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REVIEW

COLUMN LIQUID CHROMATOGRAPHY OF INTEGRAL MEMBRANE PROTEINS

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1. INTRODUCTION

A major function of biological membranes is the compartmentation of biological processes in cells and organelles. Membranes consist of phospholipid molecules and proteins. The phospholipid molecules are amphipatic: they consist of a hydrophilic head and a hydrophobic tail. As long ago as 1925, Gorter and Grendel [1] recognized that the lipids arrange themselves in aqueous solution to form a bilayer in which the hydrophilic heads are pointing into the solution environment

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TABLE 1

HYDROPHOBICITY OF INTEGRAL MEMBRANE PROTEINS

Expressed as the percentage of leucine, isoleucine, valine and methionine (LIVM), of proteins with at least one transmembrane spanning region and their relative molecular masses, compared with the reference proteins (ribonuclease, lysozyme, ovalbumin) and the average protein. Data from ref. 7.

Protein	Relative molecular mass (kD)	LIVM (%)
Sendai virus F2	13-15	29.7
F1	50	29.6
HN	67	25.7
Hepatitis B surface antigen	25	29.2
Protein E1 from Corona virus	26	33.3
H-subunit R. viridis	28	26.7
Cytochrome P-450	56	26.3
Bacteriorhodopsin	27	32.0
ω-Subunit energ. complex	8	33.3
Cardiac membrane proteolipids	11	28.2
sn-1,2-Diacylglycerol kinase	13	36.9
Moloney MuLV p15 E	20	32.2
Bovine ribonuclease	14	14.5
Lysozyme	14	17.8
Ovalbumin	43	27.0
Average protein	–	20.2

and the hydrophobic tails occupy the inside of the bilayer. In 1934, the bilayer model was extended to contain proteins [2]. There are two categories of membrane proteins, peripheral and integral. The peripheral membrane proteins are only loosely attached to the membrane and can be liberated under relatively mild conditions, e.g. high salt concentrations, chelating agents or chaotropic ions [3]. Integral membrane proteins, e.g. viral membrane proteins and receptor proteins, cross the lipid bilayer once or several times [4–6], and the hydrophobic membrane-spanning amino acid sequence of the integral membrane protein interacts strongly with the inner portion of the lipid bilayer.

The isolation of integral membrane proteins requires more drastic conditions, and detergents (also called surfactants or tensides) or organic solvents generally have to be used to extract the protein from the bilayer. The membrane-spanning portion of an integral membrane protein contains a relatively large number of hydrophobic amino acids, which is often reflected in the total amino acid composition of the protein. The percentage of large hydrophobic amino acids can be considered as an indication of the hydrophobicity of a protein [7]. This percentage is 20.2% in an average protein (from 314 families of proteins [8] and an average of 30.3% in a number of membrane proteins (Table 1). However, the size of the protein is also important, since the hydrophobicity of a small part of the protein may be compensated for by a relatively large number of hydrophilic amino acids in externally located regions of the larger integral membrane proteins. This is shown in Table 2, where the percentages of large hydrophobic amino acids in a

TABLE 2

HYDROPHOBICITY OF INTEGRAL MEMBRANE RECEPTOR PROTEINS

Expressed as the percentage of leucine, isoleucine, valine and methionine (LIVM).

Protein	Relative molecular mass (kD)		LIVM (%)	Ref.
	By SDS-PAGE	From sequence		
β-Adrenergic receptor	64	46	27.5	9
Insulin receptor α-subunit	120-130	82	22.3	10
β-subunit	90	70	23.9	10
Acetylcholine receptor	40	50	32.5	11
Muscarinic acetylcholine receptor	70	51	24.1	12
Platelet-derived growth factor receptor	180	120	20.1	13
Epidermal growth factor receptor	138	131	22.7	14
TM86 T-cell receptor		34	24.8	15
Transferrin receptor	95	85	22.3	16
Asialoglycoprotein receptor	42	33	22.3	17

number of receptor proteins are given. These integral membrane proteins are generally larger than those listed in Table 1 and have an average of 24.3% of large hydrophobic amino acids. Nevertheless, these integral membrane receptor proteins also contain strongly hydrophobic regions, which may result in aggregation and in difficulties during purification. The methodology needed to purify integral membrane proteins by column liquid chromatography, in particular by high-performance liquid chromatography (HPLC), is the subject of this paper.

2. PROPERTIES OF DETERGENTS

Detergent extraction is often the first step in the purification of an integral membrane protein. Detergents are lipid-like substances. They possess a hydrophilic head and a hydrophobic tail and are able to compete with the lipids in a bilayer. They are also more hydrophilic than the lipids. As a consequence detergent–protein complexes are soluble in aqueous solutions, and the detergent molecules, in mimicking the lipid molecules, help to maintain the native configuration of the membrane proteins during a purification procedure.

There are several categories of detergents [18–22], and some of their properties are listed in Table 3.

(a) Mild non-ionic detergents, e.g. the Triton, Brij, Emulgen and Tween series, Emulphogen, octylglucoside and dodecyl dimethylamineoxide.

(b) Bile salts, which are mild, ionic, naturally occurring detergents, e.g. cholate, taurodeoxycholate.

(c) Denaturing ionic detergents, e.g. sodium dodecyl sulphate (SDS).

(d) Mild amphoteric detergents, e.g. 3[(3-cholamidopropyl)dimethylamino]-1-propane sulphonate (CHAPS) and sulfobetaines (Zwittergent series).

TABLE 3

CRITICAL MICELLE CONCENTRATION AND MICELLAR MOLECULAR MASS OF DETERGENTS

Data are from refs. 18–22; C_xE_y: x refers to the number of carbon atoms in the alkyl chain and y to the average number of oxyethylene units; a phenyl ring is designated by ∅; *tert.*-C_8 refers to a tertiary octyl group, and $C_{18:1}$ indicates an 18-carbon chain with one double bond.

Detergent	Description	CMC (m*M*)	Micellar relative molecular mass
Ionic			
Sodium dodecyl sulphate		8.13 (H_2O)	17 000
		2.30 (0.05 *M* NaCl)	24 200
		0.51 (0.5 *M* NaCl)	38 100
Sodium cholate		13–15	900–2100
Sodium deoxycholate		4–6	1700–12 100
Sodium taurodeoxycholate		2–6	2000
Non-ionic			
Triton X-100	*tert.*-C_8∅ $E_{9.6}$	0.24–0.30	90 000
Nonidet P40	*tert.*-C_8∅ E_9	0.29	
Triton X-114	*tert.*-C_8∅ $E_{7\text{-}8}$	0.2	
Tween 80	$C_{18:1}$ sorbitan E_{20}	0.012	76 000
Emulphogen BC-720	$C_{12}E_8$	0.087	65 000
Octylglucoside	C_8 glycoside	25.0	8000
Brij 35	$C_{12}E_{23}$	0.091	49 000
Dodecyl dimethylamineoxide		2.2	17 000
Amphoteric			
CHAPS	Bile acid derivative	4–6	6150
Zwittergent 3-12	Sulfopropylammonium compound	3.6	

The choice of a suitable detergent may depend on several factors. The critical micelle concentration (CMC) is the concentration of monomer at which micelles, i.e. spherical bilayer aggregates of detergent molecules, begin to form. Triton X-100 has a low CMC, 0.24–0.30 m*M*, and is difficult to remove by dialysis. Octylglucoside has a high CMC, 25 m*M*, and can easily be removed by dialysis. Therefore, further studies to be carried out with the membrane protein may determine the choice of detergent. Some studies require a soluble protein–detergent complex in order to maintain biological activity, in which case Triton would be preferable. Complete removal of detergent generally leads to precipitation of the membrane protein. Some non-ionic detergents (Berol, Nonidet, Triton, Emulgen, Renex) absorb UV light and therefore interfere with the spectrophotometric determination of proteins at 280 nm. Removal of unbound detergent or exchange of one detergent for another has been reviewed by Furth et al. [23, 24]. We have successfully removed Triton X-100 from detergent extracts of Sendai virus [25] by incubation with Amberlite XAD-2 [26].

Extraction by ionic detergents can be applied when maintaining the biological activity is of less importance. Ionic detergents usually denature proteins, although integral membrane proteins may retain part of their native conformation [27]. The use of bile salts (cholate, deoxycholate) has the limitation that below pH 7.8 they tend to form aggregates, which precipitate [20]. At a pH approaching the pK_a, insoluble bile acid is formed. The pK_a values for deoxycholate and cho-

late are 6.2 and 5.2, respectively, and for the conjugated bile salt taurodeoxycholate the value is 1.9. In addition, deoxycholate forms a gel just above the precipitation limit [28]. Therefore it is advisable to use a conjugated bile salt that has a lower pK_a and can be used over a wider pH range [20].

3. GENERAL METHODS FOR PURIFICATION OF MEMBRANE PROTEINS

The amphiphilic properties of integral membrane proteins are the principal reason why methodologies to purify hydrophilic proteins are not applicable to their purification. As was shown in the preceding paragraph, detergent extraction can be used as a (pre)purification step to remove the integral membrane proteins from the (sub)cellular membranes of cells. Any further chromatographic purification steps will require the presence of a detergent to keep the protein in solution.

Further prepurification can be obtained by creating a phase separation in the solution containing detergent. Bordier [29] extracted proteins from a lipid bilayer by creating a two-phase system with Triton X-114. This detergent separates into an aqueous phase and a detergent phase at 30°C, and integral membrane proteins were preferentially found in the detergent phase while hydrophilic proteins showed up in the aqueous phase. This procedure was successfully applied to a number of proteins: E1 glycoprotein of a corona virus [30]; E2 protein of Semliki Forest virus [31]; G protein of vesicular stomatitis virus [32]; rat intestinal brush border membrane proteins [33]; major surface protein of Leishmania [34]. However, two well characterized integral membrane proteins, the acetylcholine receptor [35] and the T8 antigen of human T lymphocytes [36], failed to partition into the detergent phase. Parish et al. [37] recently developed a more versatile version of this prepurification technique. They used ammonium sulphate to facilitate entry of integral membrane proteins into the detergent phase. Since addition of ammonium sulphate lowers the cloud point to physiologically acceptable temperatures, this also had the advantage that the procedure could be extended to several other detergents, including the more commonly used Triton X-100. Six murine lymphocyte surface molecules partitioned into the Triton X-100 phase from 33–50% ammonium sulphate saturation.

Organic solvents can also be used to extract integral membrane proteins from the lipid bilayer. A two-phase system can be created, and membrane proteins soluble in the organic phase can be separated from proteins in the aqueous phase [3].

4. COLUMN LIQUID CHROMATOGRAPHY OF MEMBRANE PROTEINS

The following types of chromatography have been used for the purification of integral membrane proteins: size-exclusion chromatography (SEC); ion-exchange chromatography (IEC); bioaffinity chromatography (BAC); reversed-phase chromatography (RPC) and hydrophobic-interaction chromatography (HIC). For all of these, high-performance versions are available but they are not always practical. With large amounts of starting material, classical, conventional chro-

matography is often used prior to HPLC. This is particularly useful when the protein to be purified is present in minor amounts.

Bonnerjea et al. [38] analysed 100 papers on protein purification published in 1984. In 75% of the purification schemes IEC was involved, and BAC and SEC in 60% and 50%, respectively. These percentages would probably be different if membrane protein purification alone were considered. In the purification of receptor proteins, BAC is often the major purification step. Viral membrane proteins are often purified by immuno-BAC, and quite a number of other membrane proteins have been purified by reversed-phase HPLC (RP-HPLC). Relatively less popular is the application of high-performance BAC (HP-BAC). Comparative studies with BAC and HP-BAC may show if there is any advantage in using HP-BAC.

The choice of the chromatographic methodology largely depends on the properties of the membrane protein to be purified and on its ultimate use. When the structural integrity of the protein is of less importance, i.e. in amino acid sequence studies, all modes of HPLC can be used, alone or in combination. When the structure of the protein has to remain intact, mild conditions are required. In that case, buffer systems of physiological pH containing a mild non-ionic detergent are to be preferred. When monoclonal or polyclonal antibodies are available, immuno-BAC can be used. Similarly, a hormone or a virus can be attached to a solid support to isolate its receptor. SEC, IEC and HIC can be carried out under mild conditions. High concentrations of salt in HIC may lead to precipitation of membrane proteins. To be able to use lower concentrations of salt, less hydrophobic columns should be used for HIC of membrane proteins. Josić et al. [39] showed that liver membrane proteins in 0.5 *M* ammonium sulphate could be eluted from a propyl column, first by a decreasing ammonium sulphate concentration, then with water followed by a gradient of up to 1% of a non-ionic detergent.

Hydrophilic amino acid residues are generally located on the surface of a native protein, and most of the hydrophobic residues are buried in the interior. The accessibility studies of Lee and Richards [40] show that in ribonuclease-S, myoglobin and lysozyme, 25% of the total number of hydrophobic amino acid residues are accessible on the surface. The percentage of hydrophobic amino acids in integral membrane proteins is higher than in an average protein (see Table 1), therefore more hydrophobic amino acids residues will be surface-located in integral membrane proteins. As a consequence, detergents have to be present during HIC, IEC, SEC and BAC.

Other factors that may play a role in the choice of the chromatographic methodology, especially in RPC of membrane proteins, are the size of the protein and its overall hydrophobicity. In RPC, proteins are generally denatured by contact with the organic solvent, the low pH and the column ligands, and therefore all hydrophobic amino acid side-chains, including the alkyl part of the hydrophilic lysine, may interact with the column ligands. An average membrane protein may contain 10.1% more large hydrophobic amino acids and an average receptor membrane protein 4.2% more than an average protein. As a consequence, a membrane protein will have more sites available for interaction with hydrophobic column ligands, and relatively high concentrations of organic solvent will be needed for

TABLE 4

MEMBRANE PROTEINS PURIFIED BY SE-HPLC

Protein(s) from	Reference(s)
Tick-borne encephalitis virus	42,43
Sendai virus	7,27,44,45
Equine infectious anemia virus (EIAV)	46
Halobacterium halobium	47,48
ATPase	49,50
Blood platelet membrane	51
sn-1,2-Diacylglycerol kinase	52
E. coli cytochromes	53
Ia antigens	54
Liver plasma membranes/Morris hepatoma	55,56
Influenza virus	57
Platelet-derived growth factor receptor	58
Erythrocyte ghosts	59
Membrane glycoprotein antigen	60
Bovine rhodopsin	61
Glucose transporter	62
Muscarinic acetylcholine receptor	63

its elution. The same is true with regard to the size of the protein. The larger the protein, the more sites it will have available for interaction with the column. The receptor membrane proteins are less hydrophobic but larger than the other membrane proteins (cf. Tables 1 and 2), which means that they also will be difficult to purify by RPC. The purification of integral membrane proteins from Sendai virus [41] illustrates the problems encountered in RPC of membrane proteins (see Section 4.4).

4.1. Size-exclusion high-performance liquid chromatography (SE-HPLC)

There are two different approaches in SE-HPLC, which can be distinguished by the type of buffer used for elution. Either denaturing conditions are used, e.g. SDS or an organic solvent [16, 27, 42–44, 46, 47, 49, 52, 53, 57–59, 62, 63] or non-denaturing conditions, e.g. an elution buffer with a mild non-ionic detergent [45, 48–51, 54, 56, 57, 59–61]. Concentrations of detergent range from 0.003 to 0.88%, but generally are ca. 0.1% in an elution buffer of pH 6.5–7.0. A number of membrane proteins that have been subjected to SE-HPLC are listed in Table 4.

4.2. Ion-exchange high-performance liquid chromatography (IE-HPLC)

In IE-HPLC, mild conditions are used. Elution buffers of neutral or near-neutral pH contain a non-ionic detergent in concentrations ranging from 0.03 to 0.5%, or a zwitterionic detergent, e.g. 0.05% CHAPS [56], and proteins are generally eluted with an increasing concentration of sodium chloride. Table 5 lists a number of membrane proteins that have been subjected to IE-HPLC. In addition

TABLE 5

MEMBRANE PROTEINS PURIFIED BY IE-HPLC

Protein(s) from	Reference(s)
Liver plasma membranes/Morris hepatoma	56
Membranes (*E. coli*, human erythrocytes)	64
ATPase complex	65
Sendai virus	27,66
Cytochrome P-450	67,68
Bovine viral diarrhea virus	69
Chloroplast energy coupling complex	70
Blood platelet membrane	51
Leishmania membrane protein	34

to or instead of a detergent, the elution buffer may contain glycerol [67, 68] or organic solvent [70] to diminish non-specific hydrophobic interaction. The addition of up to 8 *M* urea may also be helpful in this respect [71].

4.3. Bioaffinity chromatography

BAC derives its selectivity from the specificity of the solute for a ligand coupled to a column matrix. These specificities may range from relatively broad to narrow, e.g. lectin-coupled columns will have affinity for specific sugar moieties and as a result may bind a variety of glycosylated membrane proteins. Receptor ligands are more specific, and the epidermal growth factor (EGF) receptor, for example, has been purified by chromatography on an EGF column [72].

Similarly, immuno-BAC has been shown to be highly specific and it has been successfully applied to the purification of membrane proteins (Table 6). In the conventional low-pressure mode, BAC is a relatively rapid method and so far it is not clear whether high-performance affinity columns do have a real advantage over the soft gel columns, since in both cases the columns can be used in an HPLC system. Membrane proteins have been purified by conventional BAC (Table 6), and it is to be expected that the same elution systems can be applied to perform HP-BAC. One example is the purification of the substance P receptor by HP-BAC [92]. The membrane proteins listed in Table 6 were eluted in various ways.

(a) High or low pH buffer, e.g. 0.15 *M* ethanolamine (pH 11.2) or 0.1. *M* glycine hydrochloride (pH 2.5) [72–74, 76–78, 83, 90–92].

(b) Chaotropic agent, e.g. 3 *M* potassium, sodium or ammonium thiocyanate [74, 75, 80, 81, 84, 85, 87, 88].

(c) High salt concentration, e.g. 4 *M* magnesium chloride or 3 *M* sodium chloride [79, 86].

(d) Denaturant, e.g. 6 *M* guanidine hydrochloride [92].

(e) Free ligand, e.g. a peptide [89].

A non-ionic detergent (0.1–3.3%) was generally included in the elution buffer.

TABLE 6

MEMBRANE PROTEINS PURIFIED BY BIOAFFINITY CHROMATOGRAPHY

Protein(s) from	Reference(s)
Respiratory syncytial virus	73
Canine distemper virus	74
Measles virus	75
Borna disease virus	76
Cytomegalovirus	77
Epstein-Barr virus	78,79
Varicella zoster virus	80,81
Sendai virus	82,83
Herpes simplex virus	84-86
Hepatitis A	87
Hepatitis B	88
Polyoma virus medium size tumor antigen	89
Rhinovirus receptor	90
Adenovirus attachment protein	91
Substance P neuropeptide receptor	92
Epidermal growth factor receptor	72

4.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

During the past few years RP-HPLC has become one of the most popular techniques for the purification of proteins. The technique is based on hydrophobic interaction between hydrophobic ligands attached to a column support and hydrophobic patches on the protein. Many proteins unfold on contact with the hydrophobic ligands and by being dissolved in an organic solvent of low pH. Therefore, the total number of hydrophobic groups dominates the elution process during RP-HPLC. Thus, large integral membrane proteins, which contain a relatively high number of hydrophobic groups (see Table 1), will require high concentrations of organic solvent for elution.

Detergent-extracted Sendai virus proteins were used as a model mixture for the development of HPLC methods for the purification of integral membrane proteins [7, 41]. The detergent extracts of Sendai virus contain three proteins that are associated with the lipid bilayer: the matrix protein M, the hemagglutinin-neuraminidase protein HN and the fusion protein F. A detergent extract reduced with dithiothreitol (DTT) contains membrane proteins ranging in molecular mass from 13 to 68 kD, while a non-reduced extract contains proteins with molecular masses from 65 to 272 kD.

The first extract was subjected to RP-HPLC on a Phenyl 5PW-RP column with 100-nm pores. The smallest proteins, F2 (13–15 kD) and M (38 kD), were both eluted as a sharp peak at 32.5 and 40% organic solvent concentration, respectively (see Fig. 1). This difference also shows the importance of the size of the protein, since they have an almost similar percentage of large hydrophobic amino acids (29.7 and 30.3% leucine, isoleucine, valine, methionine, LIVM). The two larger Sendai virus membrane proteins were eluted as multiple peaks at higher organic solvent concentrations, between 44.5 and 52.5%, (Fig. 1, HN, dotted area;

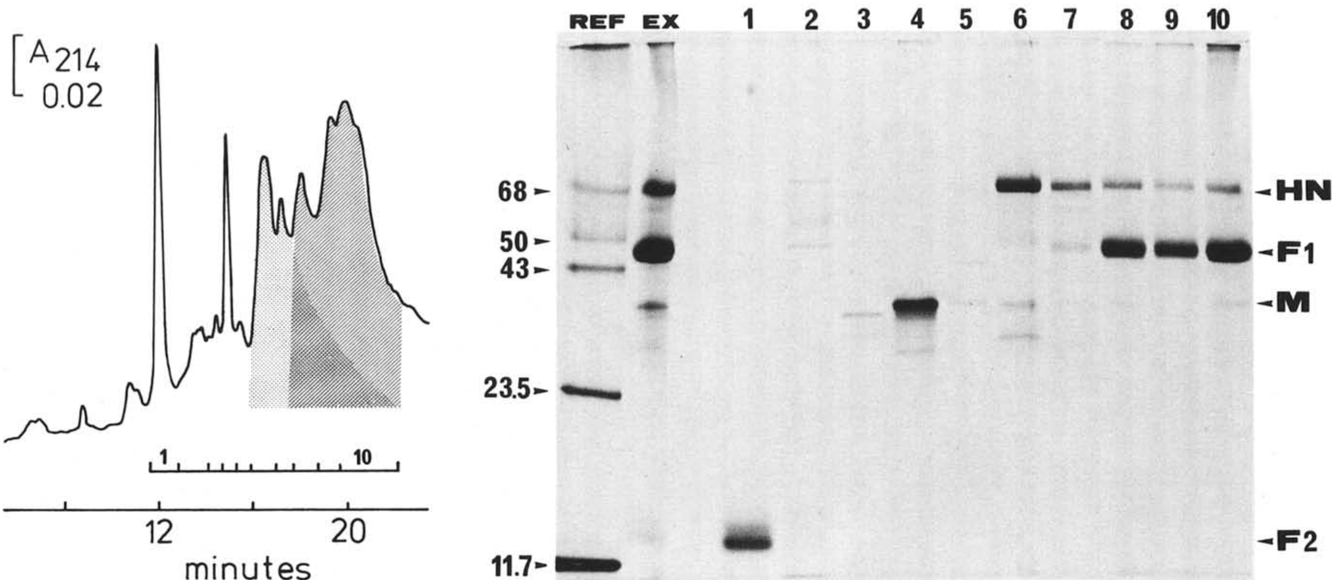

Fig. 1. RP-HPLC of a Triton X-100 extract of purified Sendai virus which was reduced with DTT. The Phenyl-5PW RP (Toyo Soda, Japan) column (50×4.6 mm I.D.) was eluted with a 24-min gradient, consisting of 15–75% acetonitrile in water containing 0.05% TFA. The flow-rate was 1 ml/min and the absorbance was monitored at 214 nm. Fractions (1–10) were analysed by SDS polyacrylamide gel electrophoresis (13% gels). The molecular mass of reference proteins (REF) is indicated in kD. EX = the Triton extract; dotted area = HN protein; hatched area = F1 protein. (From ref. 41.)

F1, hatched area). The multiple peaks may be caused by repeated precipitation and dissolution of the larger membrane proteins.

Chromatography of the non-reduced extract [7] showed that the proteins in this extract, F (65 kD) and the dimer and tetramer of HN (136 and 272 kD, respectively), could be eluted but that no separation was obtained. Again precipitation and dissolution may have been the principal cause of the broad peaks that were eluted at similar organic solvent concentrations (ca. 50%). Therefore it is expected that, as a general rule, large integral membrane proteins will be difficult to purify by RP-HPLC.

Since the hydrophobic nature of a protein is determined by the total number of hydrophobic groups, it is not unexpected that RPC of small membrane proteins (less than 50 kD) is more successful than of larger proteins. At an equal percentage of hydrophobic residues, the organic solvent concentration necessary for protein desorption will increase with protein size. Thus, if conservation of biological activity is not crucial, a reduction of protein size generally will enhance protein recovery and separation efficiency. Unfortunately, the applicability of RP columns for membrane proteins is not predictable from the performance with simple water-soluble reference proteins. In a recent study [94] we evaluated several RP-HPLC column materials, differing with respect to bonded ligands, pore size and particle size, for the purification of membrane proteins. Despite the fact that four selected columns performed equally well with hydrophilic proteins, large differences in protein recovery were observed when Sendai virus membrane proteins were applied to these columns. For instance, recovery of the M protein, which ranged from 0 to 50%, was found to be dependent both on the solvent system [0.1% trifluoroacetic acid (TFA) in water–acetonitrile or 12 m*M* hydrochloric acid in water–ethanol–*n*-butanol (4:1)] and on the column material in an interdependent way. It appeared that the optimal solvent system for the purification of these membrane proteins depended on the RP material and was different for each protein. Therefore, to find the best conditions for the purification of a particular membrane protein, one should preferably evaluate a few different solvent systems with an exploratory set of different RP materials.

In order to increase the recovery of both mass and biological activity of membrane proteins, strategies that reduce the organic modifier concentration needed for elution of a membrane protein should be employed. The use of solvents of higher eluotropic strength, e.g. 1- or 2-propanol instead of acetonitrile, results in the elution of proteins at lower organic solvent concentrations, while it increases both resolution and recoveries [25, 27, 95]. Mixed organic phases are also advantageous for this purpose. The inner-core proteins and the envelope proteins of murine leukemia virus [96] were purified with a gradient of acetonitrile at 23°C during the first part of the separation followed by a gradient of 1-propanol at 50°C in the final part. Recovery of the viral proteins was nearly quantitative. Also, improved chromatographic results were obtained with large peptides of cytochrome P-450 [97] and the platelet-derived growth factor (PDGF) receptor [58], flavivirus proteins [43] and Sendai virus membrane proteins [25, 94] by elution with acetonitrile–propanol or butanol–ethanol. These improvements are ascribed to the fact that the increase in eluent strength is greater than the increase

TABLE 7

MEMBRANE PROTEINS PURIFIED BY RP-HPLC

Protein(s) from	Reference(s)
Cardiac membrane proteolipids	102
Chloroplast energy coupling complex	70
Moloney murine leukemia virus	96
Sendai virus	25,27
Influenza virus	103
Tick-borne encephalitis virus	43
Ia antigens	54
Cytochrome P-450 fragments	97
Bacteriorhodopsin fragments	99
Platelet-derived growth factor receptor fragments	58

of the denaturing and precipitating effects of mixed organic phases [95]. Substitution of TFA by less hydrophobic ionic modifiers such as phosphoric acid or hydrochloric acid, will generally reduce protein retention as well [25, 95, 98]. Also, high concentrations of formic acid (up to 60%) have been used for RPC of membrane proteins and polio virus capsid proteins [97, 99–101]. Although short exposure appears not to be harmful [97, 101], prolonged contact with high concentrations of formic acid may result in esterification of Ser and Thr residues or in cleavage at Asp-Pro bonds.

5. SELECTED BIOMEDICAL AND BIOLOGICAL APPLICATIONS

Many of the integral membrane proteins listed in Tables 4–7 are of direct medical importance, e.g. proteins from blood platelet membranes or erythrocytes [51, 59, 62, 64]. Leishmania [34] are protozoan parasites responsible for a variety of diseases affecting humans and other mammals. Receptor proteins such as the EGF receptor [72] and the PDGF receptor [58] mediate various cellular responses. They possess intrinsic tyrosine kinase activity, which is stimulated by binding of the hormone to the receptor. Furthermore they are structurally related to oncogenes. Other receptor proteins play an important role in viral pathogenesis. Viruses attach to these receptor proteins on the surface of cells in the initial stage of the infection process, e.g. the rhinovirus receptor [90] and the adenovirus attachment protein [91]. Viral membrane proteins often play a crucial role in stimulating the defence mechanism of the infected host. Many of these proteins have been purified by BAC (Table 6).

Most of the other integral membrane proteins listed in Tables 4–7 are of indirect medical importance. They are often present in or isolated from non-human sources and may serve as model proteins or systems for their human counterparts, e.g. Sendai virus [25, 27, 66, 82, 83], which is a paramyxovirus of mice and is related to human paramyxoviridae (e.g. paramyxoviruses type 1-4, measles virus, mumps virus and respiratory syncytial virus). The Ia antigens [54] isolated from Balb/c mice cells are glycoproteins, which are expressed on subpopulations of B

and T cells, macrophages and epidermal cells, and they play a role in the regulation processes of the immune defence mechanism. The cytochrome P-450 liver enzyme system [67, 68] is involved in various reactions, including biotransformation of steroids, the activation and deactivation of drugs, and in xenobiotic toxicity, carcinogenesis and teratogenesis. The ATPase system [49, 50] is involved in active calcium ion transport, and the cardiac membrane proteolipids of canine heart [102] are important in the regulation of transmembrane calcium ion fluxes by catecholamines. Selected applications are described below.

5.1. Microbore high-performance liquid chromatography of proteins

Very often only limited amounts of biologically interesting proteins are available. For this reason there is a growing interest in high-sensitivity techniques. For instance, current gas-phase sequencing technology permits the determination of the primary structure of picomole amounts of proteins and peptides. HPLC has become a versatile tool in the isolation of proteins as well as in the detection of proteins for analytical purposes. Trace amounts of protein present in relatively large volumes of complex mixtures can be concentrated and separated by gradient elution on IE or RP columns [104]. Thus this technique would be very useful for the purification of biologically important proteins present in bulk biological samples. However, with the commonly used standard HPLC columns, the sensitivity of UV detection is limited both by the low protein concentration during elution and by baseline disturbances caused by solvent peaks. The low mass recovery on these columns with nanogram or even microgram amounts of protein, especially with the more hydrophobic proteins, is another limiting factor.

For analytical applications, the detection sensitivity can be increased by using lower flow-rates. However, to maintain the same resolution, the gradient time has to be increased [105], which may further reduce the recovery of the proteins [106]. Therefore, to obtain smaller peak volumes and a higher detection sensitivity, alternative solutions are required, such as the use of microbore columns (1–2 mm I.D.). Under conditions of isocratic elution, the peak volume is directly related to column length and decreases with the square of the column diameter [107]. Under gradient elution conditions these relationships are rather similar but more complex [108]. At first sight, the small sample volume that can be applied to microbore columns to avoid extra-column band broadening might seem a limitation. However, this is only true for isocratic systems. When gradient systems are employed for protein elution, the effect of sample volume on band broadening is totally insignificant. Under appropriate initial solvent conditions the proteins are strongly retained and are concentrated on top of the column [108, 109]. For the same reason, conventional gradient HPLC equipment can be used, although the volume of the line on the upstream side of the column should be kept as low as possible to avoid long dwell-times. In addition, peak volume may be further reduced by a reduction of column length, which also has a beneficial effect on the recovery of hydrophobic proteins [110]. In fact, the use of short microbore columns employing flow-rates of 0.1–0.2 ml/min has enabled trace enrichment from large sample volumes (more than 2.0 ml) resulting in subse-

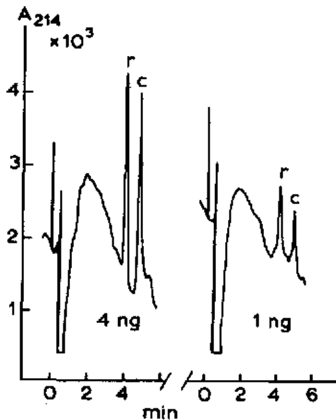

Fig. 2. RP-HPLC of 4 and 1 ng of ribonuclease (r) and cytochrome c (c) on a microbore column (50×1.0 mm I.D.) packed with TMS-250 (Toyo Soda, Japan). Proteins were eluted with a linear gradient of 20–75% acetonitrile in 8 min at a flow-rate of 0.2 ml/min. (From ref. 113.)

quent recovery of purified protein in a small volume (less than 100 μl) of volatile buffer.

Because of the small peak volume that can be obtained, the method was used to concentrate proteins and peptides from diluted solutions or from minced slices of polyacrylamide gels prior to analysis [109, 111, 112], and for instance allows direct transfer to gas-phase sequencers. Thus, as an advantageous side-effect of the small elution volumes obtainable with microbore chromatography, losses due to handling of proteins at the nanogram or microgram level after elution are reduced. Such losses occurring in concentrating methods are often due to non-specific adsorption.

By using short microbore columns with conventional gradient equipment, much smaller peak volumes (40–100 μl) and thus higher sensitivity of detection are obtained, without loss of resolution or speed compared with a conventional analytical column [109, 113, 114]. At equal linear solvent speeds, the protein peak heights were eight to nine times higher on a microbore column than on a conventional column with an I.D. of 1.0 or 4.6 mm, respectively [113].

Finally, apart from the effect of peak volume reduction, sensitivity is further improved by a reduction of solvent peaks. With the reduced solvent consumption at lower flow-rates, equally smaller amounts of solvent impurities accumulate on the column. These effects significantly contribute to an overall increase in sensitivity, enabling detection of proteins down to 1 ng (Fig. 2). This is at least twenty times more sensitive than with conventional columns.

5.2. Selected applications of bioaffinity chromatography

Similarly to IEC and RPC, BAC is a concentrating method. Tiny amounts of proteins present in relatively large volumes, e.g. cell membrane extracts, can be purified and at the same time concentrated. Cellular receptor proteins are examples of proteins that have been isolated in this way. Cohen et al. [72] isolated a

biologically active 170-kD EGF–kinase complex by bioaffinity purification. Cell membranes were solubilized by Triton X-100 and subjected to BAC on AffiGel to which EGF was covalently attached. The receptor proteins were eluted with 5 m*M* ethanolamine (pH 9.7) containing 10% glycerol and 0.2% Triton X-100.

Another group of cellular receptors plays an important role in the initial stage of viral and bacterial infections. For the purification of adenovirus attachment proteins, purified adenovirus was cross-linked by glutaraldehyde and coupled to AH-Sepharose as a natural ligand [91]. Plasma membranes of HeLa cells were extracted with 0.5% Triton X-100, and solubilized proteins were prefractionated by chromatography on wheatgerm Lectin-Sepharose 6MB. The unretained and retained fractions were separately loaded onto the adeno-AH-Sepharose 4B column. Two proteins (40 and 42 kD) with a high affinity for adenovirus were eluted with 0.1 *M* sodium chloride in a 0.1 *M* glycine hydrochloride buffer (pH 2.6) containing 0.037% Triton X-100. The amount of receptor protein in the unglycosylated fraction and the glycosylated fraction corresponded to ca. 2 and 1%, respectively, of the total solubilized membrane protein fractions.

Anti-receptor monoclonal antibodies were used for the isolation of the human rhinovirus attachment protein [90]. These anti-receptor monoclonal antibodies were shown to protect cells from infection by the major group of human rhinoviruses as well as by some other picorna viruses. A crucial step was the solubilization of the plasma membrane preparation by 0.3% sodium deoxycholate in 20 m*M* Tris–HCl (pH 7.4) in the presence of 1 m*M* phenylmethylsulphonyl fluoride. The adsorbed receptor (90 kD) was eluted in biologically active form from an immuno-bioaffinity column (AffiGel to which anti-receptor monoclonal antibodies were coupled) with 50 m*M* diethylamine (pH 11.5). A single immuno-bioaffinity step yielded a 4000-fold purification of the receptor protein. The same protein was eluted as 440-kD protein from an SE-HPLC column (Superose 12). This suggests the existence of a multicomponent complex, of which the 90-kD protein is one constituent.

A promising application of immuno-BAC is the purification of proteins obtained by recombinant-DNA technology (see below, Section 5.3.). The hepatitis-B surface antigen (HBsAg) produced in yeast cells was purified in this way [88]. Polyclonal antibodies that were purified from sera of goats that had been hyperimmunized with the human plasma HBsAg antigen were used. The specific anti-HBsAg antibodies were separated from goat antibodies and coupled to Sepharose. A clarified yeast cell extract, obtained from cells expressing HBsAg, was applied to the column, and elution of HBsAg was achieved by 3 *M* ammonium thiocyanate. The products (25 and 28 kD) were essentially pure hepatitis B antigens, the non-glycosylated and glycosylated form, respectively.

The production of antibodies against proteins by immunization with synthetic peptides comprising sequential antigenic regions of the protein which may react with the intact protein can be a useful tool in molecular biology studies. Such antibodies can be applied in an alternative type of immuno-BAC. This approach has been used in the purification of the polyoma virus medium-size antigen (medium T antigen) [89]. The immunoglobulin G (IgG) fraction of a rabbit anti-peptide antiserum was isolated and coupled to Sepharose 4B. A detergent

extract of polyoma virus-infected cells was incubated with the anti-peptide Sepharose beads. The medium T antigen was eluted by 10 m*M* sodium phosphate (pH 7.0) containing 0.15 *M* sodium chloride, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% trasylol, 1 m*M* DTT, 10 m*M* magnesium chloride and 10 μg of peptide (per 100 μl of cell extract). A large amount of anti-peptide antibodies (25 μg) was necessary for the isolation of less than 0.005 μg of medium T antigen. This suggests that anti-peptide antibodies, in comparison with monoclonal and polyvalent antibodies, do bind rather inefficiently to the antigens. Addition to the elution buffer of the combination of detergents, as well as the 30-fold excess of peptide (of the peptide binding capacity of the column), was necessary to achieve an optimal release of the medium T antigen.

5.3. Chromatography of proteins produced by recombinant-DNA techniques

Manipulation of DNA in vitro allows the expression of medically important genes, e.g. viral genes in prokaryotes such as *Escherichia coli* and eukaryotic cell culture systems. Proteins produced in this way, especially those produced in prokaryotes, may be insoluble or present in an aggregated form. The purification of eukaryotic polypeptides synthesized in *E. coli* is the subject of a recent review [115], and it was shown that the problems associated with the purification of this class of proteins are largely similar to those encountered in the purification of integral membrane proteins. Shire et al. [71] developed a procedure to purify foot-and-mouth disease virus (FMDV) VP1 surface antigen expressed as a fusion protein from *E. coli*. *E. coli* contaminants had to be removed, since they affected the chromatography and had a deleterious effect on the immunogenicity of the VP1-fusion protein. The VP1-fusion protein, obtained as a pellet, was dissolved in 7 *M* guanidine hydrochloride containing 0.1% 2-mercaptoethanol and 50 m*M* sodium phosphate (pH 7.0) and subjected to gel permeation chromatography on Sephacryl S-300 in the same buffer. The fractions containing the 31-kD VP1-fusion protein were dialysed against 8 *M* urea and 14 m*M* Tris–HCl (pH 8.5) containing 0.1% 2-mercaptoethanol (urea–Tris buffer). Subsequent chromatography on DE-52 gave 97% pure VP1-fusion protein with almost quantitative recovery in the flow-through fraction, and the *E. coli* contaminants were eluted with a 0.0–0.2 *M* sodium chloride gradient in urea–Tris buffer. The purified VP1-fusion protein appeared to be immunogenic. Direct chromatography on DE-52, without the preceding gel permeation step in guanidine hydrochloride, was not possible since 8 *M* urea failed to eliminate interactions between the VP1-fusion protein and *E.coli* contaminants. Complexes of these proteins were eluted unseparated by a sodium chloride gradient from the anion-exchange matrix. Guanidine hydrochloride (7 *M*) was shown to be effective in disrupting the interaction between certain *E. coli* proteins and the VP1-fusion protein, allowing their separation by gel permeation chromatography or prolonged high-speed centrifugation (12 h, 44 000 *g*).

DuBois [116] used IE-HPLC and RP-HPLC to purify the fusion products of the v-myb oncogene and of human T-cell lymphotropic virus type I (HTLV-I) Px and p21E from *E. coli* inclusion bodies. To purify the Px fusion protein, the

following procedures were used. Pellets of bacterial cell lysates containing the expressed fusion protein were solubilized in 7 *M* guanidine hydrochloride, Tris–HCl (pH 7.5) with 2 m*M* DTT, dialysed against 7 *M* urea, loaded onto a TSK-DEAE column and eluted with a salt gradient. Alternatively, this protein was purified by RP-HPLC on a C_3 column. The 7 *M* guanidine hydrochloride extract was dialyzed against 5 *M* guanidine hydrochloride, made 0.1% in TFA and applied to a C_3 column. The Px fusion protein (molecular mass 15 kD) was eluted with an acetonitrile gradient in 0.1% TFA as a sharp peak at 32% acetonitrile, free from contaminants with molecular masses ranging from 30 to 100 kD. These examples show that after solubilization, bacterially expressed fusion proteins can be purified by chromatographic methods. Such proteins may also retain immunoreactive epitopes. The Px fusion protein was used to detect antibodies against the gene product (p42) of HTLV-I in sera of individuals who have adult T-cell leukemia or are asymptomatic carriers of the virus [117].

6. CONCLUSIONS

At present, a wide variety of HPLC systems for purification of integral membrane proteins is available. The choice for a particular system or combination of systems will largely depend on the protein to be purified and on whether an intact protein is required for further studies.

SE-HPLC is only useful when the desired membrane protein has a large difference in molecular mass compared with the other components in a sample. In SE-HPLC of integral membrane proteins this may occur by the formation of large micellar complexes consisting of detergent molecules and the desired protein. The micelles can be separated from other proteins which are present in a monomeric form. However, SE-HPLC will generally be more useful in multidimensional chromatography, i.e. combined with another mode of HPLC.

IE-HPLC is performed under mild conditions. Elution is achieved with buffers of physiological pH containing a mild non-ionic detergent and a salt gradient. This is probably one of the most versatile HPLC modes, and it can be applied to virtually all types of membrane protein.

BAC is the most selective method, but it is restricted to those proteins to which antibodies or receptors and inhibitors are available. When the affinity of the protein for the coupled ligand is not too high, elution can be achieved under relatively mild conditions.

RP-HPLC has a denaturing effect on most proteins. Moreover, larger membrane proteins (more than 50 kD) are difficult to separate by this mode of HPLC. Hydrophobic ligands that resemble non-ionic detergents [118, 119] may be useful in the purification of intact membrane proteins, not only when they are used in the HIC mode but possibly also in an RP mode.

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8. SUMMARY

Biological membranes have as a major function the compartmentation of biological processes in cells and organelles. They consist of a bilayer of phospholipid molecules in which proteins are embedded. These integral membrane proteins, which cross the bilayer once or several times, generally have a higher than average hydrophobicity and tend to aggregate. Detergents are needed to remove integral membrane proteins from the lipid bilayer and they have to be present during further chromatographic purification. Predominantly, four modes of HPLC have been used alone or in combination for the purification of integral membrane proteins. These are based on differences of proteins in size (size-exclusion chromatography, SEC), electrostatic interaction (ion-exchange chromatography, IEC), bioaffinity (bioaffinity chromatography, BAC) and hydrophobic interaction (reversed-phase chromatography, RPC, and hydrophobic-interaction chromatography, HIC). SEC, IEC, BAC and HIC are used under relatively mild conditions, and buffer systems generally contain a non-ionic detergent. RPC generally has a denaturing effect on the protein and should preferably be used for the purification of integral membrane proteins smaller than 50 kD.

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