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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF RED BLOOD CELL MEMBRANES

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SUMMARY

Three reversed-phase (Hi-Pore RP-318, Hi-Pore RP-304, and Bio-Gel TSK Phenyl-RP+) and one hydrophobic-interaction (Bio-Gel TSK Phenyl-5PW) columns were used in a Bio-Rad chromatography system to separate the membrane proteins of human erythrocytes. A linear gradient, starting with 0.05% trifluoroacetic acid and ending with 95% acetonitrile and 0.05% trifluoroacetic acid was used. The four columns demonstrated slightly different selectivities for the proteins in ghosts. These profiles were further altered when ghosts were solubilized with 0.1% sodium dodecyl sulfate. The columns with less hydrophobic packings and larger pore sizes appear to be best suited for reversed-phase analyses of erythrocyte membrane proteins. Detergent solubilization was unnecessary for good resolution of the protein components.

INTRODUCTION

Membrane proteins are more lipophilic than other proteins and should be strongly retained by reversed-phase columns due to the lipid-like surface of reversed-phase packings. As a consequence, special and sometimes harsh elution techniques have been developed for reversed-phase high-performance liquid chromatography (RP-HPLC) of membrane proteins[1–4].

We have studied four different columns for their ability to separate membrane proteins. Two of these columns had 330-Å pores with C_4 and C_{18} stationary phases (HiPore RP-304 and Hi-Pore RP-318, respectively). The two remaining columns had 1000-Å pores and phenyl groups on the stationary surface, with (Bio-Gel TSK Phenyl-5PW) or without (Bio-Gel TSK Phenyl RP+) a second hydrophilic stationary phase. The use of this variety of reversed-phase columns under identical elution conditions provided an opportunity for evaluating each column for the analysis of erythrocyte membrane proteins.

To avoid the strong hydrophobic interactions between reversed-phase packings and membrane proteins, some studies have been carried out by high-performance hydrophobic-interaction chromatography (HPHIC) with reversed-phase techniques[5,6]. These columns exhibit weaker hydrophobic interactions with solutes than reversed-phase columns[7]. The Bio-Gel TSK Phenyl-5PW is a HPHIC column, which is nearly identical to the Bio-Gel TSK Phenyl-RP+ reversed-phase column, except for the density of hydrophobic groups on the surface of the matrix.

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RP-HPLC of membrane proteins has also been performed after the membranes were solubilized with detergent[2,8,9]. This is not always preferable, since detergents are strongly bound to membrane components, often influencing their structure and activity. Heukeshoven and Dernick[1,10] and Power *et al.*[11] have demonstrated successful separations of water-insoluble proteins by RP-HPLC. They avoided detergent solubilization and instead used formic acid, acetonitrile or guanidine hydrochloride containing dithiothreitol for solubilization. In the present study, solubilized and unsolubilized samples were injected to determine whether solubilization was necessary or helpful for separating the membrane-bound proteins.

MATERIALS AND METHODS

All reagents were of the highest purity available. Bio-Rad's protein chromatography system was used, equipped with either the Hi-Pore RP-304, Hi-Pore RP-318, Bio-Gel TSK Phenyl-5PW or the Bio-Gel TSK Phenyl-RP+ column (Bio-Rad). Eluents were passed through 0.45-μm filters before use. Separations were performed at room temperature.

Human whole blood was obtained by venipuncture from healthy volunteers. Red cell ghosts were prepared according to the method of Fairbanks *et al.*[12]. Cold (5°C) red cell ghosts containing protein at a concentration of 0.15 mg/ml were diluted 1:1 with cold 5 m*M* sodium phosphate buffer (pH 7.4). Small aliquots (0.5 ml) of the ghost preparation were placed into capped plastic vials and kept frozen (−20°C) until used. Whole ghosts were either injected directly, without filtration or they were solubilized in 0.1% sodium dodecyl sulfate (SDS) using a vortex mixer at its highest setting and 25°C to mix the suspension for 1 min.

Four different types of reversed-phase columns were studied with two types (solubilized and unsolubilized) of membrane protein samples. The columns with pore sizes of either 330-Å (Hi-Pore RP-304 and Hi-Pore RP-318) or 1000-Å (Bio-Gel TSK PhenylRP+ and Bio-Gel TSK Phenyl-5PW) are all designed for the analysis of biopolymers. To avoid additional separation effects, guard columns were not used in any of the separations.

All separations were carried out with a 30-min linear gradient from 0 to 95% acetonitrile in the presence of 0.05% trifluoroacetic acid (TFA). The system was kept at 95% acetonitrile for an additional 10 min before recycling to the initial conditions. Each separation was repeated several times and protein standards were used to test for changes in column selectivity between analytical separations. No differences in elution profiles were observed throughout this study after repeated injections.

RESULTS AND DISCUSSION

In each of the figures, chromatography of 100 μl of unsolubilized erythrocyte ghosts (a), 100 μl of erythrocyte ghosts that had been solubilized by adding 0.1% SDS and mixing in a Vortex mixer for 1 min (b) and a blank chromatogram (c) are shown.

Fig. 1 shows the results obtained with the Hi-Pore RP-318 column. For this column it appears as if more protein was eluted with the solubilized than with the unsolubilized sample, although the total areas are nearly the same ($2.5 \cdot 10^6$ *versus*

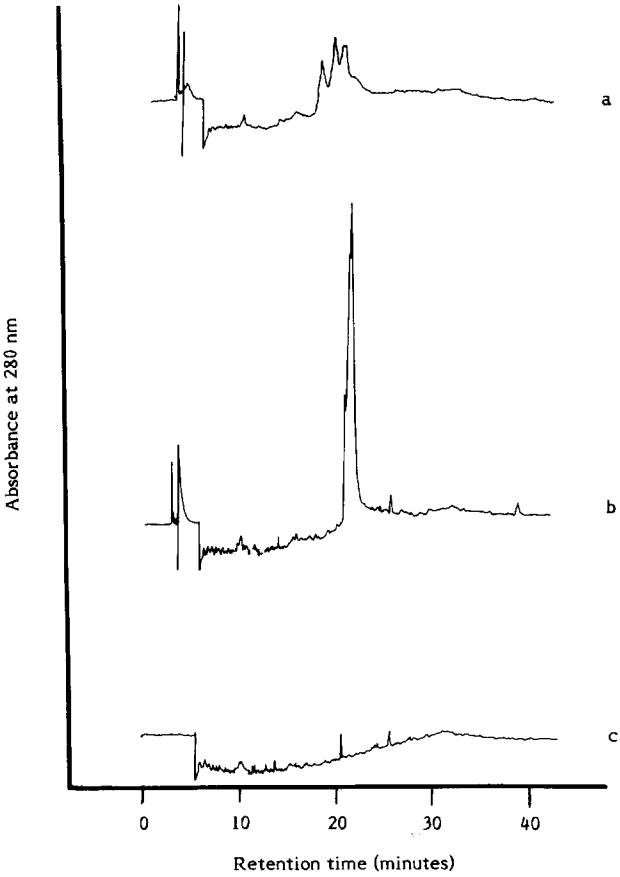

Fig. 1. RP-HPLC of unsolubilized (a) and solubilized (b) human erythrocyte ghosts separated on the Hi-Pore RP-318 column, and a blank chromatogram (c). A 30-min linear gradient from 0 to 95% acetonitrile in 0.05% trifluoroacetic acid was used. Solubilization was carried out with 0.1% sodium dodecyl sulfate and Vortex mixing for 1 min. Flow-rate, 1.0 ml/min; absorbance at 280 nm, 0.015 a.u.f.s. For each separation, 100 μl of unfiltered sample was injected at the start of the gradient.

$2.0 \cdot 10^6$ integrator counts, respectively). The unsolubilized sample appears to be better resolved than the solubilized sample.

A similar trend was observed in the Hi-Pore-RP-304 column (Fig. 2). The chromatogram of the unsolubilized sample appears to be almost identical with that in Fig. 1a. A slightly better separation of the solubilized sample (Fig. 2b) was achieved than when the more hydrophobic column was used (Fig. 1b). Differences in elution profiles between Figs. 1 and 2 may be attributed to the hydrocarbon chain length of the stationary phase, since other parameters of these two columns (including ligand density, column dimensions, support material and pore size) are nearly identical.

When a reversed-phase column with larger pore size (1000-Å) was used, such as the Bio-Gel TSK Phenyl-RP+ (Fig. 3), both the solubilized (Fig. 3b) and unsolubilized (Fig. 3a) samples gave similar profiles. The most dramatic difference between these two chromatographic patterns was near the end of the gradient, at *ca.*

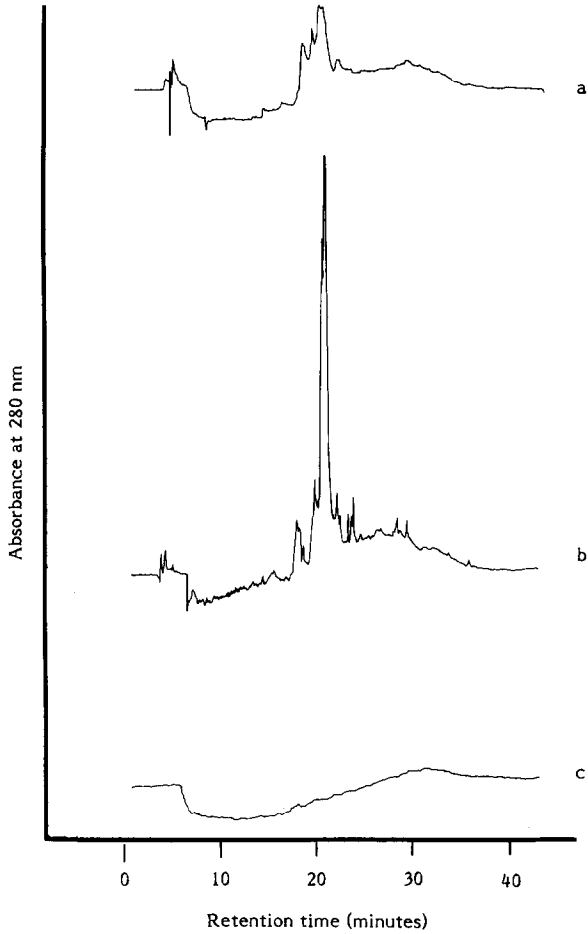

Fig. 2. RP-HPLC of unsolubilized (a) and solubilized (b) ghosts on the Hi-Pore RP-304 column, and a blank chromatogram (c). Conditions were as described in Fig. 1.

23 min. This region may correspond to unsolubilized phospholipids from the erythrocyte membrane (Fig. 3a) and from mixed micelles of lipid and detergent in the solubilized fraction (Fig. 3b). From the similarities of the remaining profiles in Fig. 3a and b, it appears that the presence of SDS had little effect on the total hydrophobic behavior of most of the membrane proteins in the human erythrocyte. This observation further implies that the differences observed between the profiles of Figs. 1–3 may largely be due to the differences in pore size (330 *vs.* 1000 Å) rather than differences in the selectivity of alkyl or phenyl groups.

The profiles of the unsolubilized fractions in Figs. 1a, 2a, and 3a are similar. The three major peaks in all these profiles are better resolved by the Bio-Gel TSK Phenyl-RP+ than by either of the Hi-Pore columns. This difference may be due to the differences in either pore size or length of the columns (75 mm, Bio-Gel; 250 mm, Hi-Pore). The longer columns may produce inferior resolution due to diffusion effects.

The columns that were used to create the profiles in Figs. 1–3 were reversed-phase columns. Fig. 4 shows results from a hydrophobic-interaction column. In this case, the same acetonitrile gradient was used as that shown in Figs. 1–3, but this column (Bio-Gel TSK Phenyl-5PW) is much less hydrophobic than comparable reversed-phase columns[7]. In these separations, both the solubilized (Fig. 4b) and unsolubilized (Fig. 4a) profiles look similar, but resolution was severely diminished when SDS was used. This is probably related to the two-phase nature (hydrophobic and hydrophilic) of the Bio-Gel TSK Phenyl-5PW support. The bifunctional material may give rise to a large variety of interactions between the sample and the stationary phase. This would be further complicated by the potentially large number of protein detergent structures that could form when the protein is solubilized by the detergent.

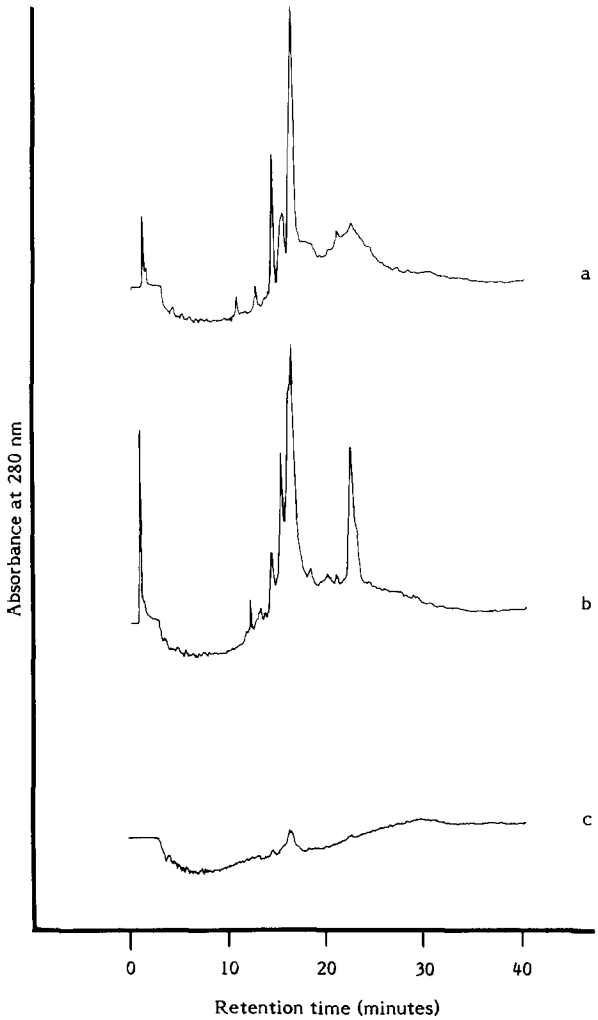

Fig. 3. RP-HPLC of unsolubilized (a) and solubilized (b) ghosts on the Bio-Rad TSK Phenyl-RP+ column, and a blank chromatogram (c). Conditions were described in Fig. 1.

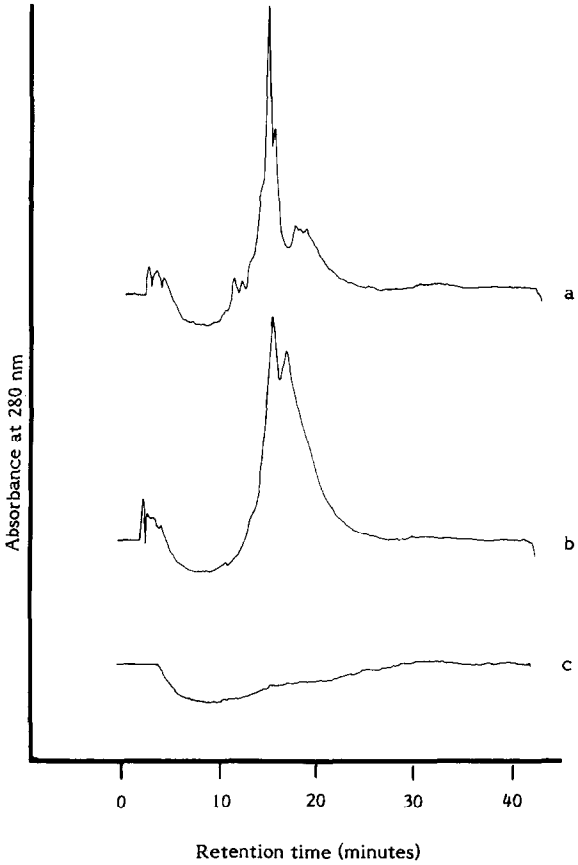

Fig. 4. RP-HPLC of unsolubilized (a) and solubilized (b) ghosts on the Bio-Gel TSK Phenyl-5PW column. A blank is shown in (c). Conditions were described in Fig. 1.

Membrane proteins are so hydrophobic that many of them could conceivably be irreversibly bound to a reversed-phase column. The recovery of membrane-bound viral proteins from this column was 60–80% when identical elution conditions were used[6]. However, protein recoveries were not measured in the present study. Such irreversible binding could influence the elution pattern of subsequent separations. To test the reproducibility of separating whole (unsolubilized) membranes on reversed-phase columns, two columns were selected, and three or four consecutive injections were made. The results are shown in Figs. 5 and 6. Both, the Hi-Pore RP-318 (Fig. 5) and the Bio-Gel TSK-Phenyl RP+ (Fig. 6) columns, gave reproducible separations after several consecutive injections. This indicates that any irreversibly bound material does not significantly interfere with subsequent analyses, but the possibility of irreversible retention of some proteins is not ruled out.

CONCLUSION

RP-HPLC appears to be a good choice for the separation or analysis of mem-

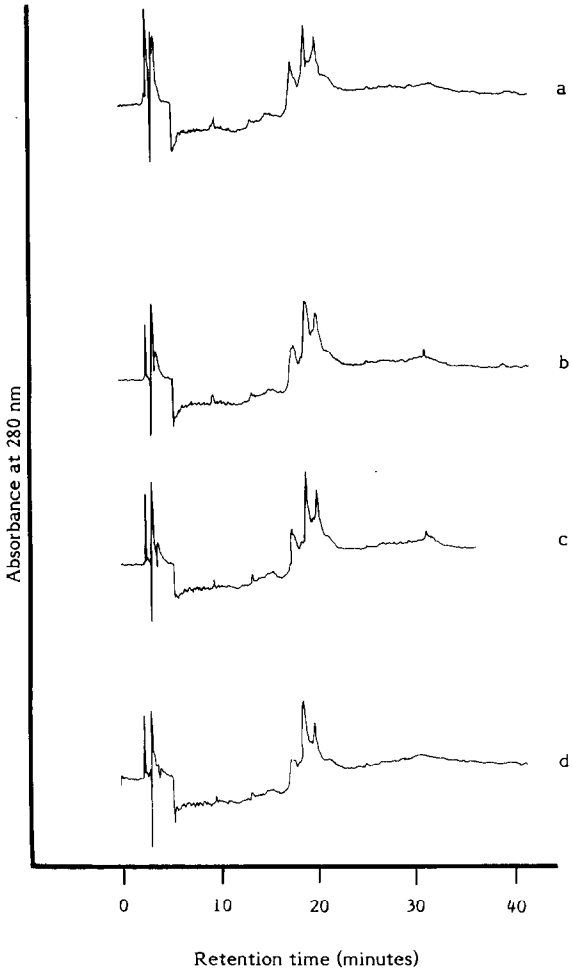

Fig. 5. Four consecutive injections of 100 μl unsolubilized ghosts on the Hi-Pore RP-318 column. Conditions were as described in Fig. 1a.

brane proteins. Of the four columns tested, the Bio-Gel TSK Phenyl-RP+ appeared to give the best results with little or no apparent advantage to previous membrane solubilization.

The selectivities of the four columns clearly differed, although it appears likely that the components which were eluted from each column were similar. Based on the total peak areas, recoveries were nearly identical for all columns tested. The large pore size (1000 Å) of the Bio-Gel TSK Phenyl-RP+ may have been largely responsible for its success in separating both solubilized and unsolubilized membrane components. One explanation for this success is that large micellar aggregates should be more easily eluted from this column than from columns with smaller pore sizes.

All separations were reproducible. Major changes in selectivity were not observed, even after several analyses.

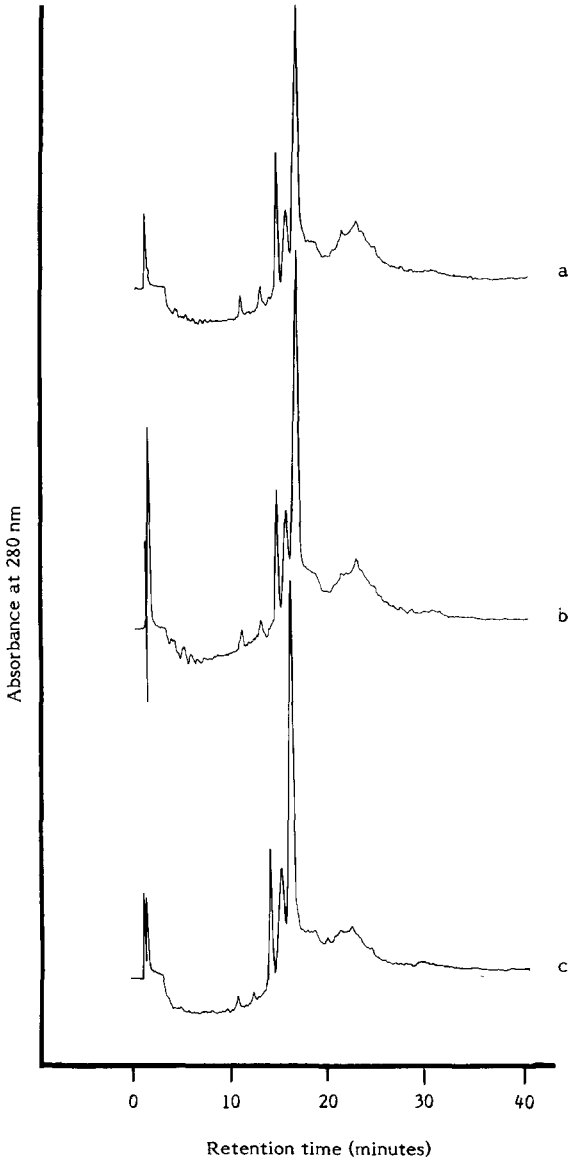

Fig. 6. Three consecutive injections of 100 μl unsolubilized ghosts on the Bio-Gel TSK Phenyl-RP+ column. Conditions were as described in Fig. 3a.

Since solubilization of the membrane was unnecessasry for the separations observed here, TFA and acetonitrile appeared to have adequately solubilized the components that were eluted from the columns. It would be interesting to determine whether membrane solubilization by other detergents or protein elution by other solvents would have any significant effect on the elution patterns observed here.

The chromatographic fractions were not analyzed either for content or dena-

turation. It is not known whether the eluted components were peripheral or integral membrane proteins, or both. The major peripheral proteins, including spectrin (α and β chains), actin and glyceraldehyde-3-phosphate-dehydrogenase, might correspond to the major peaks in this study. Further analyses of the ability of a variety of membrane proteins to be separated by RP-HPLC would be valuable.

RP-HPLC is a good analytical tool for a variety of compounds, including nucleic acids, amino acids, lipids, and proteins. RP-HPLC is not as suitable for analyzing membrane as for soluble proteins, due to their strongly hydrophobic nature. Nevertheless, the results indicate that RP-HPLC is a good analytical technique for studying the protein components of membranes.

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