Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Protein Liquid Chromatography
M. Kastner, editor

CHAPTER 12

Integral Membrane Proteins

Gjalt W. WELLING* and Sytske WELLING-WESTER

Department of Medical Microbiology, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands

CONTENTS

ABBREVIATIONS

AC	affinity chromatography
BAC	bioaffinity chromatography
BA-HPLC	bioaffinity-HPLC

* To whom corrspondence should be addressed.

References pp. 549–556

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CMC	critical micellar concentration
DOC	deoxycholate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
HIC	hydrophobic interaction chromatography
HLB	hydrophilic-lipophilic balance
HP-BAC	high performance BAC
HPLC	high performance liquid chromatography
IEC	ion exchange chromatography
IE-HPLC	ion exchange-HPLC
IMAC	immobilized metal ion affinity chromatography
MCAC	metal chelate affinity chromatography
NHS	N-hydroxysuccinimide
RPC	reversed-phase
RP-HPLC	reversed-phase-HPLC
SDS	sodium dodecyl sulfate, sodium lauryl sulfate
SEC	size exclusion chromatography
SE-HPLC	size exclusion-HPLC
TFA	trifluoroacetic acid
Tris	N-tris-(hydroxymethyl)aminomethane

12.1. 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, i.e. they consist of a hydrophilic head and a hydrophobic tail. The lipids arrange themselves in aqueous solution to form a bilayer in which the hydrophilic heads are pointing into the solution environment and the hydrophobic tails occupy the inside of the bilayer. Regarding the association with the bilayer, two major types of membrane proteins can be distinguished. The peripheral membrane proteins are loosely attached to the membrane and can be liberated under relatively mild conditions, e.g. high salt concentrations, chelating agents or chaotropic ions [1,2]. Integral membrane proteins on the other hand, e.g. viral membrane proteins and receptor proteins, cross the lipid bilayer one or more times [3–5] and the hydrophobic membrane-spanning amino acid sequence of the integral membrane protein strongly interacts with the inner portion of the lipid bilayer.

The isolation of integral membrane proteins requires more drastic conditions, and generally, detergents (also called surfactants) or organic solvents 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. This increases the total hydrophobicity of the protein, which may result in aggregation and in difficulties during purification. The methodology to purify integral membrane proteins by column liquid chromatography in particular by high-performance liquid chromatography (HPLC) is the subject of this chapter. Additional information can be found in Refs. [6] and [7].

Abbreviations p. 527

12.2. PROPERTIES OF DETERGENTS

Detergents (surfactants) are the key reagents in the purification of integral membrane proteins [8]. Solubilization of membranes including the proteins, or selective extraction by detergents is often the first step in the purification of an integral membrane protein. Detergents are lipid-like substances. Like the major constituent of the membrane, the phospholipid molecule, they contain a hydrophilic head and a hydrophobic tail. And most importantly they 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.

Since detergents are the key reagents in the purification of integral membrane proteins, we will elaborate on this subject in order to facilitate the choice for a particular detergent. From the literature it seems that there are only a few suitable detergents, e.g. Triton X-100, NP-40, octylglucoside, deoxycholate (DOC). This may be partially true for solubilization, but when chromatography is involved, other detergents may be more suitable as additives to the eluent.

There are several categories of detergents [8–17]: (a) mild non-ionic detergents, e.g. the Triton, Brij, and Tween series, Emulphogen, octylglucoside; (b) bile salts, which are mild ionic naturally occurring detergents, e.g. cholate, taurodeoxycholate; (c) denaturing ionic detergents, e.g. sodium dodecylsulfate (SDS) and cetyltrimethylammoniumbromide; (d) mild amphoteric detergents, e.g. 3-[(3-cholamidopropyl)-dimethylamino]-1-propane sulphonate (CHAPS), sulfobetaines, carboxybetaines and dodecyldimethylamineoxide (above pH 7). Some of their properties are listed in Table 12.1.

A practical approach to the extraction of an integral membrane protein from its lipid bilayer is given in Table 12.2 with Sendai virus membrane proteins as an example.

The choice of a suitable detergent may depend on several factors, i.e. critical micellar concentration (CMC), hydrophilic–lipophilic balance number (HLB), micellar molecular weight, cloud point, UV-transparency, effect on biological activity and price.

12.2.1. Critical micellar concentration (CMC)

The 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 mM), and is difficult to remove by dialysis. Octylglucoside has a high CMC (25 mM), and can easily be removed by dialysis. Therefore, any further studies to be carried out with a particular membrane protein may determine the choice of detergent. Some studies require a soluble protein–detergent complex in order to maintain biological activity. In such cases the CMC is of less importance, although the relatively high concentration of detergent present in extracts may affect the biological activity to some extent. Similarly, high concentrations of certain detergents may interfere with immunological assays, e.g. an ELISA. Excess detergent can then be removed by dialysis (when the CMC is high, e.g. >5 mM) or by size exclusion chromatography (SEC) using an eluent with a lower concentration of detergent. Complete removal of detergent generally leads to precipitation of the membrane

TABLE 12.1

CRITICAL MICELLAR CONCENTRATION AND MICELLAR MOLECULAR WEIGHT OF SELECTED DETERGENTS. IN THE DESCRIPTIONS C_xE_y, x REFERS TO THE NUMBER OF C ATOMS IN THE ALKYL CHAIN AND y TO THE AVERAGE NUMBER OF OXYETHYLENE UNITS; A PHENYL RING IS DESIGNATED BY ϕ; TERT-C8 REFERS TO A TERTIARY OCTYL GROUP AND $C_{18:1}$ INDICATES AN 18-CARBON CHAIN WITH ONE DOUBLE BOND. DATA ARE FROM REFS. [8–17]

Detergent	Description	CMC (mM)	Micellar mol. weight
Ionic			
Sodium dodecyl sulfate	(in H_2O)	8.13	17000
	(in 0.05 M NaCl)	12.30	24200
	(in 0.5 M NaCl)	0.51	38100
Cetyltrimethylammoniumbromide	$C_{16}N^+(CH_3)_3$	1.1	62000
Bile salts			
Sodium cholate		13–15	900–2100
Sodium deoxycholate		4–6	1700–12100
Sodium taurodeoxycholate		2–6	2000
Non-ionic			
Triton X-100	*tert*-$C_8\phi E_{9.6}$	0.24–0.30	90000
Nonidet P40	*tert*-$C_8\phi E_9$	0.29	
Triton X-114	*tert*-$C_8\phi E_{7-8}$	0.2	
Polyoxyethylene alkyl ether	$C_{10}E_5$	0.69	
Polyoxyethylene alkyl ether	$C_{12}E_5$	0.049	
Emulphogen BC-720	$C_{12}E_8$	0.087	65000
Lubrol PX	0.02–0.1		64000
Thesit	$C_{12}E_9$	0.09	
Brij 35	$C_{12}E_{23}$	0.091	49000
Tween 80	$C_{18:1}$sorbitan E_{20}	0.012	76000
Octylglucoside	C_8 glycoside	25.0	8000
Dodecyl-β-D-maltoside	C_{12} maltoside	0.2	50000
Hecameg	6-*O*-(*N*-heptylcarbamoyl)-methyl-*O*-D-glucopyranoside	19.5	
Mega-10	*N*-(D-gluco-2,3,4,5,6-penta-hydroxy-hexyl)-*N*-methyl-decanamide	6.2	
Amphoteric			
CHAPS	Bile acid derivative		6150
Zwittergent 3-12 (sulfobetain SB 12)	Sulfopropylammonium compound	3.6	
(*N*-dodecyl-*N*,*N*-dimethyl-ammonio)undecanoate	Alkyl carboxybetaine	0.13	
(*N*-dodecyl-*N*,*N*-dimethyl ammonio)butyrate	Alkyl carboxybetaine	4.3	
Dodecyl dimethylamine oxide	$[C_{12}N^+(CH_3)_2O^-]$ (above pH 7)	2.2	17000

protein. Removal of unbound detergent or exchange of one detergent for another has been the reviewed by Furth et al. [18,19] and Hjelmeland [20]. We have successfully removed Triton X-100 from detergent extracts of Sendai virus [21] by incubation with Amberlite XAD-2 [22].

Abbreviations p. 527

TABLE 12.2

GROWTH OF SENDAI VIRUS AND EXTRACTION WITH NON-IONIC DETERGENT

Aliquot of Sendai virus is grown in 10-day-old embryonated chicken eggs
Harvest allantoic fluid after 72 h
Centrifuge 30 min at 2000 g (removes cell debris)
Ultracentrifugation 60 min 70000 g (virus particles are pelleted)
Resuspend pellet in 10 mM Tris·HCl, pH 7.2
Determine protein concentration
Add 1 ml of 4% non-ionic detergent to 1 ml of virus suspension (20 mg of protein)
20 min room temperature
Ultracentrifugation 60 min 70000 g (remaining virus particles are pelleted)
Supernatant contains integral membrane proteins HN and F
Store at −80°C in aliquots of 200 μl

12.2.2. Hydrophilic–lipophilic balance number (HLB)

The hydrophilic–lipophilic balance number (HLB) of a detergent is an index of its hydrophilicity. It ranges from 1 (hydrophobic) to 20 (hydrophilic). The HLB of a polyoxyethylene alkyl ether detergent (the C_xE_y series) is the ratio between the hydrophilic oxyethylene part and the hydrophobic alkyl part. It can be calculated by the formula

[weight percentage of oxyethylate]/5 Ref. [23]

TABLE 12.3

PROPERTIES OF NON-IONIC DETERGENTS AND THE RELATIVE YIELDS OF HN AND F PROTEIN AFTER EXTRACTION OF SENDAI VIRUS

Detergent	MW[a]	HLB[b]	CMC[c]	Relative yield (%)[d]
C_8E_5	350	13.5	9.14	65
C_8E_6	394	14.3	8.63	64
C_8E_7	438	14.8	8.45	62
$C_{10}E_5$	378	12.5	0.69	82
$C_{10}E_6$	422	13.3	0.83	77
$C_{10}E_7$	466	14.0	0.88	74
$C_{10}E_8$	510	14.5	0.92	71
$C_{12}E_4$	362	10.7	0.047	14
$C_{12}E_5$	406	11.7	0.049	100
$C_{12}E_6$	450	12.5	0.064	90
$C_{12}E_7$	494	13.2	0.067	78
$C_{12}E_8$	538	13.7	0.067	74
$C_{14}E_5$	434	10.9	0.0092	14
Triton X-100	628	13.5	0.24	63
Octylglucoside	292	12.6	25	43

[a] Average molecular weight in Daltons.
[b] Hydrophilic–lipophilic balance calculated according to Becher [23].
[c] Critical micellar concentration determined according to De Vendittis et al. [24].
[d] The yield obtained with $C_{12}E_5$ was taken as 100%.

We have compared a number of non-ionic detergents with different HLBs and CMCs with respect to the yield of extracted protein. The integral membrane proteins of Sendai virus haemagglutinin-neuraminidase (HN) and fusion protein (F) were extracted from purified virions with 2% detergent, i.e. polyoxyethylene alkyl ethers varying by 8–14 hydrocarbon units in the alkyl chain and by 4–8 ethylene glycol units in the oxyethylene chain, Triton X-100 and octylglucoside [15]. Extraction of Sendai virus with $C_{12}E_5$ gave the highest yield, which was about one quarter of the total amount of HN and F protein. A second and third successive extraction enhances the yield. However this may cause partial disruption of the virus particles resulting in a mixture of membrane and internal proteins. To allow comparison between different detergents, the highest yield obtained with $C_{12}E_5$ was taken as 100%. The relative yields obtained with the other detergents are shown in Table 12.3. An increase in the number of ethylene glycol units in the oxyethylene chain from 5 to 8 (at a fixed alkyl chain length) and a decrease in the number of hydrocarbon units in the alkyl chain from 12 to 8 (at a fixed oxyethylene chain length) decreases the yields of HN and F protein. An increase in the alkyl chain length to C_{14} or a decrease in the oxyethylene chain to E_4 resulted in low yields. These detergents ($C_{14}E_5$ and $C_{12}E_4$) dissolved poorly in aqueous solutions and therefore are not suitable for subsequent chromatographic procedures. The yields of protein are related to the HLB of the detergents. The highest yields were obtained with polyoxyethylene alkyl ethers with HLB values ranging from 11.5 to 12.5. De Pinto et al. [25] reported maximal solubilization of mitochondrial porins with detergents when HLB values were between 10 and 13.5. Umbreit and Strominger [26] reported optimum HLBs of detergents ranging from 12 to 14. They investigated other types of non-ionic detergents for the extraction of D-alanine carboxypeptidase from *Bacillus subtilis* and phosphoacetylmuramylpentapeptide translocase and succinate dehydrogenase from *Micrococcus luteus*. Optimum HLB values may depend on the hydrophobicity of the protein to be solubilized. CMC values are of importance for detergent removal or detergent exchange [18–20,27] but they do not correlate with the solubilizing properties of the detergent.

12.2.3. Micellar molecular weight

Each detergent has an aggregation number (*N*) (not shown in Table 12.1). This is the average number of monomers in a micelle. This results in a micellar molecular weight and this value is given in Table 12.1. Proteins embedded in such micelles will present themselves either as relatively large biological macromolecules (e.g. with Triton X-100) or as much smaller ones (e.g. with octylglucoside). The latter may be important for crystallization studies [28].

12.2.4. Cloud point

The cloud point of a detergent is the temperature at which a detergent solution passes from an isotropic micellar system into a two-phase system. This property of a detergent has been employed in purification methodologies. Bordier extracted proteins from a lipid bilayer by creating a two-phase system with Triton X-114, which has a cloud point of 22°C [29]. This detergent separates into an aqueous phase and a detergent phase at 30°C and integral membrane proteins will be preferentially found in the detergent phase while

Abbreviations p. 527

TABLE 12.4

PARTITIONING OF INTEGRAL MEMBRANE PROTEINS IN A TWO-PHASE SYSTEM WITH TRITON X-114

Extraction as described in Table 12.2, with Triton X-114
Warm the supernatant containing the integral membrane proteins to 30°C
Centrifuge the turbid solution 5 min at 2500 × g; membrane proteins are in the detergent-rich lower phase

hydrophilic proteins show up in the aqueous phase (Table 12.4). 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], and the 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-rich phase. Possibly additional properties of the protein other than binding of the detergent to the transmembrane region are important in the partitioning between the two phases. Maher and Singer suggest that a membrane protein forming a transmembrane channel, e.g. the pentameric acetyl choline receptor, is not able to intercalate with the well-structured Triton X-114 micelle. They made the Triton micelle less structured by adding linoleic acid. Indeed, phase separation with this mixture yielded the acetylcholine receptor in the detergent-rich phase [35]. In a more versatile version of the phase-separation technique ammonium sulfate is used to facilitate entry of integral membrane proteins into the detergent phase [37,38]. Addition of ammonium sulfate lowers the cloud point of other detergents, e.g. from 65 to 20°C for Triton X-100. This extends the range of detergents that can be used in this approach [38]. Six murine lymphocyte surface molecules partitioned into the Triton X-100 phase from 33 to 50% ammonium sulfate saturation [37] (see Table 12.5).

12.2.5. UV-transparency

Some non-ionic detergents (e.g. Berol, Nonidet, Triton, Emulgen, Renex) absorb UV-light and therefore interfere with the spectrophotometric determination of proteins at 280 nm during chromatography. By contrast, others are indeed UV-transparent, e.g. the C_xE_y series.

12.2.6. Price

Yet another factor, which is of less importance in a solubilization/extraction procedure,

TABLE 12.5

PARTITIONING OF INTEGRAL MEMBRANE PROTEINS IN A TWO-PHASE SYSTEM USING AMMONIUM SULFATE AND NON-IONIC DETERGENT

Extraction as described in Table 12.2, with, e.g. Triton X-100
Add 40 % (w/v) ammonium sulfate solution (final concentration 10%)
Centrifuge the turbid solution 10 min at 10000 × g; membrane proteins are in the detergent-rich upper phase
Dialyze overnight against extraction buffer with 0.1% detergent

but more important for chromatography when large volumes of eluent are used, may be the price of the detergent. For example the price of octylglucoside is 300 times that of Triton X-100. Some detergents (incidentally with excellent chromatographic qualities and of high purity) are indeed free of charge from their manufacturers (e.g. Emulgen series).

12.2.7. Biological activity

Non-ionic detergents, bile salts and the mild amphoteric detergents generally do not affect the biological activity of a membrane protein. 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 [39]. The use of bile salts (cholate, deoxycholate) has the limitation that below pH 7.8 they tend to form aggregates which precipitate [9]. At a pH approaching the pK_a, insoluble bile acid is formed. The pK_a values for deoxycholate and cholate are 6.2 and 5.2, respectively and for the conjugated bile salt taurodeoxycholate, 1.9. In addition, deoxycholate forms a gel just above the precipitation limit. Therefore it is advisable to use a conjugated bile salt which has a lower pK_a and can be used over a wider pH range.

12.3. CHROMATOGRAPHIC POSSIBILITIES

To date, there are four modes of chromatography which have proven fruitful for the purification of integral membrane proteins: size exclusion chromatography (SEC), ion exchange chromatography (IEC), bioaffinity chromatography (BAC), and reversed-phase chromatography (RPC).

Purifications involving hydrophobic interaction chromatography, immobilized metal ion affinity chromatography (metal chelate affinity chromatography), hydroxyapatite chromatography or chromatofocusing for this purpose are relatively scarce. Of these, ceramic hydroxyapatite HPLC looks most promising and generally applicable to other membrane proteins. Rögner [40] used this mode of HPLC for further purification of membrane proteins from cyanobacterial thylakoid membrane in the presence of 0.03% dodecyl β-D-maltoside. In a similar way Ichimura et al. [41] separated membrane proteins from rat liver microsomes in the presence of a non-denaturing detergent.

For these four major modes of chromatography, high-performance versions are available but they are not always practical. With large amounts of starting material, classical, conventional chromatography is often used prior to HPLC. HPLC is particularly useful when the protein to be purified is present in minor (i.e. milligram) quantities or less. 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 and biological activity of the protein has to remain intact, mild conditions are essential requirements. 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 and IEC can usually be carried out under mild conditions.

Hydrophilic amino acid residues are generally located on the surface of a native

TABLE 12.6

MEMBRANE PROTEINS PURIFIED BY SE-HPLC

Protein/proteins from	References
Ca^{++}ATPase	[44–47]
Arachidonoyl-diacylglycerol kinase	[48]
Bacteriorhodopsin	[46]
Band 3 protein from erythrocyte membrane	[49]
Blood platelet membrane	[50]
Bovine rhodopsin	[51]
Cell-CAM 105 (CAM = cell adhesion molecule)	[52]
Chlorophyll-*a*/*b*-binding protein	[53]
Cytochrome b_6f complex (*Synechocystis* PCC 6803)	[40]
Reaction center Photosystem I and II	
Cytochrome *c* oxidase	[54]
Cytochromes (*E. coli*)	[55]
sn-1.2-diacylglycerol kinase	[56]
Dipeptidyl aminopeptidase	[52]
Equine infectious anemia virus (EIAV)	[57]
Erythrocyte ghosts	[58]
Erythrocytes (human) (e.g. glycophorin C)	[59]
Glucocerebrosidase (human placenta)	[60,61]
Glucose transporter	[62]
UDP-glucuronosyltransferase	[63,64]
Halobacterium halobium	[65,66]
Herpes simplex virus	[67]
Influenza virus	[68]
$\alpha_1\beta_1$-integrin	[52]
Mannitol-specific enzyme II of the *E. coli*	[69]
PEP-dependent phosphotransferase system	
Plasma membranes (liver)/Morris hepatoma	[70,71]
Membrane glycoprotein antigen	[72]
N^5-Methyltetrahydromethanopterin:coenzyme M	[73]
MHC class II-peptide antigen complexes	[74]
Monoamine transporter	[75]
Muscarinic acetylcholine receptor	[76]
Mycoplasma gallisepticum	[77]
NADH oxidase	[78]
OMPs of *Haemophilus influenzae*	[79]
Platelet-derived growth factor receptor	[80]
Prostaglandin H_2 synthase	[81]
Rat lens	[82]
Reaction center from *Rhodopseudomonas spheroides*	[46]
Sendai virus	[39,83–85]
Tick-borne encephalitis virus	[86,87]

protein and most of the hydrophobic residues are buried in the interior. Accessibility studies show that in ribonuclease-S, myoglobin and lysozyme, 25% of the total number of hydrophobic amino acid residues are accessible on the surface [42]. The percentage of hydrophobic amino acids in integral membrane proteins is at least 10% higher than

TABLE 12.7

MEMBRANE PROTEINS PURIFIED BY IE-HPLC

Protein/proteins from	References
F_1F_0-ATPase (bovine heart mitochondria)	[89]
ATPase complex	[90]
Blood platelet membrane	[50]
Bovine viral diarrhea virus	[91,92]
Chloroplast energy coupling complex	[93]
Cytochrome *c* oxidase (*Bacillus* PS3)c oxidase (@it#bacillus@/it# PS3)",4>	[94]
Cytochrome *c* oxidase subunitsc oxidase subunits",4>	[95]
Cytochrome P-450	[96–98]
Epstein-Barr virus	[99]
Erythrocytes (human)	[100]
Herpes simplex virus	[101,102]
Intestinal peptide transporter	[103]
Leishmania membrane protein	[34]
Membrane antigen (*Plasmodium falciparum*)	[104]
Membranes (*E. coli*)	[105]
N^5-Methyltetrahydromethanopterin:coenzymeM	[73]
Microsomal membrane proteins (rat liver)	[106]
Mycoplasma gallisepticum	[17,77]
OMPs (*E. coli*)	[100]
Plasma membranes (liver)/Morris hepatoma	[71]
Platelet-derived growth factor receptor	[107]
Protein tyrosine phosphatase	[108]
Sendai virus	[39,109–112]
Submandibular and parotid gland (rat)	[113]
Measles virus	[114]

in an average protein, and therefore more hydrophobic amino acid residues will be surface-located in integral membrane proteins. As a consequence, detergents have to be present during IEC, SEC and BAC. Other factors which 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 or the column ligand and therefore all hydrophobic amino acid side chains – including the alkyl part of the hydrophilic lysine – may interact with the column ligands. As a consequence, a membrane protein will have more sites available for interaction with hydrophobic column ligands than will an average hydrophilic protein. Relatively high concentrations of organic solvent will be needed for elution of membrane proteins. The same is true with regard to the size of the protein. A larger protein will have more sites available for interaction with the alkyl groups of the reversed phase than will a small protein. The purification of integral membrane proteins from Sendai virus [43] illustrates the problems encountered in RPC of membrane proteins (see Section 12.4).

Tables 12.6–12.9 contain information on the purification of membrane proteins by SE-HPLC, IE-HPLC, affinity chromatography and RP-HPLC, respectively.

Abbreviations p. 527

TABLE 12.8

MEMBRANE PROTEINS PURIFIED BY AFFINITY CHROMATOGRAPHY

Protein/proteins from	References
Adenovirus attachment protein	[120]
Alpha-factor receptor (ste2p) (*Saccharomyces cerevisiae*)	[117]
ATPase	[116]
Borna disease virus	[121]
Canine distemper virus	[122]
Cyt b_6f and LCHII	[123]
Cytochrome *c* reductase (potato mitochondria) (*Neurospora crassa*)c reductase (potato mitochondria) (@it#Neurospora crassa@/it#)",4>	[124,125]
Cytomegalovirus	[126]
Epidermal growth factor receptor	[119]
Epstein–Barr virus	[127,128]
Erythrocytes (human) (e.g. glycophorin C)	[59]
Fusogenic protein (rat brain microsomal membranes)	[129]
Hepatitis B	[130]
Herpes simplex virus	[131–133]
B27 Histocompatibility antigen (HLA)	[134]
Human lung leukotriene C_4 synthase	[135]
Measles virus	[136]
Oncoprotein bcl-2	[137]
Plasma membrane proteins (human)	[138]
Polyoma virus medium size tumor antigen	[139]
Respiratory syncytial virus	[140]
Rhinovirus receptor	[141]
Sendai virus	[142,143]
Substance P neuropeptide receptor	[144]
Sugar phosphate transporter (*E. coli*)	[118]
Varicella zoster virus	[145,146]
Plasma membranes (liver)/Morris hepatoma	[147]

12.4. APPLICATION EXAMPLES

12.4.1. Size exclusion HLPC (SE-HPLC)

There are two different approaches in SE-HPLC of integral membrane proteins which can be distinguished by the type of eluent. Either eluents providing denaturing conditions are used, e.g. SDS or an organic solvent [39,44,55–58,62,65,68,70,75,76,80,82,84,85–88], or the membrane protein is eluted under non-denaturing conditions, e.g. with an elution buffer containing a mild non-ionic detergent [40,43,45–54,59–61,63,64,66,67,69,71–74,77–79,81,85].

Concentrations of detergent generally range from 0.003 to 1% in an elution buffer of pH 6.5–7.0, although higher concentrations especially of cholate (4%), deoxycholate (1.5%) and octylglucoside (1.25%) have also been used [80,69]. Some membrane proteins which have been subjected to SE-HPLC are listed in Table 12.6. Suitable eluents to start with may depend on further studies. When the protein should retain its native conformation, a non-ionic detergent, e.g. 0.88% octylglucoside in 0.06 M sodium phosphate, pH 6.5 containing 0.15 M NaCl can be used. These conditions were applied

TABLE 12.9

MEMBRANE PROTEINS PURIFIED BY RP-HPLC

Protein/proteins from	References
Bacteriorhodopsin fragments	[148]
Caprine arthritis-encephalitis lentivirus	[149]
Cardiac membrane proteolipids	[150]
Chloroplast energy coupling complex	[93]
Cytochrome P-450 fragments	[151]
Cytochrome *c* oxidase subunitsc oxidase subunits",4>	[95]
Herpes simplex virus	[152]
Influenza virus	[153]
Moloney murine leukemia virus	[154]
Mycoplasma hyopneumoniae	[155]
Myelin glycoprotein P_0	[156]
Platelet-derived growth factor receptor fragments	[80]
Rhodopsin (large hydrophobic peptides)	[157]
Sendai virus	[21,39,83]
Tick-borne encephalitis virus	[84]

for the purification of bovine rhodopsin [51]. When the protein may be denatured, 0.1% SDS in 0.05 M sodium phosphate, pH 6.5 is a suitable eluent to start with. Samples can be treated as in polyacrylamide gel electrophoresis with or without reduction and boiling in 4% SDS prior to SE-HPLC [40] (see Table 12.10) or by adding SDS to the sample (final concentration 4%) without boiling [88]. Immunological activity of particular membrane proteins is retained by the latter procedure despite the presence of SDS. The separation of Sendai virus membrane proteins is given as an example. Sendai virus contains two integral membrane proteins, the haemagglutinin-neuraminidase protein (HN, relative molecular mass, $M_r = 68\,000$) and the fusion protein F ($M_r = 65\,000$). Both proteins are present in a non-ionic detergent extract in multimeric forms. Dimeric HN and tetrameric HN and F are observed. Upon addition of 4% SDS (final concentration) and 3 min at 100°C, HN remains in its multimeric form and F is

TABLE 12.10

SE-HPLC OF A DETERGENT EXTRACT OF INTEGRAL MEMBRANE PROTEINS. MANUFACTURERS CAN BE LOCATED USING THE DIRECTORY IN PART D OF THIS BOOK

Add 4 mg SDS to 100 µl of a detergent extract
3 min in boiling water
Centrifuge 5 min in Eppendorf centrifuge
Inject supernatant into SE-HPLC system (with, e.g. a Superose 6 column, Amersham Pharmacia Biotech) running at 0.5 ml/min with detection at 280 nm
Collect 1 ml fractions (we use 70 × 11 mm Minisorp tubes (Nunc)) and take aliquots of 50 µl for SDS-PAGE
Dialyze against water (cover tubes with a square piece of dialysis membrane tubing and close them by fitting a slice of silicone tubing over the dialysis membrane) or store at −80°C
Compare elution volume of each peak with those in a reference run. Useful for a reference run are similarly-treated BSA (68 kDa) (its dimer is present in small amounts), ovalbumin (43 kDa) and trypsin-inhibitor (20 kDa), 50 µg of each protein in 100 µl; absorbance monitoring at 0.1 or 0.2 AUFS

Abbreviations p. 527

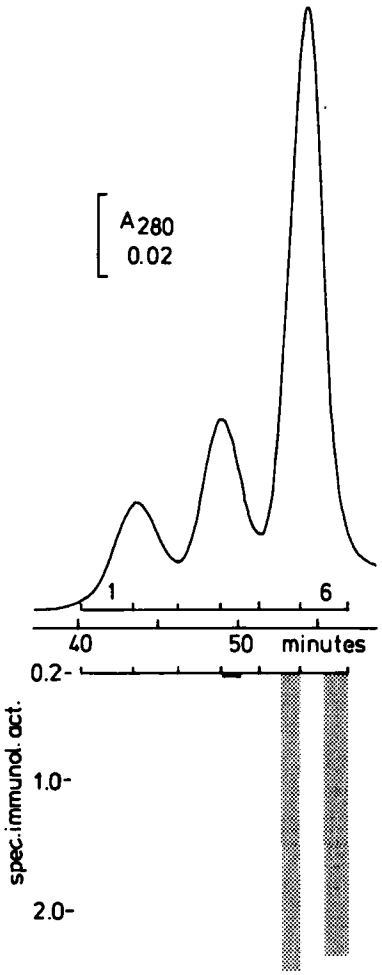

Fig. 12.1. Size exclusion HPLC of a Sendai virus envelope extract on two tandemly-linked Superose-6 (300 × 10 mm i.d.) columns (Amersham Pharmacia Biotech). The extract was dissolved in 4% SDS and the column was eluted with 0.1% SDS in 50 mM sodium-phosphate, pH 6.5 at a flow rate of 0.5 ml/min. The absorbance was monitored at 280 nm. Fractions (1–6) were analyzed on 8% SDS-gels. EX: the Sendai virus envelope extract. The positions of the HN_4, HN_2 and the F protein are indicated. The specific immunological activity is indicated below the fractions. The left column represents the reactivity with Mabs directed against intact HN protein (in fraction 4 the immunological activity is slightly above the cut-off level of the OD_{492} of the ELISA). The right column represents the reactivity with Mabs directed against intact F protein (dotted area) (from Ref. [88]).

present as a monomer. These special properties allowed purification based on size with 0.1% SDS present in the eluent (Fig. 12.1). When the sample was not heated at 100°C, F remained largely in its multimeric form and separation of HN and F protein was not possible. A monoclonal antibody directed against F protein still reacted with the protein despite the SDS-treatment. It is also possible to use a salt-free eluent. We used 45% acetonitrile in 0.1% HCl for SE-HPLC of Sendai virus proteins [84].

TABLE 12.11

IE-HPLC OF A DETERGENT EXTRACT OF INTEGRAL MEMBRANE PROTEINS

An anion exchange HPLC column is equilibrated in buffer A: 20 mM Tris·HCl, pH 7.8 containing 0.1% (w/v) of non-ionic detergent (we prefer $C_{10}E_5$)
Detergent extract (preferably the same detergent as in buffer A) containing 500–1000 μg of protein and diluted 1:1 (v/v) with buffer A, is injected into the IE-HPLC system
The column is run at 1 ml/min; absorbance monitored at 280 nm
10 min isocratic elution
24 min linear gradient from buffer A to 0.5M NaCl in the same buffer
Collect 1 ml fractions (we use 70 × 11 mm Minisorp tubes (Nunc) and take aliquots of 50 μl for SDS-PAGE
Dialyze against water (cover tubes with a square piece of dialysis membrane tubing and close the tubes by fitting a slice of silicone tubing over the dialysis membrane) or store at −80°C

12.4.2. Ion exchange HPLC (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 [71], and proteins are generally eluted with an increasing concentration of NaCl. Table 12.7 lists a number of membrane proteins that have been subjected to IE-HPLC. In addition to, or even instead of, a detergent, the elution buffer may contain glycerol [96,97] or organic solvent [93] to diminish unspecific hydrophobic interaction. The addition of up to 8 M urea may also be helpful in this respect [115].

Since most proteins are either neutral or acidic, an anionic column support would be the first choice when information about the membrane proteins to be purified is not available. A suitable buffer to start with is 20 mM Tris·HCl, pH 7.8 containing 0.1% (w/v) of a non-ionic detergent (see Table 12.11). IE-HPLC can also be utilized in a two-step procedure to separate hydrophilic protein (fragments) of membrane proteins from intact membrane

TABLE 12.12

SPECIFIC ELUTION OF INTEGRAL MEMBRANE PROTEINS BY A TWO-STEP IE-HPLC PROCEDURE

An anion exchange HPLC column is equilibrated in buffer A: 20 mM Tris·HCl, pH 7.8 containing 0.01% (w/v) $C_{10}E_5$ (*low detergent concentration*)
A 2% $C_{10}E_5$ detergent extract is diluted to 0.01% with 20 mM Tris·HCl, pH 7.8 and applied to the IE-HPLC column
The column is run at 1 ml/min; absorbance monitored at 280 nm
10 min isocratic elution after application of the sample
12 min linear gradient from buffer A to 0.5M NaCl in the same buffer (buffer B)
15 min isocratic elution with buffer A
30 min equilibration with 20 mM Tris·HCl, pH 7.8 containing 0.1% (w/v) $C_{10}E_5$ (buffer C) (*high detergent concentration*)
N.B. blank run, no application of sample; 12 min linear gradient from buffer C to 0.5 M NaCl in the same buffer (buffer D)
Collect 1 ml fractions (we use 70 × 11 mm Minisorp tubes (Nunc)) and take aliquots of 50 μl for SDS-PAGE
Dialyze against water (cover tubes with a square piece of dialysis membrane tubing and close them by fitting a slice of silicone tubing over the dialysis membrane) or store at −80°C

proteins (Table 12.12). At low detergent concentrations, hydrophilic proteins are eluted with the salt gradient, while a subsequent blank run with the same gradient at higher detergent concentration results in elution of the integral membrane proteins [112].

This procedure was applied for the purification of integral membrane proteins from Sendai virus [112], Herpes simplex virus [101] and a membrane antigen from *Plasmodium falciparum* [104]. The purification of Sendai virus membrane proteins is shown as an example in Figs. 12.2 and 12.3. What is special about the sample is that in addition to intact integral membrane proteins HN and F, it also contains HN and F fragments that lack the hydrophobic membrane spanning region. Elution pattern (Fig. 12.2) and corresponding gel (Fig. 12.3) for (b) (0.1% detergent; second run, no sample applied) should be compared with (c) (0.052% detergent; first run, sample applied). In contrast to gel b, gel c contains many polypeptide bands which are presumably hydrophilic fragments of the membrane proteins.

The samples contain 1% of the detergent and consist at least partly of micelles with the hydrophobic transmembrane region of the protein in the hydrophobic bilayer of the micelle. They are subjected to IE-HPLC with an eluent containing a relatively low concentration of detergent (below the CMC). The small amount of detergent monomers in the eluent will try to establish equilibrium with the micellar membrane protein-detergent complex that is attached to the ion exchange ligands of the column support. Together with the right salt concentration this may result in partial elution of the membrane proteins (A, B and D in Fig. 12.3a). In the second run the detergent concentration will be sufficiently high to pull all remaining integral membrane proteins A, B, and D into the eluent at the appropriate salt concentration. This may result in relatively pure membrane protein, because all hydrophilic proteins which did not need a detergent for solubilization were already eluted during the first run (e.g. the truncated form of HN designated by C in Fig. 12.3a). Table 12.13 illustrates the purification of glycoprotein D from Herpes simplex virus type 1 (HSV-gD) by this procedure [101]. It actually shows that this approach is not entirely successful in the sense that there is no exclusive elution of the integral membrane protein HSV-gD in the second (high detergent concentration) run. In the first run, 20–30% of the eluted protein is HSV-gD. We argued that this might be partly due to the high detergent concentration (1%) in the sample that was applied to the column. This almost certainly will affect the retention of membrane protein during the first run. Indeed, dilution of the samples prior to chromatography resulted in less HSV-gD during the first run (unpublished results).

12.4.3. Bioaffinity chromatography (BAC)

Bioaffinity chromatography (BAC, or simply affinity chromatography, AC) 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. Another example is metal chelate affinity chromatography (MCAC), also known as immobilized metal ion affinity chromatography (IMAC). This type of affinity chromatography is often used for the purification of proteins obtained by recombinant-DNA technology. A chelating group (e.g. nitriloacetic acid) is coupled to a chromatographic matrix and the column is charged with Ni^{2+}, Cu^{2+} or Zn^{2+}. Tagging of the

target protein with up to six histidines (resulting in a His-tail) allows purification of the protein [116–118], although the specificity of the metal chelating ligand is not only directed to His but also to surface-located Cys and Trp. Receptor-ligands are more specific and, e.g. the epidermal growth factor (EGF) receptor has been purified by chromatography on an EGF-column [119]. Similarly, immuno-BAC has shown to be highly specific and it has been successfully applied in the purification of membrane proteins. In the conventional

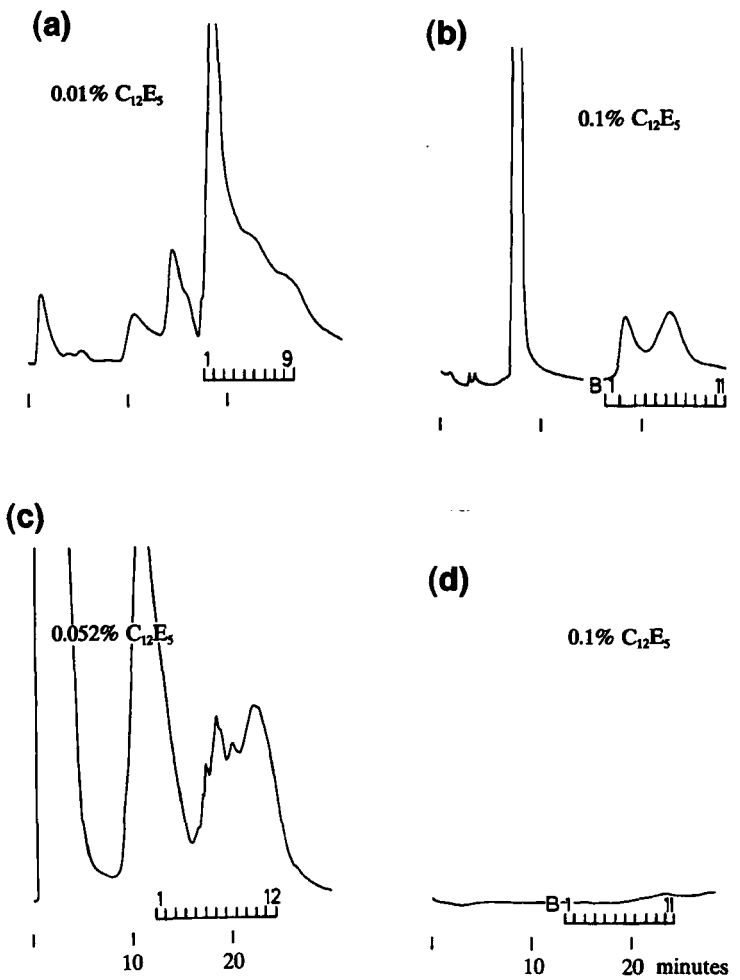

Fig. 12.2. IE-HPLC elution profiles of a $C_{12}E_5$ extract of Sendai virus containing HN and F proteins in the presence of 0.01% (a) or 0.52% (c) $C_{12}E_5$. Anion exchange HPLC was performed with a Mono Q HR 5/5 column (Amersham Pharmacia Biotech). After isocratic elution, retained proteins were eluted with a linear 12-min gradient from 20 mM Tris·HCl (pH 7.8) containing the different detergent concentrations to 0.5 M sodium chloride in the same buffer. The flow rate was 1 ml/min and the absorbance was monitored at 280 nm. Chromatography in the presence of the above detergent concentrations was followed by a second chromatography (blank run) using a sodium chloride gradient in the presence of 0.1% $C_{12}E_5$ to elute residual HN and F proteins (b,d). Fractions were collected during the gradient elution as indicated and subjected to SDS-PAGE (see Fig. 12.3) (from Ref. [112]).

Abbreviations p. 527

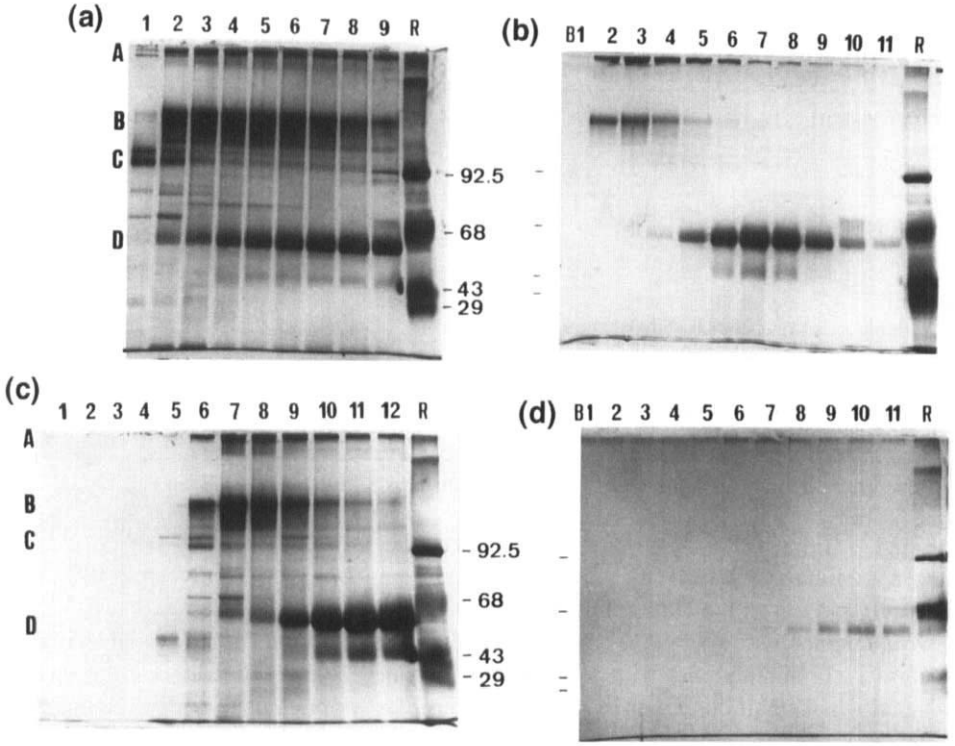

Fig. 12.3. SDS-PAGE analysis on 12% gels under non-reducing conditions of the fractions collected during chromatography in the presence of different concentrations of $C_{12}E_5$: 0.01% (a) lanes 1–9, followed by (b) a blank run with 0.1%, lanes B1–11; 0.052% (c) lanes 1–12, followed by (d) a blank run with 0.1%, lanes B1–11. Polypeptides were visualized by silver staining. A, B, C and D are the tetrameric, dimeric and truncated forms of HN protein, and F protein, respectively. The molecular masses ($\times 10^{-3}$) of reference (R) proteins are given on the right. E: $C_{12}E_5$ extract of Sendai virus (from Ref. [112]).

low-pressure mode, BAC is a relatively rapid method and so far it is not clear whether application of high-performance affinity columns does have a real advantage over using the soft gel columns, since in both cases, the columns can be implemented in an HPLC-system. Most of the membrane proteins in Table 12.8 have been purified by conventional BAC. The same elution systems can be applied in HP-BAC (BA-HPLC). Two examples of HP-BAC are the purification of the substance P receptor [144] and of human plasma membrane proteins [138].

The membrane proteins in Table 12.8 were eluted by any of a large number of agents. These include: (a) high or low pH buffers, e.g. 0.15 M ethanolamine, pH 11.2, 0.05 M diethylamine, pH 11.5, or 0.1 M glycine HCl, pH 2.5 [119–122,126,127,137,140,141,143, 144]; (b) chaotropic agents, e.g. 3 M K, Na, or NH_4SCN [122,130–132,134,136,142,145, 146]; (c) high salt concentrations, e.g. 4 M $MgCl_2$ or 3 M NaCl [123,128,134]; (d) denaturants, e.g. 6 M guanidine·HCl or 8 M urea [144]; (e) 1 M α-methylpyranoside [129,138,147]; (f) free ligand, e.g. a peptide [139], or (g) a compound which reduces the ligand resulting in a decreased affinity of ligand and protein [124,125]; and (h) 150–

TABLE 12.13

PURIFICATION OF GLYCOPROTEIN D OF HERPES SIMPLEX VIRUS TYPE 1 FROM INFECTED Sf21 CELLS

Purification step	Percentage gD present in purification step (%)[a]
Starting material	
Extract of infected cells	10–15
IE-HPLC	
Flow-through (isocratic elution with 0.005% $C_{10}E_5$ in buffer)	0
Salt gradient: 0–0.5 M NaCl with 0.005% $C_{10}E_5$ in buffer	20–30
Isocratic elution with 0.005% $C_{10}E_5$ in buffer	0
Isocratic elution with 0.1% $C_{10}E_5$ in buffer	Trace
Salt gradient: 0–0.5 M NaCl with 0.1% $C_{10}E_5$ in buffer	>90

[a] The percentage gD is based on the analysis of the fractions by SDS-PAGE and a gD-specific ELISA.

300 mM imidazole [116–118]. A non-ionic detergent (0.1–3.3%) or glycerol (10%) was generally present in the elution buffer.

When monoclonal antibodies or antigen-binding fragments thereof are available, immunoaffinity chromatography with ready-to-use activated column supports can be recommended when small quantities of protein are desired. A prerequisite is that the ligand is

TABLE 12.14

IMMUNOBAC OF A DETERGENT EXTRACT OF MEMBRANE PROTEINS

Dissolve 0.5–10 mg monoclonal antibody (preferably purified IgG) in 1 ml 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, and measure A_{280} at a suitable dilution

Wash out the isopropanol present in a 1 ml NHS-activated HiTrap (Amersham Pharmacia Biotech) column with ice-cold 1 mM HCl

Apply monoclonal antibody solution to the column and leave column (sealed) for 30 min at room temperature

Wash with 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, measure A_{280} of washes and determine how much of the initial protein is coupled to the column

For deactivation of remaining NHS-groups and washing, wash with:
- 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 (buffer A)
- 0.1 M acetate, 0.5 M NaCl, pH 4.0 (buffer B)
- Buffer A
- Leave column for 30 min
- Buffer A
- Buffer B
- Buffer A
- Starting buffer: 20 mM Tris·HCl, pH 8.0 with 0.5 M NaCl and 0.1% of a non-ionic detergent
- Elution buffer: 50 mM diethylamine in 20 mM Tris·HCl, pH 8.0 and 0.1% of a non-ionic detergent
- Starting buffer

Apply protein mixture containing membrane protein in starting buffer to column and wash with this buffer until A_{280} is back to baseline

Elute membrane protein with elution buffer and neutralize samples by adding 1 M Tris·HCl, pH 8.0

Abbreviations p. 527

stable in the eluents mentioned under (a)–(c) above. A general immunoaffinity chromatography procedure is listed in Table 12.14.

12.4.4. Reversed-phase HPLC (RP-HPLC)

RP-HPLC can also be used for the purification of integral membrane proteins. This chromatography is based on hydrophobic interaction between hydrophobic ligands attached to a column support and hydrophobic patches on the protein. Many proteins unfold upon 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, containing a relatively high number of hydrophobic groups will require high concentrations of organic solvent for elution. Detergent-extracted Sendai virus proteins served as a model mixture for the development of HPLC-methods for the purification of integral membrane proteins [43,83]. When Sendai virus particles are extracted with non-ionic detergent (as described in Table 12.2) and 1 M NaCl is present during the incubation, then the detergent extract contains three proteins that are associated with the lipid bilayer: the matrix protein M, the haemagglutinin-neuraminidase protein HN, and the fusion protein F.

When such a detergent extract is reduced with DTT, it contains membrane proteins ranging in molecular mass from 13 000 to 68 000, while a non-reduced extract contains proteins with molecular masses from 65 000 to 272 000. The first extract was subjected to RP-HPLC on a Phenyl 5PW-RP column with 100-nm pores. The smallest proteins, F2 (13–15 kDa) and M (38 kDa), were both eluted as a sharp peak at 32.5 and 40% organic solvent concentration, respectively (see Fig. 12.4). This difference also shows the importance of the size of the protein. The two larger Sendai virus membrane proteins were eluted

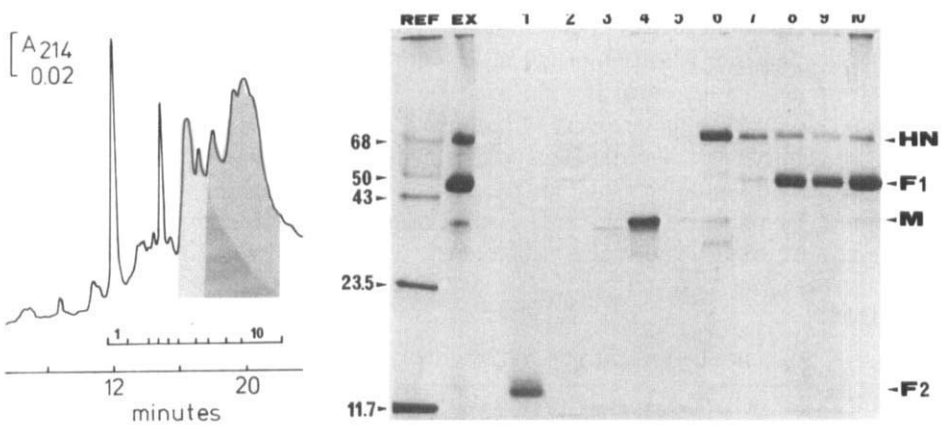

Fig. 12.4. RP-HPLC of a Triton X-100 extract of purified Sendai virus reduced with DTT. The Phenyl-5PW RP (Toyo Soda, Japan, now TosoHaas) column (50 × 4.6 mm i.d.) was eluted with a 24-min gradient, consisting of 15–75% acetonitrile in water containing 0.05% TFA. The flow rate was 1 ml/min and the absorbance was monitored at 214 nm. Fractions (1–10) were analyzed by SDS polyacrylamide gel electrophoresis (13% gels). The M_r ($\times 10^{-3}$) of reference proteins (REF) is indicated on the left. EX: the Triton extract; dotted area: HN protein; hatched area: F1 protein This manufacturer can be located using the Directory in Part D of this volume (from Ref. [43]).

at higher organic solvent concentration, between 44.5 and 52.5% as multiple peaks (Fig. 12.4, dotted area: HN; hatched area: F1). The multiple peaks may be caused by repeated precipitation and dissolution of the larger membrane proteins. Chromatography of the non-reduced extract [80] showed that the proteins in this extract, F (65 kDa), the dimer and tetramer of HN (136 and 272 kDa, respectively) could be eluted but that no separation was obtained. Again precipitation and dissolution may have been the principal cause of the broad peaks which were eluted at a similar organic solvent concentration (around 50%). Therefore, we expect that large integral membrane proteins will be more 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 RP-HPLC of small membrane proteins (less than 50 kDa) is more successful than that of larger proteins. At an equal percentage of hydrophobic residues, the organic solvent concentration necessary for protein desorption will increase with protein size. If conservation of biological activity is not crucial, a reduction of protein size generally will enhance protein recovery and separation efficiency. Several integral membrane proteins which have been purified by RP-HPLC are listed in Table 12.9.

The applicability of RP-columns for membrane proteins is not predictable from the performance with simple water-soluble reference proteins. We have evaluated several RP-HPLC column materials, differing with respect to bonded ligands, pore size and particle size, for the purification of membrane proteins [158]. These columns performed equally well with hydrophilic proteins; however their performance differed when Sendai virus membrane proteins were subjected to chromatography on 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% TFA in water/acetonitrile or 12 mM HCl in water/ethanol-*n*-butanol (4:1)) and on the column material in an interdependent way. 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. A 30–100 nm pore-size RP-material and an acetonitrile gradient in 0.05% TFA for elution is probably a good system to start with (see Table 12.15).

However, 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 explored. The use of solvents of higher eluotropic strength, for example 1-propanol or 2-propanol instead of acetonitrile, results in the elution of proteins at lower organic solvent concentrations, while it increases both resolution and

TABLE 12.15

RP-HPLC OF A DETERGENT EXTRACT OF REDUCED MEMBRANE PROTEINS

500 mg Amberlite XAD-2 is added to 1 ml detergent extract
20 min 37°C
After sedimentation, add dithiothreitol to supernatant (20 mM final concentration)
Repeat Amberlite XAD-2 incubation in the presence of dithiothreitol
Pass turbid supernatant through a 2-μm filter to remove Amberlite fines
Inject into RP-HPLC system (RP-HPLC support with at least 30-nm pores)
The column is run at 1 ml/min; absorbance monitored at 214 nm
24 min linear gradient from 10 to 75% acetonitrile in water containing 0.05% TFA
Collect fractions, dilute with water for freeze-drying

Abbreviations p. 527

recoveries [21,39,160]. To this end, also mixed organic phases are advantageous. The inner core proteins and the envelope proteins of murine leukemia virus [154] were successively 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. Improved chromatographic results were also obtained with large peptides of cytochrome P-450 [151] and the platelet-derived growth factor receptor [80], of flavivirus proteins [87] and Sendai virus membrane proteins [21,158] by elution with mixtures of acetonitrile/propanol or butanol/ethanol. These improvements are ascribed to the fact that the increase in eluent strength is larger than the increase of the denaturing and precipitating effects of mixed organic phases [159]. Substitution of TFA by less hydrophobic ionic modifiers like phosphoric acid or hydrochloric acid will generally reduce protein retention as well [21,159,160]. Also, high concentrations of formic acid (up to 60%) have been used for RPC of membrane proteins and polio virus capsid proteins [148,151,161,162]. Although short exposure appears not to be harmful [151,162], prolonged contact with high concentrations of formic acid may result in esterfication of Ser and Thr residues or in cleavage at Asp-Pro bonds.

12.5. WHERE TO GO FROM HERE

Several HPLC systems for purification of integral membrane proteins are available. The choice for a particular system or combination of systems will depend largely on the protein to be purified and whether a biologically active protein is required for further studies.

SE-HPLC is of limited use as a single method of purification. It is only useful when the desired membrane protein has a large difference in molecular weight compared to 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 is more useful in multidimensional chromatography, combined with other modes 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 one of the most versatile HPLC modes that can be applied to virtually all types of membrane proteins.

BAC is the most selective method but 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 (>50 kDa) are difficult to separate by this mode of HPLC. Hydrophobic ligands which resemble non-ionic detergents [163,164] 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 RPC mode. However for relatively small membrane proteins which do not have to retain their conformation, RP-HPLC is an excellent purification method resulting in proteins without any salt or detergent remaining.

Yet another useful approach to purify membrane proteins may be the application of

immobilized artificial membranes as a HPLC matrix. Several cytochrome P450 isozymes have been partially purified in this way [165].

12.6. TROUBLESHOOTING

Membrane proteins may contain one or more membrane-spanning regions predominantly consisting of hydrophobic amino acids. As a consequence, they are more hydrophobic than an average protein and therefore difficult to dissolve in aqueous solutions without the addition of any surfactant (detergent). Many of the problems in chromatographic purification of membrane proteins can be ascribed to solubility problems. There are several remedies for this depending on the chromatographic procedures used, the hydrophobicity of the protein, and whether the protein should retain its native conformation or not.

When extraction with a detergent results in a turbid solution or a solution that is too viscous for chromatography, another detergent may be the remedy. We have used $C_{12}E_5$ and $C_{10}E_5$ for extraction and subsequent HPLC. The less hydrophobic $C_{10}E_5$ with an HLB value of 12.5 was more useful in this respect than $C_{12}E_5$ which has an HLB value of 11.7 (see Table 12.3). More recently, we obtained similar results with a new amphoteric detergent DDMAU [17, 166].

When it is not important to retain the conformation, the protein will almost certainly dissolve in 4% SDS for 3 min in boiling water. Subsequent chromatography is then limited to SE-HPLC, where the buffer contains for example 0.1% SDS. SE-HPLC with non-ionic and amphoteric detergent in the eluent will almost certainly result in multiple peaks containing different multimeric forms of the proteins and therefore is not suitable in most cases for purification.

When detergents other than ionic detergents are used, the CMC should also be taken into account. A detergent with a high CMC, e.g. octylglucoside may have the advantage that it can be removed more easily by dialysis since it has a small micellar molecular weight (see Table 12.1). However, high concentrations (above the CMC) have to be used during chromatography in order to obtain a satisfactory separation. Moreover, it is generally advisable to have a mild detergent present in order to maintain biological activity.

RP-HPLC of membrane proteins may present another type of problem. The increasing concentration of organic solvent used for elution may result in on-column precipitation and low recoveries. Occasionally, the protein may dissolve again, but yields are generally low. This problem will occur more frequently with proteins with $M_r > 50\,000$. Such proteins will have a larger number of hydrophobic groups interacting with the reversed-phase column ligands and therefore a higher concentration of organic solvent will be needed for elution. A possible remedy may be elution with mobile phases containing a lower concentration of organic modifier of higher elutropic strength, e.g. propanol instead of acetonitrile. Proteins will then be eluted at a lower organic solvent concentration.

12.7. 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 one or more 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 HPLC, SE-HPLC), electrostatic interaction (ion exchange HPLC, IE-HPLC), bioaffinity (affinity chromatography, BAC), and hydrophobic interaction (reversed-phase HPLC, RP-HPLC). SE-HPLC, IE-HPLC and BAC are used under relatively mild conditions and buffers systems generally contain a non-ionic detergent. RP-HPLC generally has a denaturing effect on the protein and should preferably be used for the purification of integral membrane proteins smaller than 50 kDa.

12.8. REFERENCES

1. J. Van Renswoude and C. Kempf, Purification of integral membrane proteins, Methods Enzymol., 104 (1984) 329–339.
2. L.M. Hjelmeland, Solubilization of native membrane proteins, Methods Enzymol., 182 (1990) 253–264.
3. N. Unwin and R. Henderson, The structure of proteins in biological membranes, Sci. Am., 250 (1984) 56–66.
4. J. Deisenhofer, O. Epp, K. Miki, R. Huber and H. Michel, X-ray structure analysis of a membrane protein complex. Electron density map at 3 Å resolution and a model of the chromophores of the photosynthetic reaction center from *Rhodopseudomonas viridis*., J. Mol. Biol., 180 (1984) 385–398.
5. W. Kühlbrandt, D.G. Wang and Y. Fujiyoshi, Atomic model of plant light-harvesting complex by electron crystallography, Nature, 367 (1994) 614–621.
6. G. von Jagow and H. Schägger (Eds.), A Practical Guide to Membrane Protein Purification, Academic Press, San Diego, CA, 1994.
7. J.B.C. Findlay, Purification of membrane proteins, in: E.L.V. Harris and S. Angal (Eds.), Protein Purification Applications, a Practical Approach, IRL Press, Oxford, 1990, p. 59.
8. J.M. Neugebauer, Detergents: an overview, Methods Enzymol., 182 (1990) 239–253.
9. A. Helenius and K. Simons, Solubilization of membrane proteins, Biochim. Biophys. Acta, 415 (1975) 29–79.
10. C. Tanford and J.A. Reynolds, Characterization of membrane proteins in detergent solutions, Biochim. Biophys. Acta, 457 (1976) 133–170.
11. A. Helenius, D.R. McCaslin, E. Fries and C. Tanford, Properties of detergents, Methods Enzymol., 56 (1979) 734–749.
12. L.M. Hjelmeland and A. Crambach, Solubilization of functional membrane proteins, Methods Enzymol., 104 (1984) 305–318.
13. A. Gonenne and R. Ernst, Solubilization of membrane proteins by sulfobetaines, novel zwitterionic surfactants, Anal. Biochem., 87 (1978) 28–38.
14. D. Plusquellec, G. Chevalier, R. Talibart and H. Wróblewski, Synthesis and characterization of 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside, a new surfactant for membrane studies, Anal. Biochem., 179 (1989) 145–153.
15. J. van Ede, J.R.J. Nijmeijer, S. Welling-Wester, C. Örvell and G.W. Welling, Comparison of non-ionic detergents for extraction and ion exchange high-performance liquid chromatography of Sendai virus integral membrane proteins, J. Chromatogr., 476 (1989) 319–327.
16. J.E.K. Hildreth, *N*-D-Gluco-*N*-methylalkanamide compounds, a new class of non-ionic detergents for membrane biochemistry, Biochem. J., 207 (1982) 363–366.
17. C. Brenner, G. Jan, Y. Chevalier and H. Wróblewski, Evaluation of the efficacy of zwitterionic dodecyl

carboxybetaine surfactants for the extraction and the separation of Mycoplasma membrane protein antigens, Anal. Biochem., 22 (1995) 515–523.
18. A.J. Furth, Removing unbound detergent from hydrophobic proteins, Anal. Biochem., 109 (1980) 207–215.
19. A.J. Furth, H. Bolton, J. Potter and J.D. Priddle, Separating detergent from proteins, Methods Enzymol., 104 (1984) 318–328.
20. L.M. Hjelmeland, Removal of detergents from membrane proteins, Methods Enzymol., 182 (1990) 277–282.
21. R. van der Zee, S. Welling-Wester and G.W. Welling, Purification of detergent-extracted Sendai virus proteins by reversed-phase high-performance liquid chromatography, J. Chromatogr., 266 (1983) 577–584.
22. C.A. Kruse, E.B. Spector, S.D. Cederbaum, B.J. Wisniesky and G. Popjak, Microinjection of arginase into enzyme-deficient cells with the isolated glycoproteins of Sendai virus as fusogen, Biochim. Biophys. Acta, 645 (1981) 339–345.
23. P. Becher, in: M.J. Schick (Ed.), Non-ionic Surfactants, Marcel Dekker, New York, 1967, p. 608.
24. E. De Vendettis, G. Palumbo, G. Parlato and V. Bocchini, A fluorimetric method for the estimation of the critical micelle concentration of surfactants, Anal. Biochem., 115 (1981) 278–286.
25. V. De Pinto, R. Benz and F. Palmieri, Interaction of non-classical detergents with the mitochondrial porin; a new purification procedure and characterization of the pore-forming unit, Eur. J. Biochem., 183 (1989) 179–187.
26. J.N. Umbreit and J.L. Strominger, Relation of detergent HLB number to solubilization and stabilization of D-alanine carboxypeptidase from *Bacillus subtilis* membranes, Proc. Natl. Acad. Sci. USA, 70 (1973) 2997–3001.
27. P.C. Adams, F.D. Roberts, L.W. Powell and J.W. Halliday, Chromatographic detergent exchange in the preparation of membrane receptor protein, J. Chromatogr., 427 (1988) 341–344.
28. W. Kühlbrandt, Three-dimensional crystallization of membrane proteins, Quart. Rev. Biophys., 21 (1988) 429–477.
29. C. Bordier, Phase separation of integral membrane proteins in Triton X-114 solution, J. Biol. Chem., 256 (1981) 1604–1607.
30. P. Rottier, D. Brandenburg, J. Armstrong, B. van der Zeijst and G. Warren, Assembly in vitro of a spanning membrane protein of the endoplasmic reticulum: the E1 glycoprotein of coronavirus mouse hepatitis virus A59, Proc. Natl. Acad. Sci. USA, 81 (1984) 1421–1425.
31. H. Garoff, C. Kondor-Koch, R. Petterson and B. Burke, Expression of Semliki Forest virus proteins from cloned complementary DNA. II. The membrane-spanning glycoprotein E2 is transported to the cell surface without its normal cytoplasmic domain, J. Cell. Biol., 97 (1983) 652–658.
32. M. Pesonen, W. Ansorge and K. Simons, Transcytosis of the G protein of vesicular stomatitis virus after implantation into the apical plasma membrane of Madin–Darby Canine Kidney cells. I. Involvement of endosomes and lysosomes, J. Cell. Biol., 99 (1984) 796–802.
33. C. Tiruppathi, D.H. Alpers and B. Seetharam, Phase separation of rat intestinal brush border membrane proteins using Triton X-114, Anal. Biochem., 153 (1986) 330–335.
34. J. Bouvier, R.J. Etges and C. Bordier, Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes, J. Biol. Chem., 260 (1985) 15504–15509.
35. P.A. Maher and S.J. Singer, Anomalous interaction of the acetylcholine receptor protein with the non-ionic detergent Triton X-114, Proc. Natl. Acad. Sci. USA, 82 (1985) 958–962.
36. P.M. Snow, G. Keizer, J.E. Coligan and C. Terhorst, Purification and *N*-terminal amino acid sequence of the human T cell surface antigen T8, J. Immunol., 133 (1984) 2058–2066.
37. C.R. Parish, B.J. Classon, Tsagaratos, I.D. Walker, L. Kirszbaum and I.F.C. McKenzie, Fractionation of detergent lysates of cells by ammonium sulfate-induced phase separation, Anal. Biochem., 156 (1986) 495–502.
38. B. Fricke, Phase separation of non-ionic detergents by salt addition and its application to membrane proteins, Anal. Biochem., 212 (1993) 154–159.
39. G.W. Welling, J.R.J. Nijmeijer, R. Van der Zee, G. Groen, J.B. Wilterdink and S. Welling-Wester, Isolation of detergent-extracted Sendai virus proteins by gel-filtration, ion exchange and reversed-phase high-performance liquid chromatography and the effect on immunological activity, J. Chromatogr., 297 (1984) 101–109.

40. M. Rögner, Purification of membrane protein complexes isolated from a cyanobacterial thylakoid membrane by high-performance liquid chromatography, J. Chromatogr., 512 (1990) 219–226.
41. T. Ichimura, N. Ikuta, Y. Uda, T. Horigome and S. Omata, Separation of membrane proteins solubilized with a nondenaturing detergent and a high salt concentration by hydroxyapatite high-performance liquid chromatography, Anal. Biochem., 224 (1995) 250–255.
42. B. Lee and F.M. Richards, The interpretation of protein structures: estimation of static accessibility, J. Mol. Biol., 55 (1971) 379–400.
43. G.W. Welling, R. van der Zee and S. Welling-Wester, High-performance liquid chromatography of Sendai virus membrane proteins, Trends Anal. Chem., 5 (1986) 225–230.
44. H. Lüdi and W. Hasselbach, State of aggregation of detergent-solubilized sarcoplasmic reticulum adenosine triphosphatase investigated by high-performance liquid chromatography, J. Chromatogr., 297 (1984) 111–117.
45. J.P. Andersen, B. Vilsen, H. Nielsen and J.V. Møller, Characterization of detergent-solubilized sarcoplasmic reticulum Ca^{2+}-ATPase by high-performance liquid chromatography. Biochemistry, 25 (1986) 6439–7447.
46. M. le Maire, L.P. Aggerbeck, C. Montheilhet, J.P. Andersen and J.V. Møller, The use of high-performance liquid chromatography for the determination of size and molecular weight of proteins: a caution and a list of membrane proteins suitable as standards, Anal. Biochem., 154 (1986) 525–535.
47. T. Nakao, T. Ohno-Fujitani and M. Nakao, Sodium and potassium ion-dependent change in oligomerization of Na, K-ATPase in $C_{12}E_8$ detected by low-angle laser light scattering technique in combination with high performance porous silica gel chromatography, J. Biochem., 94 (1983) 689–697.
48. J.P. Walsh, R. Suen, R.N. Lemaitre and J.A. Glomset, Arachidonoyl-diacylglycerol kinase from bovine testis. Purification and properties, J. Biol. Chem., 269 (1994) 21155–21164.
49. J.R. Casey and R.A. Reithmeier, Detergent interaction with band 3, a model polytopic membrane protein, Biochemistry, 32 (1993) 1172–1179.
50. J.L. McGregor, P. Clezardin, M. Manach, S. Gronlund and M. Dechavanne, Tandem separation of labelled human blood platelet glycoproteins by anion exchange and gel fast protein liquid chromatography, J. Chromatogr., 326 (1985) 179–190.
51. L.J. DeLucas and D.D. Muccio, Purification of bovine rhodopsin by high-performance size exclusion chromatography, J. Chromatogr., 296 (1984) 121–128.
52. K. Löster, O. Baum, W. Hofmann and W. Reutter, Characterization of molecular aggregates of $\alpha_1\beta_1$-integrin and other rat liver membrane proteins by combination of size exclusion chromatography and chemical cross-linking, J. Chromatogr., 711 (1995) 187–199.
53. K-D. Irrgang, G. Renge and J. Vater, Isolation, purification and partial characterization of a 30-kDa chlorophyll-*a/b*-binding protein from spinach, Eur. J. Biochem., 201 (1991) 515–522.
54. U. Brandt, H. Schägger and G. Von Jagow, Purification of cytochrome-*c* oxidase retaining its pulsed form, Eur. J. Biochem., 182 (1989) 705–711.
55. K. Kita, H. Murakami, H. Oya and Y. Anraka, Quantitative determination of cytochromes in the aerobic respiratory chain of *Escherichia coli* by high-performance liquid chromatography and its application to analysis of mitochondrial cytochromes, Biochem. Int., 10 (1985) 319–326.
56. C.R. Loomis, J.P. Walsh and R.M. Bell, *sn*-1,2-diacylglycerol kinase of *Escherichia coli*, J. Biol. Chem., 260 (1985) 4091–4097
57. R.C. Montelaro, M. West and C.J. Issel, High performance gel permeation chromatography of protein in denaturing solvents and its application to the analysis of enveloped virus polypeptides, Anal. Biochem., 114 (1981) 398–406.
58. R.S. Matson and S.C. Goheen, Use of high-performance size exclusion chromatography to determine the extent of detergent solubilization of human erythrocyte ghosts, J. Chromatogr., 359 (1986) 285–295.
59. S. Khamlichi, M.J. Loirat, D. Blanchard, M. le Maire, P. Bailly, J.P. Cartron and O. Bertrand, Influence of the size of the polar head of non-ionic detergents on membrane proteins immunoaffinity purification, J. Biochem. Biophys. Methods, 29 (1994) 123–134.
60. F.Y.M. Choy, Purification of human placental glucocerebrosidase using a two-step high-performance hydrophobic and gel permeation column chromatography method, Anal. Biochem., 156 (1986) 515–520.
61. G.J. Murray, R.J. Youle, S.E. Gandy, G.C. Zirzow and J.A. Barranger, Purification of β-glucocerebrosidase by preparative-scale high-performance liquid chromatography: the use of ethyleneglycol-containing buffers for chromatography of hydrophobic glycoprotein enzymes, Anal. Biochem., 147 (1985) 301.

62. M. Wallstén and P. Lundahl, Binding of sodium dodecyl sulfate to an integral membrane protein and to a water-soluble enzyme; determination by molecular-sieve chromatography with flow scintillation detection, J. Chromatogr., 512 (1990) 3–12.
63. D.J. Clarke, J.N. Keen and B. Burchell, Isolation and characterization of a new hepatic bilirubin UDP-glucuronosyltransferase; absence from Gunn rat liver, FEBS Lett., 299 (1992) 183–186.
64. J. Roy Chowdury, N. Roy Chowdury, C.N. Falany, T.R. Tephly and I.M. Arias, Isolation and characterization of multiple forma of rat liver UDP-glucuronate glucuronosyltransferase, Biochem. J., 233 (1986) 827–837.
65. T. Konishi and M. Sasaki, Separation of membrane proteins of *H. halobium* by gel permeation high-performance liquid chromatography, Chem. Pharm. Bull., 30 (1982) 4208–4212.
66. R. Pabst, T. Nawroth and K. Dose, Time-dependent monomerization of bacteriorhodopsin in Triton X-100 solutions analyzed by high-performance liquid chromatography, J. Chromatogr., 285 (1984) 333–341.
67. T.J. McCarry and M.N. Al-Ahdal, Isolation of glycoprotein D from Herpes simplex virus type 1 by gel filtration high performance liquid chromatography, Biomed. Chromatogr., 3 (1989) 221–225.
68. D.H. Calam and J. Davidson, Isolation of influenza viral proteins by size exclusion and ion exchange high-performance liquid chromatography: the influence of conditions on separation, J. Chromatogr., 296 (1985) 285–292.
69. J.S. Lolkema, H. Kuiper, R.H. ten Hoeve-Duurkens and G.T. Robillard, Mannitol-specific enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system of *Escherichia coli*: physical size of enzyme II^{mtl} and its domains IIBA and IIC in the active state, Biochemistry, 32 (1993) 1396–1400.
70. D. Josiæ, H. Baumann and W. Reutter, Size exclusion high-performance liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins: a comparison, Anal. Biochem., 142 (1984) 473–479.
71. D. Josiæ, W. Hofmann, B. Wieland, R. Nuck and W. Reutter, Anion exchange high-performance liquid chromatography of membrane proteins from liver and Morris hepatomas, J. Chromatogr., 359 (1986) 315–322.
72. P. Lambotte, J. Van Snick and T. Boon, Partial purification of a membrane glycoprotein antigen by high-pressure size exclusion chromatography without loss of antigenicity, J. Chromatogr., 297 (1984) 139–145.
73. P. Gärtner, A. Ecker, R. Fischer, D. Linder, G. Fuchs and R.K. Thauer, Purification and properties of N^5-methyltetrahydromethanopterin:coenzyme M methyltransferase from *Methanobacterium thermoautotrophicum*, Eur. J. Biochem., 213 (1993) 537–545.
74. H. Kalbacher and H. Kropshofer, J. Chromatogr., 548 (1991) 343.
75. C. Sagné, M-F. Isambert, J-P. Henry and B. Gasnier, SDS-resistant aggregation of membrane proteins: application to the purification of the vesicular monoamine transporter, Biochem. J., 316 (1996) 825–831.
76. K. Haga and T. Haga, Purification of the muscarinic acetylcholine receptor from porcine brain, J. Biol. Chem., 260 (1985) 7927–7935.
77. G. Jan, C. Brenner and H. Wróblewski, Purification of *Mycoplasma gallisepticum* membrane proteins p52, p67 (pMGA), and p77 by high-performance liquid chromatography, Protein Expression Purification, 7 (1996) 160–166.
78. R. Reinards, J. Kubicki and H-D. Ohlenbusch, Purification and characterization of NADH oxidase from membranes of *Acholeplasma laidlawii*, a copper-containing iron-sulfur flavoprotein, Eur. J. Biochem., 120 (1981) 329–337.
79. A.W. Cripps, D.C. Taylor, F.J. Wallace and R.L. Clancy, Respiratory immunity stimulated by intestinal immunization with purified non-typeable *Haemophilus influenzae* antigens, J. Infect. Dis., 165 (suppl 1) (1992) S199–S201.
80. P. Tempst, D.D.-L. Woo, D.B. Teplow, R. Aebersold, L. Hood and S.B.H. Kent, Microscale structure analysis of a high-molecular weight, hydrophobic membrane glycoprotein fraction with platelet-derived growth factor-dependent kinase activity, J. Chromatogr., 359 (1986) 403–412.
81. J.E. Harlan, D. Picot, P.J. Loll and R.M. Garavito, Calibration of size exclusion chromatography: use of a double Gaussian distribution function to describe pore sizes, Anal. Biochem., 224 (1995) 557–563.
82. M.S. Swamy and E.C. Abraham, Glycation of lens membrane intrinsic proteins, Curr. Eye Res., 11 (1992) 833–842.
83. G.W. Welling, K. Slopsema and S. Welling-Wester, Purification strategies for Sendai virus membrane proteins, J. Chromatogr., 397 (1987) 165–174.
84. G.W. Welling, G. Groen, K. Slopsema and S. Welling-Wester, Combined size exclusion and reversed-

phase high-performance liquid chromatography of a detergent extract of Sendai virus, J. Chromatogr., 326 (1985) 173–178.

85. G.W. Welling, K. Slopsema and S. Welling-Wester, Size exclusion high-performance liquid chromatography of Sendai virus membrane proteins in different detergents; a comparison of different columns, J. Chromatogr., 359 (1986) 307–314.
86. G. Winkler, F.X. Heinz and C. Kunz, Exclusive use of the high-performance liquid chromatographic techniques for the isolation, 4 dimethylamino-azobenzene-4′-sulphonylchloride amino acid analysis and 4-*N*,*N*-dimethylaminobenzene-4′-isothiocyanate-phenylisothiocyanate sequencing of a viral membrane protein, J. Chromatogr., 297 (1984) 63–73.
87. G. Winkler, F.X. Heinz, F. Guirakhoo and C. Kunz, Separation of flavivirus membrane and capsid proteins by multi-step high-performance liquid chromatography optimized by immunological monitoring, J. Chromatogr., 326 (1985) 113–119.
88. S. Welling-Wester, B. Kazemier, C. Örvell and G.W. Welling, Effect of detergents on the structure of integral membrane proteins of Sendai virus studied with size exclusion high-performance liquid chromatography and monoclonal antibodies, J. Chromatogr., 443 (1988) 255–266
89. J.E. Walker, R. Lutter, A. Dupuis and M.J. Runswick, Identification of the subunits of F_1F_0-ATPase from bovine heart mitochondria, Biochemistry, 30 (1991) 5369–5378.
90. H.S. Van Walraven, M. Gravesen and R. Kraayenhof, Purification of a high molecular weight membrane protein by fast protein liquid chromatography: the ATPase complex of a thermophilic cyanobacterium, J. Biochem. Biophys. Methods, 9 (1984) 163–169.
91. P. Kårsnäs, J. Moreno-Lopez and T. Kristiansen, Bovine viral diarrhea virus: purification of surface proteins in detergent-containing buffers by fast protein liquid chromatography, J. Chromatogr., 266 (1983) 643–649.
92. J.G. Mohanty and Y. Elazhary, High-performance liquid chromatographic separation and immunological characterization of soluble bovine viral diarrhea virus antigen, J. Chromatogr., 435 (1988) 149–158.
93. N.E. Tandy, R.A. Dilley and F.E. Regnier, High-performance liquid chromatographic purification of the hydrophobic ω subunit of the chloroplast energy coupling complex, J. Chromatogr., 266 (1983) 599–607.
94. R.A. Kirken, A.J. Lincoln, P.S. Fink and L.J. Prochaska, High yield purification of a four subunit caa_3-type cytochrome oxidase from the thermophilic bacterium *Bacillus* PS3 using fast protein liquid chromatography, Protein Expression Purification, 6 (1995) 707–715.
95. Y-C. Liu, L.H. Sowdal and N.C. Robinson, Separation and quantitation of cytochrome *c* oxidase subunits by Mono-Q fast protein liquid chromatography and C18 reverse-phase high-performance liquid chromatography, Arch. Biochem. Biophys., 324 (1995) 135–142.
96. A.N. Kotake and Y. Funae, High-performance liquid chromatography technique for resolving multiple forms of hepatic membrane-bound cytochrome P-450, Proc. Natl. Acad. Sci. USA, 77 (1980) 6473–6475.
97. S.K. Bansal, J.H. Love and H.L. Gurtoo, Resolution of multiple forms of cytochrome P-450 by high-performance liquid chromatography, J. Chromatogr., 297 (1984) 119–127.
98. M. Kastner and T. Schulz, Ion exchange fast protein liquid chromatography; optimization of the purification of cytochrome P-450 from marmoset monkeys, J. Chromatogr., 397 (1987) 153–163.
99. E.M. David and A.J. Morgan, Efficient purification of Epstein–Barr virus membrane antigen gp340 by fast protein liquid chromatography, J. Immunol., Methods, 108 (1988) 231–236.
100. H. Ikigai, T. Nakae and Y. Kato, Analysis of membrane proteins by ion exchange high-performance liquid chromatography, J. Chromatogr., 322 (1985) 212–219.
101. R.A. Damhof, M. Feijlbrief, S. Welling-Wester and G.W. Welling, Purification of the integral membrane glycoproteins D of Herpes simplex virus types 1 and 2, produced in the recombinant baculovirus expression system, by ion exchange high-performance liquid chromatography, J. Chromatogr., 676 (1994) 43–49.
102. G. Kessie, D.M. Dela Cruz, M.A. Taha, F.J. Al-Shammary, A.F. Tawfik and M. N. Al-Ahdal, Processing of envelope polypeptides of Herpes simplex virus type 1. Demonstration of variation in different cell lines by high-performance liquid chromatography and radioimmunoprecipitation, J. Chromatogr., 652 (1994) 234–239.
103. W. Kramer, F. Girbig, U. Gutjahr and I. Leipe, Application of high-performance liquid chromatography to the purification of the putative intestinal peptide transporter, J. Chromatogr., 521 (1990) 199–210.

104. D.L. Narum, G.W. Welling and A.W. Thomas, Ion exchange-immunoaffinity purification of a recombinant baculovirus *Plasmodium falciparum* apical membrane antigen, PF83/AMA-1, J. Chromatogr., 657 (1993) 357–363.
105. P. Lundahl, E. Greijer, H. Lindblom and L. Fägerstam, Fractionation of human red cell membrane proteins by ion exchange chromatography in detergent on Mono Q, with special reference to the glucose transporter, J. Chromatogr., 297 (1984) 129–137.
106. Y. Kato, T. Kitamura, K. Nakamura, A. Mitsui, Y. Yamasaki and T. Hashimoto, High-performance liquid chromatography of membrane proteins, J. Chromatogr., 391 (1987) 395–407.
107. L. Rönnstrand, M.P. Beckmann, B. Faulders, A. Östman, B. Ek and C-H. Heldin, High-performance liquid chromatography of the receptor for platelet-derived growth factor from porcine uterus, J. Biol. Chem., 262 (1987) 2929–2932.
108. B. Colas, C. Cambillau, L. Buscail, M. Zeggari, J-P Esteve, V. Lautre, F. Thomas, N. Vaysse and C. Susini, Stimulation of a membrane tyrosine phosphatase activity by somatostatin analogues in rat pancreatic acinar cells, Eur. J. Biochem., 207 (1992) 1017–1024.
109. G.W. Welling, G. Groen and S. Welling-Wester, Isolation of Sendai virus F protein by anion exchange high-performance liquid chromatography in the presence of Triton X-100, J. Chromatogr., 266 (1983) 629–632.
110. S. Welling-Wester, R.M. Haring, H. Laurens, C. Örvell and G.W. Welling, Comparison of ion exchange high-performance liquid chromatography columns for purification of Sendai virus integral membrane proteins, J. Chromatogr., 476 (1989) 477–485.
111. G.W. Welling, Y. Hiemstra, M. Feijlbrief, C. Örvell, J. van Ede and S. Welling-Wester, Comparison of detergents for extraction and ion exchange high-performance liquid chromatography of Sendai virus membrane proteins, J. Chromatogr., 599 (1992) 157–162.
112. S. Welling-Wester, M. Feijlbrief, D.G.A.M. Koedijk, M.A. Braaksma, B.R.K. Douma and G.W. Welling, Effect of different amounts of the non-ionic detergents $C_{10}E_5$ and $C_{12}E_5$ present in eluents for ion exchange high-performance liquid chromatography of integral membrane proteins of Sendai virus, J. Chromatogr., 646 (1993) 37–44.
113. D.O. Quissell and L.M. Deisher, Purification and partial characterization of analogous 26-kDa rat submandibular and parotid gland integral membrane phosphoproteins that may have a role in exocytosis, Arch. Oral Biol., 4 (1992) 289–295.
114. D. Gerlier, F. Garnier and F. Forquet, Haemagglutinin of measles virus: purification and storage with preservation of biological and immunological properties, J. Gen. Virol., 69 (1988) 2061–2069.
115. S.J. Shire, L. Bock, J. Ogez, S. Builder, D. Kleid and D.M. Moore, Purification and immunogenicity of fusion VP1 protein of foot and mouth disease virus, Biochemistry, 23 (1984) 6474–6480.
116. M.A. Harrison, P.C. Jones, Y.I. Kim, M.E. Finbow and J.B. Findlay, Functional properties of a hybrid vacuolar H(+)-ATPase in Saccharomyces cells expressing the Nephrops 16-kDa proteolipid, Eur. J. Biochem., 221 (1994) 111–120.
117. N.E. David, M. Gee, B. Andersen, F. Naider, J. Thorner and R.C. Stevens, Expression and purification of the *Saccharomyces cerevisiae* α-factor receptor (Ste2p), a 7-transmembrane-segment G-protein-coupled receptor, J. Biol. Chem., 272 (1997) 15553–15561.
118. E. Tamai, M-C. Fann, T. Tsuchiya and P.C. Maloney, Purification of UhpT, the sugar phosphate transporter of *Escherichia coli*, Protein Expression Purification, 10 (1997) 275–282.
119. S. Cohen, H. Ushiro, C. Stocheck and M. Chinkers, A native 170 000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles, J. Biol. Chem., 257 (1982) 1523–1531.
120. U. Svensson, R. Persson and E. Everitt, Virus-receptor interaction in the Adenovirus system I. Identification of virion attachment proteins of the HeLa cell plasma membrane, J. Virol., 38 (1981) 70–81.
121. B. Haas, H. Brecht and R. Rott, Purification and properties of an intranuclear virus-specific antigen from tissue infected with Borna disease virus, J. Gen. Virol., 67 (1986) 235–241.
122. E. Norrby, G. Utter, C. Orvell and M.J.G. Appel, Protection against canine distemper virus in dogs after immunization with isolated fusion protein, J. Virol., 58 (1986) 536–541.
123. A. Gal, G. Hauska, R. Herrmann and I. Ohad, Interaction between light harvesting chlorophyll-*a/b* protein (LHCII), kinase and cytochrome b_6/f complex, J. Biol. Chem., 265 (1990) 19742–19749.
124. H-P. Braun and U.K. Schmitz, Affinity purification of cytochrome *c* reductase from potato mitochondria, Eur. J. Biochem., 208 (1992) 761–767.
125. H. Weiss and B. Juchs, Isolation of a multiprotein complex containing cytochrome *b* and c_1 from *Neuro-*

spora crassa mitochondria by affinity chromatography on immobilized cytochrome *c*, Eur. J. Biochem., 88 (1978) 17–28.

126. E. Gonczol, F. Hudecz, J. Ianacono, B. Dietzschold, S. Starr and S. A. Plotkin, Immune responses to isolated human cytomegalovirus envelope proteins, J. Virol., 58 (1986) 661–664.
127. G.R. Nemerow, M.F. Siaw and N.R. Cooper, Purification of the Epstein–Barr virus/C3d complement receptor of human B lymphocytes: antigenic and functional properties of the purified protein, J. Virol., 58 (1986) 709–712.
128. B. Vroman, J. Luka, M. Rodriguez and G.P. Pearson, Characterization of a major protein with a molecular weight of 160 000 associated with the viral capsid of Epstein–Barr virus, J. Virol., 53 (1985) 107–113.
129. M. Rakowska, J. Zborowski and L. Corazzi, A fusogenic protein from rat brain microsomal membranes: partial purification and reconstitution into liposomes, J. Membrane Biol., 142 (1994) 35–42.
130. D.E. Wampler, E.B. Buynak, B.J. Harder, A.C. Herman, M.R. Hilleman, W.J. McAleer and E.M. Scolnick, Hepatitis-B vaccine purification by immunoaffinity chromatography, in: R.M. Chanock and R.A. Lerner (Eds.), Modern Approaches to Vaccines, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984, p. 251.
131. R.J. Eisenberg, M. Ponce de Leon, L. Pereira, D. Long and G.H. Cohen, Purification of glycoprotein gD of Herpes simplex virus types 1 and 2 by use of monoclonal antibody, J. Virol., 41 (1982) 1099–1104.
132. M. Zweig, S.D. Showalter, D.J. Simms and B. Hampar, Antibodies to a synthetic oligopeptide that reacts with Herpes simplex virus type 1 and 2 glycoprotein C, J. Virol., 51 (1984) 430–436.
133. D.J. Vaughan, L.M. Banks, D.J. Purifoy and K.L. Powell, Interactions between Herpes simplex virus DNA-binding proteins, J. Gen. Virol., 65 (1984) 2033–2041.
134. J.V. Babashak and T.M. Phillips, Isolation of a specific membrane protein by immunoaffinity chromatography with biotinylated antibodies immobilized on avidin-coated glass beads, J. Chromatogr., 476 (1989) 187–194.
135. J.F. Penrose, J. Spector, B.K. Lam, D.S. Friend, K. Xu, R.M. Jack and K.F. Austen, Purification of human lung leukotriene C_4 synthase and preparation of a polyclonal antibody, Am. J. Respir. Crit. Care Med., 152 (1995) 283–289.
136. T.M. Varsanyi, G. Utter and E. Norrby, Purification, morphology and antigenic characterization of measles virus envelope components, J. Gen. Virol., 65 (1984) 355–366.
137. S. Haldar, N. Jena, G.C. DuBois, S. Takayama, J.C. Reed, S.S. Fu and C.M. Croce, Purification and characterization of the bcl-2 protein, Arch. Biochem. Biophys., 315 (1994) 483–488.
138. J.N. Kinkel, B. Anspach, K.K. Unger, R. Wieser and G. Brunner, Separation of plasma membrane proteins of cultured human fibroblasts by affinity chromatography on bonded microparticulate silicas, J. Chromatogr., 297 (1984) 167–177.
139. G. Walter, A.M. Hutchinson, T. Hunter and W. Eckhart, Purification of polyoma virus medium-size tumor antigen by immunoaffinity chromatography, Proc. Natl. Acad. Sci. USA, 79 (1982) 4025–4029.
140. E.E. Walsh, M.W. Brandriss and J.J. Schlesinger, Purification and characterization of the respiratory syncytial virus fusion protein, J. Gen. Virol., 66 (1985) 409–415.
141. J.E. Tomassini and R.J. Colonno, Isolation of a receptor protein involved in attachment of human rhinoviruses, J. Virol., 58 (1986) 290–295.
142. M.N. Al-Ahdal, I. Nakamura and T.D. Flanagan, Cytotoxic T-lymphocyte reactivity with individual Sendai virus glycoproteins, J. Virol., 54 (1985) 53–57.
143. R. van der Zee and G.W. Welling, Detection of Sendai virus protein by reversed-phase high-performance liquid chromatography combined with immuno-chromatography, J. Chromatogr., 327 (1985) 377–380.
144. J.P. McGillis, M.L. Organist and D.G. Payan, High-performance immunoaffinity chromatographic isolation of the substance P neuropeptide receptor. Presented at the 10th Int. Symp. on Column Liquid Chromatography, San Francisco, CA, May 18–23, 1986, abstract 3105.
145. Z. Wroblewska, D. Gilden, M. Green, M. Devlin and A. Vafai, Affinity-purified Varicella–Zoster virus glycoprotein gp1/gp3 stimulates the production of neutralizing antibody, J. Gen. Virol., 66 (1985) 1795–1799.
146. W.E. Friedrich and C. Grose, Glycoprotein gp118 of varicella-zoster virus: Purification by serial affinity chromatography, J. Virol., 49 (1984) 992–996.
147. D. Josi, W. Hofmann, R. Habermann and W. Reutter, High-performance concanavalin A affinity chromatography of liver and hepatoma membrane proteins, J. Chromatogr., 444 (1988) 29–39.

148. M.J. Liao, K.S. Huang and H.G. Khorana, Regeneration of native bacteriorhodopsin structure from fragments, J. Biol. Chem., 259 (1984) 4200–4204.
149. D.P. Knowles, W.P. Cheevers, T.C. McGuire, A.L. Brassfield, W.G. Harwood and T.A. Stem, Structure and genetic variability of envelope glycoproteins of two antigenic variants of caprine arthritis-encephalitis lentivirus, J. Virol., 65 (1991) 5744–5750.
150. J-P. Capony, M.L. Rinaldi, F. Guilleux and J.G. Demaille, Isolation of cardiac membrane proteolipids by high pressure liquid chromatography; a comparison of reticular and sarcolemmal proteolipids, phospholamban and calciductin, Biochim. Biophys. Acta, 728 (1983) 83–91.
151. G.E. Tarr and J.W. Crabb, Reversed-phase high-performance liquid chromatography of hydrophobic proteins and fragments thereof, Anal. Biochem., 131 (1983) 99–107.
152. G.R. Whittaker and D.M. Meredith, Purification of the structural proteins of herpes simplex virus type 1 by reversed-phase high-performance liquid chromatography, Arch. Virol., 114 (1990) 271–276.
153. M.A. Phelan and K.A. Cohen, Gradient optimization principles in reversed-phase high-performance liquid chromatography and the separation of influenza virus components, J. Chromatogr., 266 (1983) 55–66.
154. L.E. Henderson, R. Sowder, T.D. Copeland, G. Smythers and S. Oroszlan, Quantitative separation of murine leukemia virus proteins by reversed-phase high-pressure liquid chromatography reveals newly described *gag* and *env* cleavage products, J. Virol., 52 (1984) 492–500.
155. R.P. Lee, S.W. Doughty, K. Ashman and J. Walker, Purification of hydrophobic integral membrane proteins from *Mycoplasma hyopneumoniae* by reversed-phase high-performance liquid chromatography, J. Chromatogr. 737 (1996) 273–279.
156. K.R. Brunden, C.T. Berg and J.F. Poduslo, Isolation of an integral membrane glycoprotein by chloroform-methanol extraction and C_3-reversed phase high-performance liquid chromatography, Anal. Biochem., 164 (1987) 474–481.
157. D.R. Barnidge, E.A. Dratz, J. Sunner and A.J. Jesaitis, Identification of transmembrane tryptic peptides of rhodopsin using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Protein Science, 6 (1997) 816–824.
158. R. van der Zee, T. Hoekzema, S. Welling-Wester and G.W. Welling, Comparison of reversed-phase column materials for high-performance liquid chromatography of proteins, J. Chromatogr., 368 (1986) 283–289.
159. J.P. Chang, W.R. Melander and C. Horvath, Reversed-phase chromatography of proteins with diphenyl-silica columns and hydro-organic eluents containing two organic solvents, J. Chromatogr., 318 (1985) 11–21.
160. J. Luiken, R. van der Zee and G.W. Welling, Structure and activity of proteins after reversed-phase high-performance liquid chromatography, J. Chromatogr., 284 (1984) 482–486.
161. J. Heukeshoven and R. Dernick, Reversed-phase high-performance liquid chromatography of virus proteins and other large hydrophobic proteins in formic acid containing solvents, J. Chromatogr., 252 (1982) 241–254.
162. J. Heukeshoven and R. Dernick, Characterization of a solvent system for separation of water-insoluble poliovirus proteins by reversed-phase high-performance liquid chromatography, J. Chromatogr., 326 (1985) 91–101.
163. N.T. Miller, B. Feibush and B.L. Karger, Wide-pore silica-based ether-bonded phases for separation of proteins by high-performance hydrophobic-interaction and size exclusion chromatography, J. Chromatogr., 316 (1985) 519–536.
164. J.-P. Chang, Z. El Rassi and C. Horvath, Silica-bound polyethyleneglycol as stationary phase for separation of proteins by high-performance liquid chromatography, J. Chromatogr., 319 (1985) 396–399.
165. C. Pidgeon, J. Stevens, S. Otto, C. Jefcoate and C. Marcus, Immobilized artificial membrane chromatography: rapid purification of functional membrane proteins, Anal. Biochem., 194 (1991) 163–173.
166. S. Welling-Wester, M. Feijlbrief, D.G.A.M. Koedijk and G.W. Welling, Detergent extraction of herpes simplex virus type 1 glycoprotein D by zwitterionic and non-ionic detergents and purification by ion-exchange high-performance liquid-chromotography, J. Chromotogr. A, 816 (1998) 29–37.