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FRACTIONATION OF MEMBRANE PROTEINS BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY AND BY CHROMATOGRAPHY ON AGAROSE EQUILIBRATED WITH A WATER–ALCOHOL MIXTURE OF LOW OR HIGH pH

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CONTENTS

1. INTRODUCTION

In other papers at this Symposium it has been shown that hydrophobic interaction chromatography can be used for the purification of water-soluble proteins, and that low-molecular-weight substances can be separated from proteins on neutral amphiphilic (amphipathic) beds. It is perhaps not so well known that hydrophobic interaction chromatography can also be used for the purification of particles, for instance viruses and even whole cells[1]. In fact, in collaboration with Prof. Torkel Wadström and Dr. Cyril Smith, we are using this chromatographic technique to classify bacteria. However, the applications considered in this paper deal with proteins from biological membranes.

2. COMMENTS ON THE SOLUBILIZATION OF MEMBRANE PROTEINS

The proteins in biological membranes form complexes with each other and with the constituents of the lipid bilayer. The interactions between the components in these complexes involve electrostatic, hydrophobic, Van der Waals and hydrogen bonds, all of which must be broken before complete solubilization of the membrane can take place. Accordingly, membrane proteins are not soluble in conventional buffer systems. Any purification study must therefore start with a search for a suitable solubilizing medium.

If irreversible denaturation of the proteins can be tolerated, the problem is very simple in most instances, as one can then choose buffers containing sodium dodecyl sulphate (SDS) (which, however, has been reported to be reversibly denaturing for

some enzymes)[2,3]. The problem is considerably more difficult if one must also preserve biological activity, for instance an enzyme activity. In such instances one must choose bile salts, for instance sodium deoxycholate (DOC), or neutral detergents such as Tween 20 or Triton X-100. Of these, Triton X-100 has been most widely used. However, its high UV absorption is a great disadvantage in fractionation studies. Therefore, we often use the non-UV-absorbing detergent G 3707 (Atlas Chemicals, Everberg, Belgium), which seems to be as efficient as Triton X-100[4]. As none of these detergents is as efficient as SDS, it is often necessary to supplement the medium with certain additives in order to suppress certain interactions. In such studies it is important to bear in mind that the addition of salt decreases the electrostatic but increases the hydrophobic interaction and *vice versa*. It is also known that different salts affect the hydrophobic bond to different extents (experiments have shown that the salts can be arranged in a Hofmeister series as to their effect on this bond[5–7]). For these reasons, it is often only at a certain concentration that a given salt (buffer) effects solubilization.

From these considerations, it is evident that one should investigate the solubilizing effect of a series of different additives at different concentrations and at different pHs. In this connection, it should be stressed that a clear, non-opalescent solution of membrane proteins is not necessarily free of large complexes. If the protein of interest forms part of such a complex it can, of course, never be isolated in a pure form. It can be difficult to decide whether such complexes exist, as they often contain lipids and therefore do not easily sediment in the ultracentrifuge even at high g values.

3. GENERAL ASPECTS OF THE PURIFICATION OF MEMBRANE PROTEINS

Even if we have managed to get the protein into solution in a free form, many problems will arise in connection with purification studies, *e.g.*,

(1) Most proteins solubilized in SDS have the same surface charge density (ζ-potential). They therefore cannot be separated by carrier-free electrophoresis or electrophoresis in a non-sieving supporting medium such as agarose. The same applies to a buffer containing DOC although to a lesser extent.

(2) In the presence of neutral detergents, the proteins will be less charged and therefore often migrate relatively slowly in an electrical field.

(3) The micelles of most detergents have relatively high molecular weights (in the range 20,000–80,000) and it is therefore virtually impossible to remove them by dialysis. An exception is the bile salts, with micellar molecular weights around 3000. They also differ from some other detergents in that they have a relatively high critical micellar concentration (CMC) of about 5 mM in water, which also contributes to a comparatively rapid removal by dialysis[8,9]. The recently introduced detergent octyl glycoside is extremely easy to remove by dialysis as it has a very high CMC (about 25 mM).

(4) As the detergents are bound to the membrane proteins in large amounts (often 1.4 g of SDS per gram of protein[10]), the relative differences in molecular weights between complexes of protein and detergent are often too small to permit a fractionation by chromatographic molecular sieving. Also in this instance the bile salts are preferable to other detergents owing to the low molecular weights of their micelles (bile salts and neutral detergents seem to be bound to proteins in the form of micelles).

(5) It is well known that different polymers are incompatible. Solutions of, for instance, non-crosslinked dextran and polyacrylamide will precipitate proteins. It is therefore not surprising that many hydrophobic membrane proteins precipitate or form complexes with other proteins when applied to chromatographic columns of crosslinked dextran or polyacrylamide. Also, the aggregates that often are visible at the top of analytical polyacrylamide gel electrophoresis columns can have their origin in similar incompatibilities. In some instances it is therefore preferable to utilize methods that do not require the presence of supporting media.

From the above considerations, it is evident that conventional fractionation methods have an inherent weakness when used for the separation of membrane proteins. We have therefore felt the need for novel techniques to isolate these water-insoluble biopolymers. Hydrophobic interaction chromatography was developed with this in mind, but we soon realized that this method was also useful for common water-soluble proteins. When employed with membrane proteins one should remember that the detergents added to the medium to keep the membrane proteins in solution interact with the non-polar ligands of the bed material (in addition to the hydrophobic "patches" on the surface of the proteins), thereby decreasing the adsorption of the proteins to the bed. If excessively high detergent concentrations are used no adsorption will take place unless the hydrophobicity of the ligands is increased. When all ligands have reacted with the detergent molecules (in the free form or in the form of micelles), one has created a new adsorbent with properties different from those of the original bed.

4. APPLICATIONS

The general aspects and comments given above on the solubilization of membrane proteins with the aid of detergents should be borne in mind in the following discussion of applications. Relatively few experiments have been reported in which hydrophobic interaction chromatography has been used for the fractionation of proteins from biological membranes. Weiss and Bücher[11] employed a cation exchanger with lipophilic ligands to separate mitochondrial membrane proteins. Hjertén[12] purified a protein (called T_b) from the membrane of *Acholeplasma laidlawii* by chromatography on Sepharose to which phenylethylamine had been attached by the cyanogen bromide method[13]. As the nitrogen in the amine partly retains its positive charge after the coupling, the separations obtained on such a bed are based upon both hydrophobic and electrostatic interactions. The same is true for the bed material used by Simmonds and Yon[14] for the fractionation of proteins from erythrocyte membranes.

However, upon coupling alcohols to Sepharose by the glycidyl ether method, no charges are introduced[1]. These neutral amphiphilic columns, the advantages of which have recently been pointed out[15], have also been used for the purification of membrane proteins from *Acholeplasma laidlawii*[15,16]. Another example is given in Fig. 1, which shows the chromatographic behaviour of erythrocyte membrane proteins on dodecyl-Sepharose in the presence of sodium dodecyl sulphate. Desorption was achieved by first decreasing the buffer concentration and finally by increasing the SDS concentration. The protein distribution in the effluent was determined by absorption measurements at 280 nm and the phospholipid distribution was measured by phos-

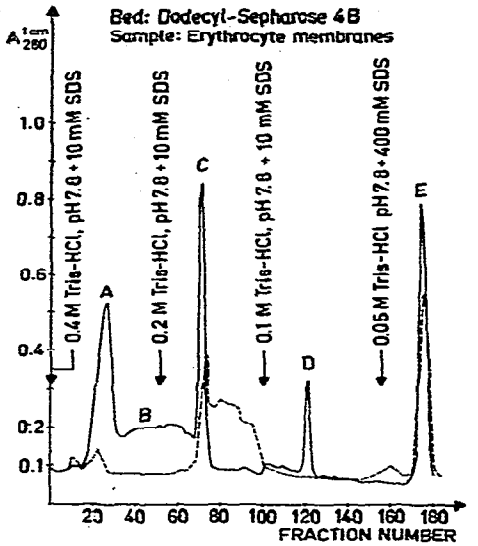

Fig. 1. Hydrophobic interaction chromatography of human erythrocyte membrane proteins on dodecyl-Sepharose. Sample amount: 10 ml (45 mg) in 0.4 *M* Tris–HCl, pH 7.8, +20 m*M* SDS. Column dimensions: 28 × 1.4 cm. Flow-rate: 13 ml/h. Fraction volume: 2.2 ml. Temperature: 21°. The broken curve corresponds to phosphate measurements.

phate determinations. The materials corresponding to the different peaks were analysed by polyacrylamide gel electrophoresis in the presence of SDS. As is evident from Fig. 2, this analysis revealed that peak A corresponded to highly purified glycoproteins (PAS-1 and PAS-2, the former being a dimer of the latter[4,17]). A similar result

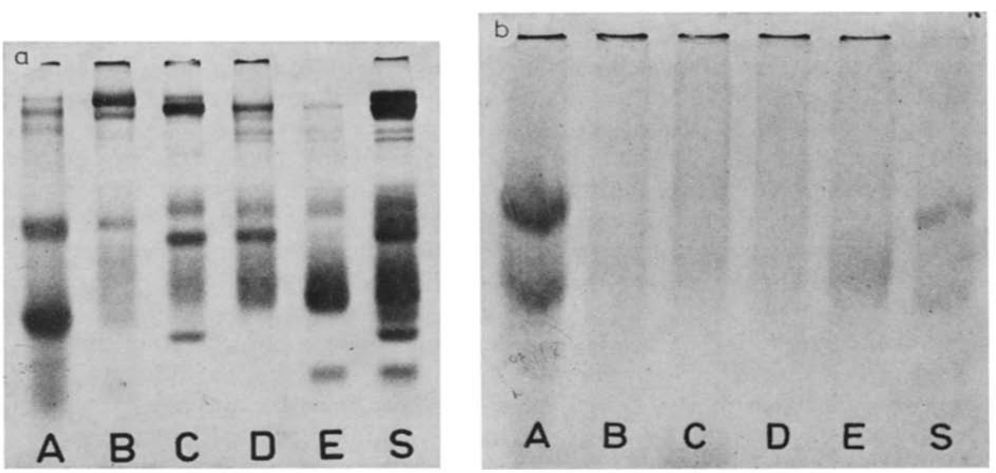

Fig. 2. Analysis of the chromatogram in Fig. 1 by SDS electrophoresis. Gel: polyacrylamide of the composition $T = 6\%$, $C = 3\%$ (these notations are defined in ref. 18). Buffer: 0.05 *M* glycine–NaOH, pH 9.8, +0.02 *M* SDS. (a) Staining for proteins with Coomassie Brilliant Blue; (b) PAS staining for glycoproteins. The sample denoted by S corresponds to the unfractionated starting material, which was somewhat degraded, probably by proteolysis.

has been reported by Simmonds and Yon[14]. Fig. 2 also indicates that the other fractions contained non-glycoproteins of different degrees of purity. In the paper on the behaviour of proteins of Sepharose at low pH, it was mentioned that membrane proteins are strongly adsorbed to this adsorbent equilibrated with butanol–acetic acid–water[19]. This observation prompted us to try to devise a new separation method. Even though it still is in the developmental stage, it has already been used for a practical fractionation problem, namely, the isolation of the glycoproteins from erythrocyte membranes (see Fig. 3). Fig. 4 shows that all of the glycoproteins are collected in fraction A, which contained no other proteins.

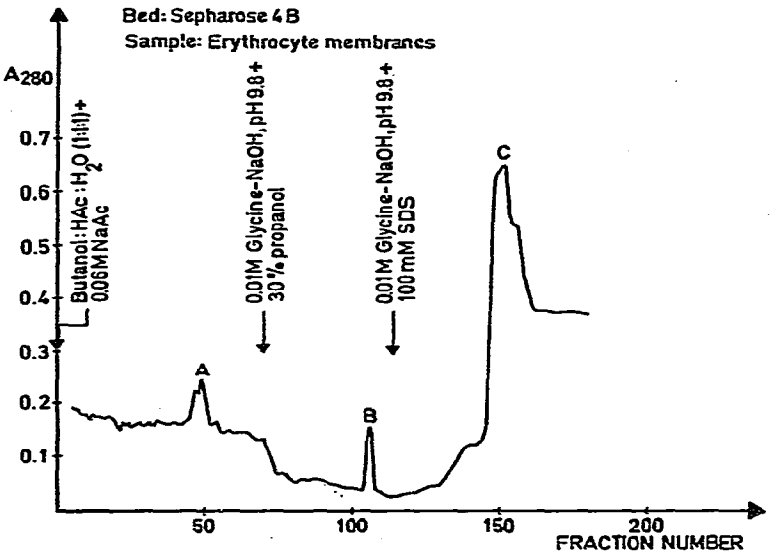

Fig. 3. Chromatography of human erythrocyte membrane proteins on Sepharose 4 B equilibrated with butanol–acetic acid–water (1:1:1). Sample amount: 2 ml (18 mg) solubilized in 4 ml of butanol–acetic acid–water (1:1:1) containing 0.06 *M* sodium acetate. Column dimensions: 70 × 2 cm. Temperature: 21°. Flow-rate: 6 ml/h. Fraction volume: 6 ml. The column was equilibrated with the same medium as was used to solubilize the sample.

5. DISCUSSION

The above experiments have shown that membrane proteins can be separated by hydrophobic interaction chromatography. The resolving power is, however, much lower than that of polyacrylamide gel electrophoresis, as shown by comparison of Figs. 1 and 2a. Similar differences in resolution between electrophoresis and chromatography are obtained when membrane proteins are chromatographed on gel beads of dextran, polyacrylamide and agarose. Can these differences originate partly from the tendency of (membrane) proteins to form complexes and to precipitate in the presence of polymers (see point 5 above)? If such is the case, there should be a greater tendency for aggregation in chromatography on gel beads than in electrophoresis in homogeneous, coherent gels. This might well be so, as proteins migrating in the pores of a polyacrylamide gel do not come into contact with each other to the same extent

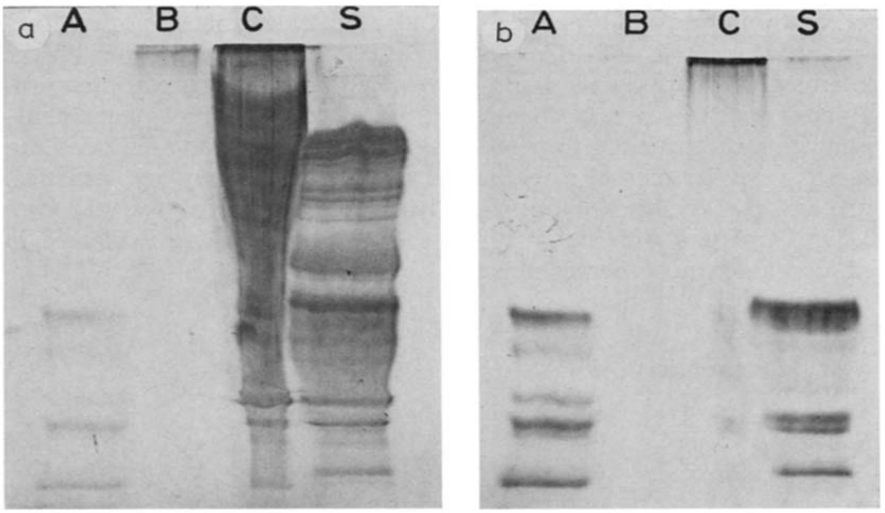

Fig. 4. Analysis of the chromatogram shown in Fig. 3 by pore-gradient electrophoresis in SDS. The gradient gels, obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), had the notation PA A4/30. Buffer: 0.05 *M* glycine–NaOH, pH 9.8, +5 m*M* SDS. (a) Staining for protein with Coomassie Brilliant Blue; (b) PAS staining for glycoprotein. The sample denoted by S corresponds to the unfractionated starting material.

as they do in chromatography (contact is, of course, a prerequisite for the formation of aggregates*). A similar hypothesis can also explain why proteins precipitate much more easily when they are submitted to isoelectric focusing in a sucrose gradient than in a polyacrylamide gel.

It is a characteristic of several chromatographic methods, including hydrophobic interaction chromatography, that most often only one peak is obtained with each buffer used for desorption (see Fig. 1). However, when Tween 20, a neutral detergent, is used several peaks usually appear in each elution step. (see Fig. 5 in ref. 15 and Fig. 4 in ref. 16). The reason for this has not been explored, but the effect should be borne in mind as this type of elution is highly desirable.

From this paper, it is evident that the presence of detergents causes several difficulties in connection with the purification of membrane proteins. We have, however, made the very important observation that many membrane proteins are soluble in conventional buffers in the absence of detergents, provided that the proteins are extensively purified (see the paper by Moore[21], who has made a similar observation). This means that in the last stages of a purification scheme one need not always use detergents, which facilitates the purification. It should also be pointed out that many membrane proteins are soluble in the absence of detergents after carboxylation of the amino groups, for instance with dimethylmaleic acid[20]. By changing the pH the protein can easily be decarboxylated. Detergents can also be avoided by solubilizing the membranes in butanol–acetic acid (morpholine)–water (Fig. 3).

* Point 5 above should also be considered in a discussion of aggregate formation.

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7. SUMMARY

Some general aspects of the solubilization and purification of non-water-soluble membrane proteins are given. Hydrophobic interaction chromatography can be used for the fractionation of such proteins provided that the detergent concentration and the hydrophobicity of the ligands are properly selected.

Columns of agarose equilibrated with butanol (or propanol)–acetic acid (or morpholine)–water have also been used for the fractionation of membrane proteins, but media suitable for desorption of all proteins have not yet been found.

REFERENCES

1 S. Hjertén, J. Rosengren and S. Påhlman, *J. Chromatogr.*, 101 (1974) 281.
2 K. Weber and D. J. Kuter, *J. Biol. Chem.*, 246 (1971) 4504.
3 J. Lenard, *Biochem. Biophys. Res. Commun.*, 45 (1971) 662.
4 D. Grant and S. Hjertén, *Biochem. J.*, 164 (1977) 465.
5 P. H. von Hippel and T. Schleich, in S. N. Timasheff and G. D. Fasman (Editors), *Structure and Stability of Biological Macromolecules*, Marcel Dekker, New York, 1969, pp. 417–574.
6 E. E. Schrier and E. B. Schrier, *J. Phys. Chem.*, 71 (1967) 1851.
7 S. Påhlman, J. Rosengren and S. Hjertén, *J. Chromatogr.*, 131 (1977) 99.
8 A. Helenius and K. Simons, *Biochim. Biophys. Acta*, 415 (1975) 29.
9 K. Shinoda, T. Yamaguchi and R. Hori, *Bull. Chem. Soc. Jap.*, 34 (1961) 237.
10 J. A. Reynolds and C. Tanford, *J. Biol. Chem.*, 245 (1970) 5161.
11 H. Weiss and T. Bücher, *Eur. J. Biochem.*, 17 (1970) 561.
12 S. Hjertén, *J. Chromatogr.*, 87 (1973) 325.
13 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
14 R. J. Simmonds and R. J. Yon, *Biochem. J.*, 163 (1977) 397.
15 S. Hjertén, in N. Catsimpoolas (Editor), *Methods of Protein Separation*, Vol. 2, Plenum Publ., New York, 1976, p. 233.
16 S. Hjertén, *Protides Biol. Fluids*, 23 (1975) 667.
17 L. S. G. Marton and J. E. Garvin, *Biochem. Biophys. Res. Commun.*, 52 (1973) 1457.
18 S. Hjertén, *Arch. Biochem. Biophys.*, Suppl. 1 (1962) 147.
19 S. Hjertén, *J. Chromatogr.*, 159 (1978) 47.
20 P. Lundahl and S. Hjertén, *Protides Biol. Fluids*, 21 (1973) 27.
21 S. Moore, *J. Chromatogr.*, 159 (1978) 3.