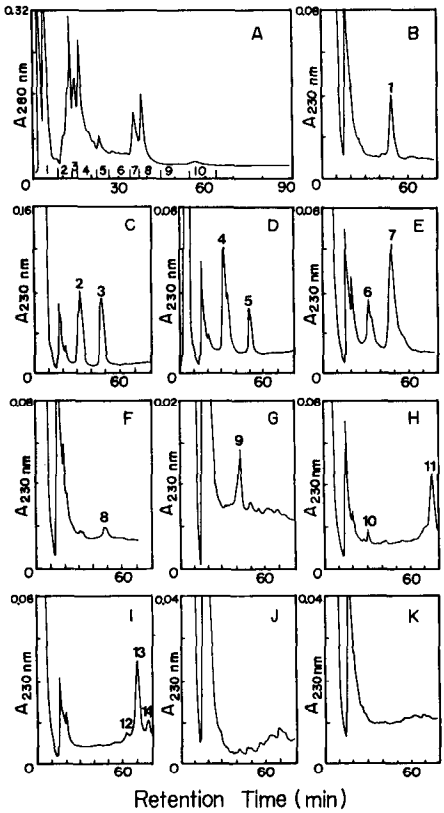

Fig. 6. Two-step purification of membrane proteins. (A) Anion-exchange HPLC of erythrocyte membrane proteins. Rat erythrocyte membrane proteins (4.5 mg) were solubilized with Zwittergent 14 and separated with a TSKgel DEAE-5PW column. The eluate was fractionated into ten fractions, as shown. (B–K) Hydroxyapatite HPLC of fractions 1–10, respectively. Fractions were treated with SDS and analysed by TSKgel HA-1000 column chromatography. Proteins separated as peaks 1–14 in B–K were as follows: 1, 3, 5 and 7 = haemoglobin β_1- and β_2-chains; 2, 4 and 6 = haemoglobin α_1- and α_2-chains; 8 = unknown; 9 = actin; 10 = 30 000 dalton protein; 11 = spectrin β-chain; 12 = unknown; 13 = spectrin α-chain; 14 = spectrin β-chain.

resolution being obtained in all instances (Fig. 5). Sodium azide could also be used instead of cytidine for correction of the baseline (see Fig. 4B and C).

Two-step analysis of erythrocyte membrane proteins

Although the above method showed very high resolution, it was not sufficient to separate all proteins in a complex protein mixture from each other. Therefore, a two-step separation method was examined. It is known that in the hydroxyapatite chromatography of protein–SDS complexes there is a positive correlation of the retention time with log (molecular mass) and Log Σ (hydrophobicity of amino acids), but not with the isoelectric point[3]. Therefore, we chose ion-exchange chromatography, the separation mode of which is different from that of the above chromatography, as the fractionation step prior to hydroxyapatite chromatography. Erythrocyte

membranes were solubilized with Zwittergent 14 and then fractionated into ten fractions by DEAE column chromatography (Fig. 6A). Then the fractions were analysed by hydroxyapatite chromatography.

As expected, the combination of DEAE and hydroxyapatite chromatography allowed a better separation of membrane proteins from each other than the one-step method. This two-step method will be useful for the analysis of more complex membrane protein mixtures. In this system, Zwittergent 14 was used to solubilize the erythrocyte membrane proteins. However, one of the intrinsic membrane proteins, anion carrier protein, could not be solubilized completely by the detergent. Hence improvements in the solubilization conditions are needed to make this method more suitable for whole membrane protein analysis. Investigations along these lines are in progress.

ACKNOWLEDGEMENT

We thank Kazuko Hasegawa for preparing this manuscript.

REFERENCES

1 G. W. Welling, R. van der Zee and S. Welling-Wester, *J. Chromatogr.*, 418 (1987) 223.
2 B. Moss and E. N. Rosenblum, *J. Biol. Chem.*, 247 (1972) 5194.
3 T. Horigome, T. Hiranuma and H. Sugano, *Eur. J. Biochem.*, 186 (1989) 63.
4 A. Gonenne and R. Ernst, *Anal. Biochem.*, 87 (1978) 28.
5 W. T. Norton and S. E. Poduslo, *J. Neurochem.*, 21 (1973) 749.
6 F. Hashimoto, T. Horigome, M. Kanbayashi, K. Yoshida and H. Sugano, *Anal. Biochem.*, 129 (1983) 192.
7 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
8 T. Horigome, T. S. Golding, V. E. Quarmby, D. B. Lubahn, Sr., K. McCarty and K. S. Korach *Endocrinology*, 121 (1987) 2099.