USE OF HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY TO DETERMINE THE EXTENT OF DETERGENT SOLUBILIZATION OF HUMAN ERYTHROCYTE GHOSTS

ROBERT S. MATSON* and STEVEN C. GOHEEN

Bio-Rad Laboratories, 1414 Harbour Way South, Richmond, CA 94804 (U.S.A.)

SUMMARY

We have studied the effects of various detergents and their concentration during human erythrocyte membrane solubilization. Detergents were selected on the basis of their low UV absorption at 280 nm, making them useful for high-performance liquid chromatography. High performance size-exclusion chromatography was then utilized to monitor the efficiency of solubilization.

Sodium dodecyl sulfate solubilized more of the erythrocyte membrane proteins than any of the other detergents studied. 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfate solubilized some, but Tween 20 and reduced Triton X-100 solubilized fewer of the human erythrocyte membrane proteins. In conclusion, high-performance size exclusion chromatography provides a rapid method for determining whether membrane proteins have been effectively solubilized.

INTRODUCTION

Detergents have frequently been used in the purification of membrane proteins, but the selection of a detergent for this purpose has been largely empirical. This may be because the molecular architecture of individual membranes is so varied and the complexity of lipid–lipid, lipid–protein, and protein–protein interactions are so great that it is unlikely that a single detergent could efficiently solubilize all proteins while preventing them from being severely denatured. For example, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfate (CHAPS) is noted to be less efficient at solubilizing the erythrocyte membrane than octyl glucoside. Sodium dodecyl sulfate (SDS) appears to solubilize most membrane proteins effectively, but frequently causes denaturation. Thus, no universally acceptable detergent for membrane solubilization exists, and many detergents should be screened to find the one that is most suitable. This can be a tedious and time consuming process. One should examine different types of detergents (ionic, zwitterionic or non-ionic) as well as their optimal concentration for solubilizing the membrane. Selecting the best buffer conditions adds to the complexity of the search.

Membrane proteins have only recently been separated by high-performance size-exclusion chromatography (HPSEC)[1-12]. HPSEC of membrane proteins was used

earlier to separate free detergents from solubilized proteins or partially solubilized aggregates[2]. In similar studies, carried out concurrently on apolipoproteins, either urea[11] or SDS[12] were used for solubilization in an attempt to optimize elution patterns for high-performance liquid chromatography (HPLC). Pabst et al.[5] studied the influence of various detergent concentrations on the solubilization of bacteriorhodopsin by Triton X-100. They discovered that large amounts of Triton were necessary to prevent the formation of dimers and trimers of the membrane protein. Five different detergents were tested by Lüdi and Hasselbach[1] for their ability to solubilize adenosine triphosphatase (ATPase). Their investigations included the use of SDS, dodecyl octaethylene glycol monoether, sodium deoxycholate, Triton X-100 and myristoyl glycerophosphocholine. The solubility of ATPase was measured by its appearance as a sharp peak within the included volume of an HPSEC column[1]. ATPase was found to be soluble by this criterion only above the critical micelle concentration (CMC) for each detergent. However, the results also indicated that multimers of ATPase were formed under each solubilization condition. The presence of multimers may have been minimized if a large excess of detergent had been used, but none of their solubilization conditions included a detergent concentration as much above the CMC as in the study by Pabst et al.[5]

Preparative HPSEC of membrane proteins was first reported by Muccio and DeLucas[3], who purified bacteriorhodopsin using octyl glucoside for solubilization. They obtained the best resolution at the lowest of a range of flow-rates[3]. Additional solubilization strategies that employ HPSEC have been developed which do not involve detergents. For example, Murray et al.[4] used HPSEC for large scale purifications of hydrophobic proteins. In this case, ethylene glycol was used for solubilization instead of detergents. Acetonitrile has also been used for analytical HPSEC of hydrophobic proteins[8-10], even though the proteins studied may or may not have been membrane-bound.

Recently, a general HPLC procedure for the purification and analysis of all plasma membrane proteins was suggested by Josic et al.[7]. Their analytical methods included not only HPSEC, but also reversed-phase HPLC (RP-HPLC) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Unfortunately, they emphasized the overall procedure, but not the solubilization technique.

In the studies on HPSEC of membrane proteins that have been discussed, detergents were the most common solubilizing agents[1-3,5-7,12]. Membrane solubilization conditions are critical for optimizing the yield of active proteins from membranes[13]. Therefore, an optimal solvating method should be determined before attempting HPLC separations. So far, a general technique for selecting the best solubilization conditions for HPLC has not been clarified.

In our present paper, human erythrocyte ghosts were selected as a model system to determine feasibility of using HPSEC to monitor the solubilization of membrane proteins. In this system, several different detergents were selected to test for their ability to solubilize the erythrocyte membrane proteins. For each detergent, solubilization conditions were altered only by varying the detergent concentration and the efficiency of solubilization was monitored by HPSEC.

MATERIALS AND METHODS

Tween-20, SDS and CHAPS were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Reduced Triton X-100 was from Aldrich (Milwaukee, WI, U.S.A.). All reagents were of the highest purity available. Buffers were passed through 0.45 μm filters prior to use. Human whole blood was obtained from healthy volunteers and processed immediately to obtain erythrocyte membrane ghosts by hypotonic lysis according to the method of Fairbanks et al.[14]. Aliquots of ghosts were stored until needed at $-20°$C after resuspension in 0.1 M sodium phosphate buffer (pH 6.5), containing 0.1 M sodium chloride and 0.02% sodium azide.

Membrane solubilization was accomplished at room temperature (23°C) by employing detergent (SDS, reduced Triton X-100, Tween-20 or CHAPS) at 0.1–50 × CMC in 0.1 M sodium chloride and 0.1 M sodium phosphate buffer (pH 6.5). CMC values for these detergents have been reported[17–19]. Usually, 100 μl of ghost suspension containing ca. 15 μg protein was mixed with an equal volume of a concentrated detergent solution to obtain the desired detergent concentration for solubilization. The sample was next mixed in a vortex mixer for 1 min at the highest speed.

A 20-μl aliquot was then injected into a Bio-Rad Quick Check Analyzer System, containing a Bio-Sil TSK 250 (300 × 7.5 mm) size-exclusion column, equilibrated under isocratic conditions with each detergent at 0.5 × CMC to 0.8 × CMC in sodium phosphate saline buffer (pH 6.5) at a flow-rate of 0.1 or 0.2 ml/min at 23°C. This system had a void volume of 5.0 ml. Monitoring was conducted at 280 nm.

In some instances, 200 μl of sample was injected and individual fractions were collected for analysis of apparent molecular weight distributions by SDS-PAGE according to the Laemmli method[15] using a mini Protean II electrophoresis system (Bio-Rad). Protein silver staining was performed using the Bio-Rad silver stain kit. Protein concentrations were determined by means of the Bio-Rad Protein Assay according to the manufacturer's instructions.

RESULTS AND DISCUSSION

In our study, we employed HPSEC for a rapid assessment of the degree of solubilization of membrane proteins by four detergents. For each of these, a series of concentrations were used for solubilization. For the purpose of this study, we define a soluble protein as one that is eluted after the void volume of a Bio-Sil TSK 250 column in the presence of a detergent (or other solubilizing media). More commonly, a soluble membrane protein is defined as one that remains in the supernatant after ultracentrifugation in the presence of detergent. The size-exclusion definition is stricter since some protein-detergent-micelle aggregates may be soluble according to the ultracentrifugation criterion, while being chromatographically inseparable. If solubilization conditions are being determined for subsequent chromatography, then the gel filtration definition is probably more suitable. It is also well known that many proteins may unfold extensively in the presence of some detergents, and others may be too large to be eluted within the exclusion limits of a specific HPSEC column. For these proteins, columns with a different range of exclusion limits can be selected.

Therefore, extensively denaturated proteins will appear mostly in the void volume along with large aggregates. Thus, large protein aggregates found in the void volume (V_0) cannot be separated by this technique. Those would be considered insoluble, while micelles containing more than one protein that appear within the included volume of the column (V_i) represent solubilized proteins. The efficiency of solubilization is determined by the ability of the detergent to form micelles containing membrane proteins without significant denaturation, such that they can be separated on the basis of molecular size. The resulting fractions could then be further analyzed by ancillary methods such as SDS-PAGE.

Detergents were selected on the basis of their potential suitability for HPLC applications and not necessarily on the basis of their demonstrated ability to solubilize red cell ghosts. The results indicate that two of the four detergents solubilized some ghost proteins (Figs. 1-4). Those detergents which failed to solubilize proteins clearly, appeared to allow some of the membrane to be eluted from the HPSEC column at the void volume (Figs. 1 and 2). However, as expected, there were considerable differences in the efficiency of the four detergents.

Tween 20 has a low absorbance at 280 nm, and it had some effect on the ability
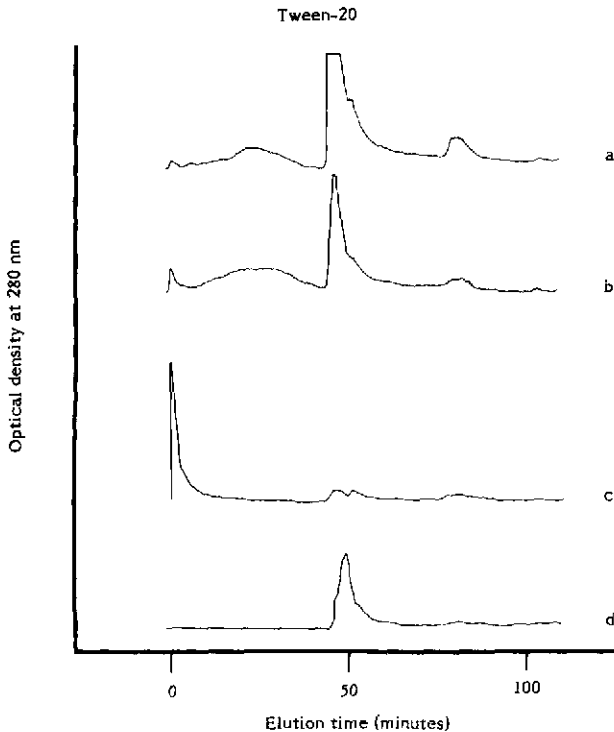

Fig. 1. HPSEC of Tween 20 solubilized membrane proteins: red blood cell ghost membranes were treated with Tween 20 as described under Methods at (a) 50 ×, (b) 5 ×, (c) 1 ×, and (d) 0.1 × the CMC; where 0.81 mM corresponds to 1 × the CMC[17]. In each case, 20 μl was injected into a Bio-Sil TSK 250 size-exclusion column previously equilibrated in elution buffer consisting of 0.1 M sodium phosphate buffer, (pH 6.5) containing 0.1 M sodium chloride and Tween-20 at 0.5 × the CMC. The flow-rate was at 0.1 ml/min, detection at 280 nm.

of red blood cell (RBC) ghost proteins to penetrate the HPSEC column (Fig. 1). When 50 × the CMC of Tween 20 (Fig. 1a) was used to solubilize the RBC ghosts, the maximum amount of protein was eluted from the column, as suggested by the area under the 280 nm absorption peak. However, most if not all of it was insoluble and eluted at the void volume of the Bio-Sil TSK 250 column. When 5 × the CMC of Tween 20 was used in membrane solubilization, less protein was eluted from the column, indicating that the proteins were totally insoluble, or they formed aggregates which were so large that they could not be eluted, or the amount of Tween was too small (Fig. 1b). More Tween 20 for solubilization (up to 1000 × the CMC, data not shown) did not enhance the amount of protein that was solubilized, making the last explanation unlikely. It also appears that at the CMC (Fig. 1c), micelles containing protein were less able to penetrate the gel column than at 0.1 × the CMC (Fig. 1d). These findings support the contention that Tween 20 is a poor detergent, at least for human RBC ghosts at pH 6.5 because the protein–detergent complexes that formed were insoluble.

A more popular non-ionic surfactant is Triton X-100. This detergent has been used extensively for membrane solubilization, although it absorbs ultraviolet light so strongly that it is a poor choice if UV absorbance is used for detection. Consequently, reduced Triton X-100 has been developed, which has a very low absorbance at 280 nm but otherwise properties similar to the unreduced form[16].

Fig. 2 shows the elution profiles obtained for RBC ghosts that had been treated with (a) 10, (b) 5, (c) 1 and (d) 0.1 × the CMC or reduced Trition X-100. All of these profiles look similar, but when compared with the results in Fig. 1, it appears that less protein was eluted from the HPSEC column with Triton X-100 than with Tween-20.

Relative to the above studies using the non-ionic surfactants, a more successful detergent for solubilizing RBC ghosts appears to be CHAPS (Fig. 3). This gentle zwitterionic detergent has been used successfully for the separation of a large number of membrane proteins, including microsomal cytochrome P-450[13]. For the erythro-
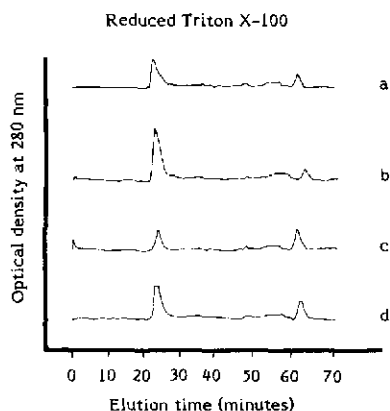

Fig. 2. HPSEC of reduced Triton X-100 solubilized membrane proteins: red blood cell ghost membranes were solubilized in reduced Triton X-100 at (a) 10 ×, (b) 5 ×, (c) 1×, and (d) 0.1 × the CMC, where 0.25 mM Triton corresponds to 1 × the CMC[17]. Chromatographic conditions were as described in Fig. 1 with the exception that the phosphate buffer contained 0.5 × the CMC reduced Triton X-100 and the flow-rate was 0.2 ml/min.
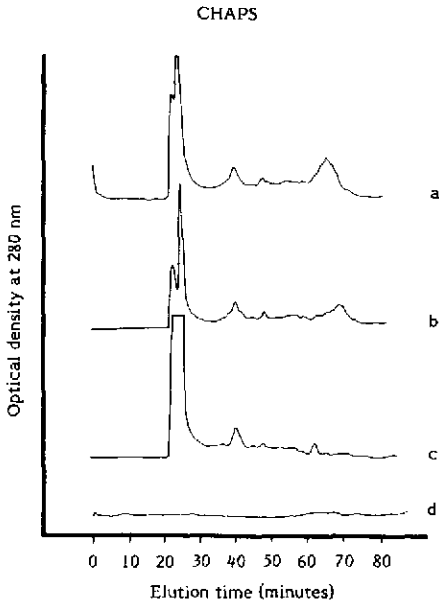
CHAPS

Fig. 3. HPSEC of CHAPS-solubilized membrane proteins: red blood cell ghost membranes were solubilized in CHAPS at (a) 10 ×, (b) 5 ×, (c) 1 ×, and (d) 0.1 × the CMC, where 1.4 mM CHAPS corresponds to 1 × the CMC[18]. Chromatographic conditions were as described in Fig. 2, except the phosphate buffer contained 0.5 × the CMC of CHAPS.

cyte membrane, the use of excess CHAPS above the CMC for solubilizing most of the membrane proteins was unnecessary. However, at 0.1 × the CMC of CHAPS, apparently none of the proteins in the human erythrocyte became soluble.

One of the most efficient detergents for solubilizing erythrocyte membrane proteins was SDS. Disruption of RBC ghosts was accomplished by using this detergent at (a) 10, (b) 5, (c) 1 and (d) 0.1 × the CMC (Fig. 4). Proteins were most completely solubilized above 5 × the CMC of SDS. Higher concentrations gave similar results (Fig. 4a), but when solubilization was attempted at and below the CMC of SDS, most proteins apparently eluted as aggregates (Fig. 4c and d).

From these initial studies, it was clear that SDS (Fig. 4) and CHAPS (Fig. 3) gave the best separations of RBC ghost proteins. To verify that gel chromatography gave results corresponding to normal molecular weight distributions, semi-preparative separations were attempted on the Bio-Sil TSK 250 analytical column, and fractions were collected for electrophoretic studies. Fig. 5 shows the separation of 200 μl of RBC ghosts that had been solubilized in 10 × the CMC of SDS. Electrophoretic analysis of the collected fractions verified that the protein–detergent complexes were being separated according to molecular weight. For example, Fig. 6 shows that the first fraction contained high-molecular-weight complexes running near the void volume of the Bio-Sil TSK 250 column ($\geqslant$300 000 daltons) which corresponded to protein bands greater than 200 000 daltons by SDS-PAGE (Table I). These were identified as spectrin bands 1 and 2 (at molecular weights of 260 000 and 225 000 daltons, respectively) according to the assignments of Dzandu et al.[20]. In the third fraction, SDS–HPSEC molecular weight complexes in the 50 000–100 000 dalton
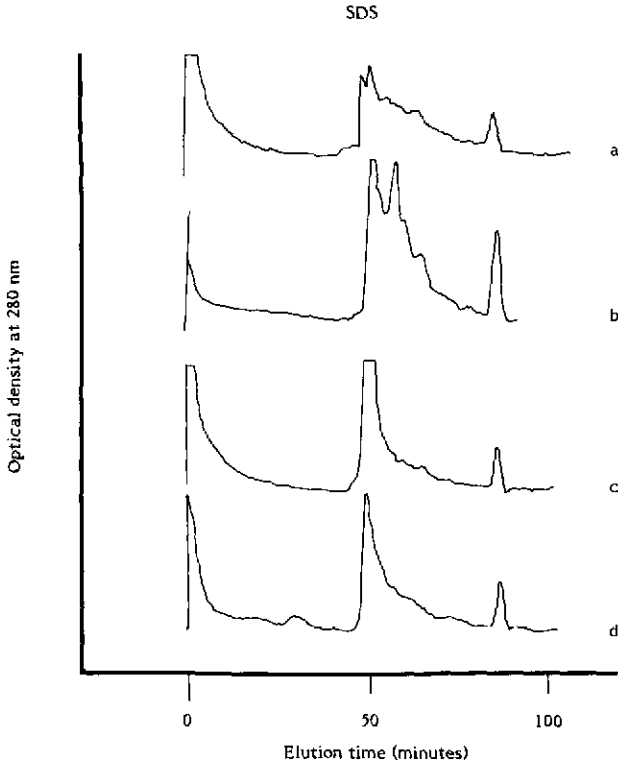
Fig. 4. HPSEC of SDS solubilized membrane proteins: red blood cell ghost membranes were solubilized in SDS at (a) 10 ×, (b) 5 ×, (c) 1 ×, and (d) 0.1 × the CMC, where 0.92 mM SDS corresponds to 1 × the CMC[19]. Chromatographic conditions were as described in Fig. 1, except that the phosphate buffer contained SDS at 0.8 × the CMC.

range were observed, while SDS-PAGE revealed several protein bands in the 27 000–52 000 dalton range that may represent bands 5, 6 and 7[20].

With few exceptions, membrane proteins were separated according to their molecular weight. There was, however, a significant discrepancy between the assigned molecular weights of the membrane proteins by HPSEC and the molecular weights of the same proteins as determined by SDS-PAGE (Table I). In nearly all cases, the molecular weight, as measured by HPSEC, appeared to be twice that from SDS-PAGE analysis. The major exception to this was the appearance of band 4.5 in fractions 4, 5 and 6. The molecular weight of this band appeared to be much lower in HPSEC (< 18 000) than in SDS-PAGE (62 000).

The discrepancy in molecular weights between SDS-PAGE and HPSEC analysis of the membrane proteins cannot be entirely explained by the results of this study. One possible explanation is that the various membrane proteins exist as monomers under SDS-PAGE and dimers (or oligomers) under HPSEC conditions. Another is that the shape of the membrane proteins is much larger than that of the soluble proteins under the HPSEC conditions used here. This possibility is less likely than the first because of the low probability that so many proteins would behave as if they
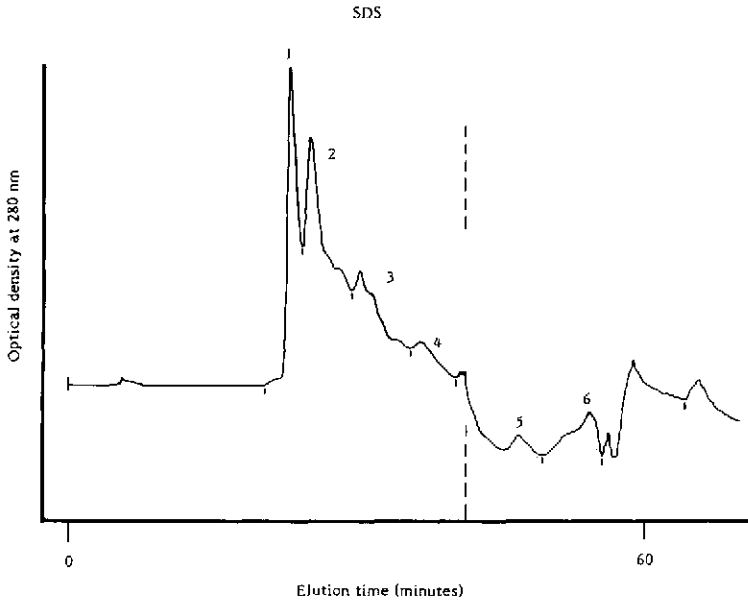
SDS

Fig. 5. Fractionation of SDS-solubilized membrane proteins: In this experiment, SDS at 10 × the CMC was used to solubilize the ghost membrane proteins. Then 200 μl was injected (26 μg protein) into the HPSEC column under the conditions described in Fig. 4. Peak fractions (1–6) were collected as indicated and subsequently analyzed by SDS-PAGE as described under Methods. Finally, 200 μl of a protein molecular weight standard mixture diluted in elution buffer, was injected under the same conditions (flow-rate, 0.2 ml/min) and the relative retention values were used to calibrate the size-exclusion properties of the column with 0.8 × the CMC of SDS detergent. For the chromatogram shown, the sensitivity was initially at 0.32 a.u.f.s. After fraction 4, the scale was set at 0.02 a.u.f.s., as indicated by the dashed line.

were essentially twice their molecular weight (Table I). A third explanation is that some specific proteins form complexes and aggregate together to form mixed micelles which elute at twice the molecular weight of the monomer. This is also unlikely since the separation in HPSEC is clearly by size (Fig. 6) and not of mixed molecular weight aggregates. The apparent ability of erythrocyte membrane proteins to form dimers in the presence of SDS is well known[21]. However, it seems odd that the soluble proteins that were used to calibrate the Bio-Sil TSK-250 column did not also form dimers under the same solubilization and elution conditions. This apparent discrepancy may be explained by the role of the large hydrophobic region in membrane proteins in creating the dimers. Soluble proteins may be less likely to form dimers in the presence of SDS due to their hydrophilic behavior.

It is also possible that the protein–detergent micelles formed during solubilization are unstable under HPSEC elution conditions in which the detergent concentration falls below its CMC. In this case, the proteins may reassociate into dimers or oligomers. For example, band 3 is a stable homodimer in detergent extracts as well as ghosts but may associate to tetramers or higher oligomers in the membrane or in solution. On the other hand, glycophorin A is stable as a dimer, even in the presence of SDS[21]. The appearance of band 4.5 in later fractions of the HPSEC chromatograms (Table I) may be due to non-specific binding of this protein to the gel. The
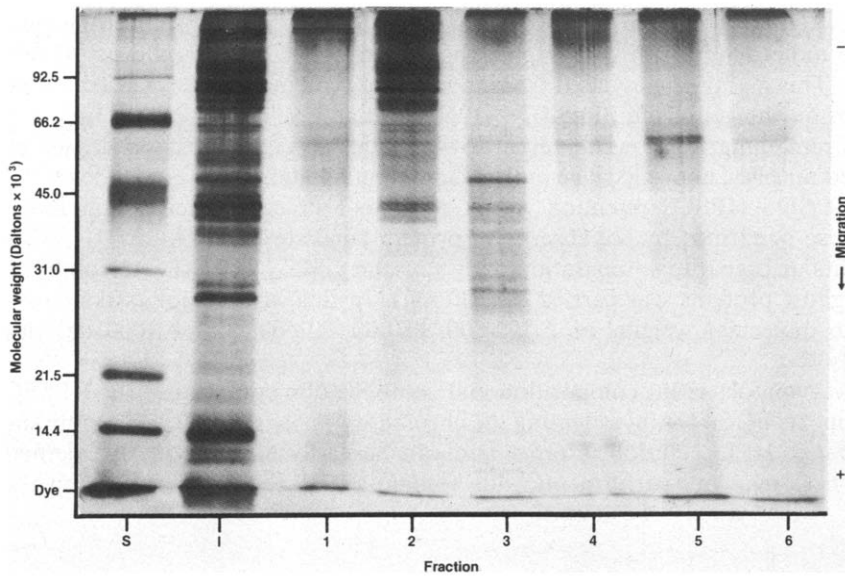
Fig. 6. SDS-PAGE of silver-stained human erythrocyte ghost proteins fractionated by SDS–HPSEC: a 10% Laemmli SDS-gel[15] was run at a constant 200 V for 45 min. Samples (20 μl) were applied in each lane. The gel was 0.75 mm in thickness. Lanes: S = molecular weight standards 14 400–92 500 daltons, I = solubilized ghosts in SDS at 10 × the CMC used for the HPLC injection described in Fig. 5, 1–6 represent aliquots of the corresponding fractions collected from the Bio-Sil TSK-250 separation shown in Fig. 5. For example, lane 1 represents fraction 1 in Fig. 5.

TABLE I

PROTEIN ASSIGNMENTS AND MOLECULAR WEIGHTS OF THE HPSEC FRACTIONS AFTER SDS SOLUBILIZATION

Fraction numbers correspond to those collected in Fig. 5. Protein assignments for erythrocyte ghost proteins are for the SDS-PAGE method of Laemmli[15] for silver-stained proteins according to Dzandu et al.[20]. Hb = hemoglobin monomer. Molecular weight assignments for the HPSEC fractions were made according to the elution times of the Bio-Rad Gel Filtration Standard that was solubilized and eluted in the presence of SDS. This was done in the same manner as the erythrocyte sample shown in Fig. 5.

Fraction number	Protein assignment number	SDS–HPSEC Mol. wt. (kdalton)	SDS-PAGE Mol. wt. (kdalton)
1	1,2	≥ 300	> 200
			110
2	2,3,4.1,4.2	220	80–95
	6	135	44
3	5,6,7	100	52
		79	44
		50	27
4	4.5, Hb	38	13
		18	
5	4.5	< 18	62
6	4.5	< 18	62

molecular weight of this band, as determined by SDS-PAGE (62 000 dalton), is similar to the molecular weight of the phosphorylated dimer of glycophorin (62 000 daltons)[20]. This highly glycosylated trans-membrane protein may be retarded by the diol groups on the gel of the Bio-Sil TSK-250 column.

The remaining fractions represent low-molecular-weight substances, such as lipid, mixed micelles, and salts. The molecular-weight assignments were made on the basis of the SDS–HPSEC retention values of protein molecular weight standards. This was also confirmed by the absence of protein bands in SDS-PAGE.

A similar preparative separation with 5 × the CMC of CHAPS to solubilize the RBC ghost proteins was carried out (Fig. 7). In this case, major peaks corresponded to molecular weights of ≥300 000, 88 000, 22 500, and <1000 daltons, respectively.

By varying detergent composition and solubilization conditions, our HPSEC method appears useful for investigating the chromatographic solubility of membrane proteins. Since HPLC elution is rapid and can be easily automated, the method described here may out-perform previous techniques. In the past, optimizing the
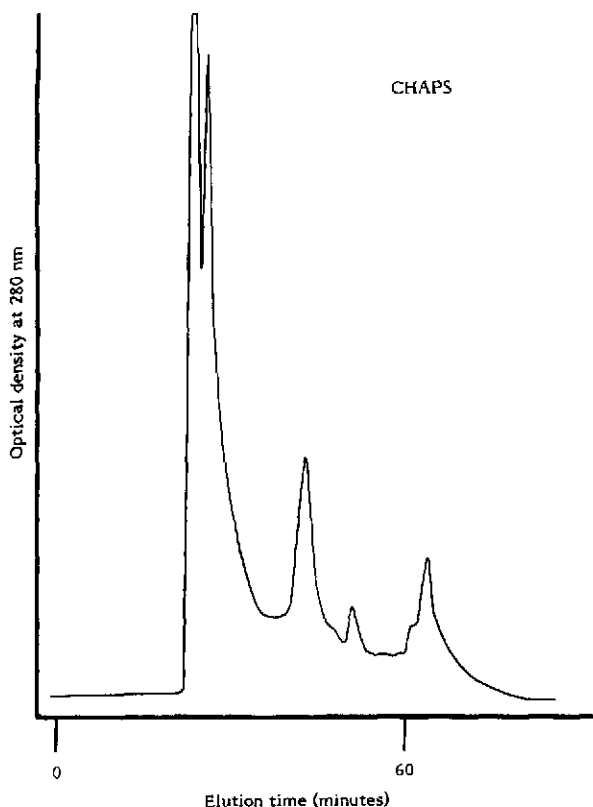

Fig. 7. Fractionation of CHAPS-solubilized membrane proteins: ghost membrane proteins were solubilized in CHAPS at 5 × the CMC and analyzed by HPSEC, essentially as described in Fig. 5, except that CHAPS was used in place of SDS detergent. The elution buffer contained 0.5 × the CMC in CHAPS. The sensitivity was at 0.08 a.u.f.s.

solubilization conditions was tedious, because complex assays were necessary for analyzing the supernatants of a large number of samples after ultracentrifugation. But even determining the solubility by ultracentrifugation gave limited information because it did not indicate whether the fraction would separate chromatographically as a single protein, or be eluted from either an open column or a HPLC column. HPLC allows accurate and reasonably complete screening of several solubilization and elution conditions within a short time.

For our study, linear flow-rates of 5.3 cm/h were used in a 300 × 7.5 mm column to optimize resolution. In earlier studies using a similar column, the same linear flow-rate (5 cm/h) gives adequate resolution[3]. An increase in flow-rate could have further reduced the analysis time. During our development of the solubilization test for membrane proteins, as described in this paper, we avoided filtering or centrifuging the samples before injecting them into the HPLC system. This procedure is not generally acceptable due to the fear of clogging the column. Although we did not experience problems such as increased backpressure, it would be wise to centrifuge samples before HPLC analysis, injecting only the supernatants.

ACKNOWLEDGEMENTS

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