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PURIFICATION STRATEGIES FOR SENDAI VIRUS MEMBRANE PROTEINS

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SUMMARY

Viral membrane proteins extracted from Sendai virions with the non-ionic detergents decylpolyethyleneglycol-300 and Triton X-100 were used as a model mixture of hydrophobic membrane proteins. The detergent extract contained the fusion protein (F) and the tetrameric and dimeric forms of the hemagglutinin-neuraminidase protein (HN). These proteins were purified by size-exclusion high-performance liquid chromatography (HPLC) in the presence of 0.1% sodium dodecyl sulphate, by ion-exchange and metal chelate affinity HPLC in the presence of 0.1% decylpolyethyleneglycol, and by reversed-phase HPLC without prior removal of the detergent. The tetramer of HN and F could be purified by size-exclusion HPLC after dissociation of a micellar aggregate containing tetrameric HN and multimeric F. The F and HN proteins could be purified by ion-exchange HPLC. Pure F protein could be obtained after metal chelate affinity HPLC. The F protein and the dimer and tetramer of HN could be eluted from a large-pore (100 nm) reversed-phase column, but they were eluted as broad, overlapping peaks. Only after reduction of the virion extract, the relatively small (13–15 kilodaltons) F_2 protein could be obtained in pure form.

INTRODUCTION

When membrane proteins are purified by high-performance liquid chromatography (HPLC), they generally behave differently from the hydrophilic reference proteins used to investigate the performance of the many currently available HPLC-sorbents. Many proteins are much more hydrophobic than the reference proteins and, as a consequence, show a tendency to aggregate. Because of this, they are often difficult to purify. The strategy for purifying such a protein strongly depends on its properties and its ultimate application. We have used Sendai virus proteins as model compounds for the development of HPLC methods for the purification of hydrophobic membrane proteins. Size-exclusion, ion-exchange and reversed-phase HPLC were used to purify these proteins[1,2].

Sendai virus is a paramyxovirus of mice and belongs to the same genus as the human para-influenza viruses. The Sendai virus envelope is composed of a lipid bi-

layer in which two integral membrane proteins are embedded, the hemagglutinin-neuraminidase (HN, M_r = 68 000) and the fusion protein (F, M_r = 65 000). These proteins are present as spikes on the outside of the virus particle, extending into the solvent. The HN protein is found in the monomeric, a dimeric and a tetrameric form, while the F protein consists of two components F_1 (M_r = 50 000) and F_2 (M_r = 13 000–15 000) which are connected by one disulfide bridge. Recently, the base sequences of the Sendai virus proteins were determined[3-5]; the molecular weights based on the amino acid sequences, are 61 495 and 63 408 for F and HN, respectively. However, these figures do not include carbohydrate groups and, therefore, we will use the earlier mentioned higher molecular weights, which are based on data from sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE).

In the present study, we investigated the suitability of various HPLC methods for the purification of hydrophobic membrane proteins. This study included repeated size-exclusion HPLC, reversed-phase HPLC with a large-pore (100 nm) packing material and metal chelate affinity and ion-exchange HPLC in the presence of the non-UV absorbing detergent decylpolyethyleneglycol-300 (decyl-PEG).

EXPERIMENTAL

Virus isolation and detergent extraction

Sendai virus was grown in 10-day-old embryonated chicken eggs. Allantoic fluid was collected after 48 h of virus growth at 36°C. Debris was pelleted at 2000 *g* for 20 min and virions were pelleted at 22 000 rpm (70 000 *g*) for 30 min. The virions were suspended in 5 m*M* Tris–HCl (pH 7.2) and extracted with decyl-PEG-300 4/5 (Janssen Chimica, Beerse, Belgium or Kwant-Hoog Vacolie Recycling and Synthese, Bedum, The Netherlands) or Triton X-100 (BDH, Poole, U.K.) at a final concentration of 2% (w/w) for 20 min at room temperature. The detergent to viral protein ratio was 2 (w/w). After centrifugation for 90 min at 100 000 *g*, the extracted viral proteins HN and F were present in the supernatant, which was stored in 200 µl portions at −80°C.

HPLC (size-exclusion, ion-exchange, metal-chelate and reversed-phase)

Chromatography was performed with a system consisting of a Waters M 6000A pump (Waters, Etten-Leur, The Netherlands) or a LKB 2150 pump (LKB, Zoetermeer, The Netherlands), a Rheodyne 7125 injector (Inacom, Veenendaal, The Netherlands), and a Waters 441 detector or a Pye Unicam LC-UV detector (Philips, Eindhoven, The Netherlands). Gradients were generated by a low-pressure mixing system, existing of an Acorn computer (Acorn, Cambridge, U.K.), interfaced with a three-way solenoid valve (Lee, Westbrook, CT, U.S.A.)[6].

Size-exclusion HPLC was performed on a TSK 4000SW column (600 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan) by elution with 0.1% SDS in 50 m*M* sodium phosphate (pH 6.5). Samples (200 µl detergent extract) were subjected to chromatography without any pretreatment. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm.

Anion-exchange HPLC was carried out on a Mono Q HR 5/5 (50 × 5 mm I.D.) column (Pharmacia, Uppsala, Sweden), which was eluted with a 24-min gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* Tris–HCl (pH 7.8), containing

0.1% (w/w) decyl-PEG. The flow-rate was 1 ml/min and the absorbance was moni-
tored at 280 nm.

Metal chelate affinity HPLC was carried out on a TSK Chelate-5PW (75 ×
7.5 mm I.D.) column (Toyo Soda). Two different gradient programs were used for
elution. The first program was essentially as described by Kato et al.[7], except that
we added 0.1% (w/w) decyl-PEG to the buffers. Briefly: the column was washed with
10 ml 0.05 M EDTA in 0.5 M sodium chloride and then equilibrated with 5 ml of
20 mM Tris–HCl (pH 8.0), containing 0.5 M sodium chloride and 0.1% decyl-PEG
(buffer A). Then the column was loaded with 5 ml of 0.2 M zinc chloride and equi-
librated again with 15 ml of buffer A. The column was eluted with a 30 min-gradient
from 0 to 0.1 M glycine in buffer A at a flow-rate of 0.5 ml/min. In the second
program, both buffers contained 0.2 M sodium acetate (pH 7.0) and 0.1% (w/w)
decyl-PEG. In addition, buffer A contained 0.5 M sodium chloride, and buffer B
contained 0.5 M ammonium chloride. The column was equilibrated with buffer A,
and subsequently loaded with 5 ml of 0.2 M zinc chloride in buffer A and 10 ml of
buffer B. Before application of the sample, the column was re-equilibrated with buffer
A and eluted with a 30-min gradient from buffer A to buffer B at a flow-rate of 0.5
ml/min. The absorbance was monitored at 280 nm.

Reversed-phase HPLC was performed by eluting detergent extracts from a
Phenyl 5PW-RP column (50 × 4.6 mm I.D., Toyo Soda) with a 24-min gradient
from 15 to 75% acetonitrile in water containing 0.05% trifluoroacetic acid. The flo-
w-rate was 1 ml/min, and the absorbance was monitored at 214 nm. In some cases,
the extracts were reduced by incubation at 37°C for 20 min in 20 mM dithiotreitol
(DTT).

Fractions were collected in 11 × 70 mm minisorp-tubes (Nunc, Roskilde, Den-
mark). After reversed-phase HPLC, the organic solvent was removed by evaporation
in a SpeedVac centrifuge (Savant Instruments, Hicksville, NY, U.S.A.) and the re-
maining aqueous solution was freeze-dried. Fractions obtained after size-exclusion,
anion-exchange, and metal chelate HPLC were dialyzed against water by covering
the minisorp-tubes with a square piece of dialysis tubing and closing them by fitting
a slice of silicone tubing (1.5 cm diameter) over the dialysis membrane. After dialysis,
the fractions were freeze-dried in the tubes.

Analysis of column eluates

The eluate fractions were analyzed on 8, 10, or 12.5% SDS-polyacrylamide
gels[8]. Polypeptide bands were visualized by silver-staining[9].

RESULTS

Size-exclusion HPLC

When a Triton X-100 extract of Sendai virus is boiled and reduced prior to
chromatography in 0.1% SDS, the HN and F_1 proteins cannot be separated (Fig.
1, +red). Without reduction, the di and tetrameric forms of HN remain intact, and
separation is obtained between F and these forms of HN (Fig. 1, –red and ref. 1). In
the present study, the detergent extract was subjected to size-exclusion HPLC without
any pretreatment. The elution pattern is shown in Fig. 2A together with an analysis
by SDS-PAGE. F protein is present in all fractions, and the dimer and tetramer of
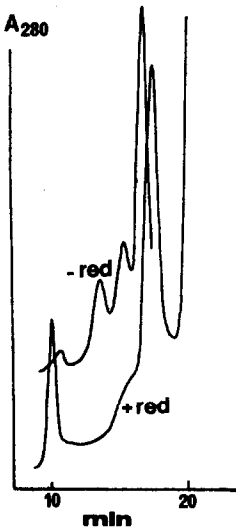
Fig. 1. Size-exclusion HPLC of a Triton X-100 extract of purified Sendai virions after reduction with DTT
(+red) or without reduction (−red). Column, TSK 4000SW; eluent, 50 mM sodium phosphate (pH 6.5),
containing 0.1% SDS; flow-rate, 1 ml/min; UV detection at 280 nm. The tetramer and dimer of HN, and
the F_1 protein were eluted at 13.7, 15.4, and 16.9 min, respectively (−red pattern) and the F protein at
17.5 min (+red pattern).

HN are mainly present in fractions 3–5 and 1–3, respectively. Fraction 1, containing
tetrameric HN and F, was dialyzed against water, freeze-dried and again subjected
to size-exclusion HPLC on TSK 4000SW under the same conditions. The elution
pattern and the SDS-PAGE analysis in Fig. 2B show that an excellent separation of
the tetramer HN from the F protein is achieved.

Anion-exchange HPLC

Anion-Exchange HPLC was carried out as described earlier[1], except that 0.1%
of the non-UV absorbing detergent decyl-PEG was used instead of Triton X-100.
The separation was as before[1]: HN was eluted first in several sharp peaks, followed
by a broad peak containing the F protein.

Metal chelate affinity HPLC

Two different gradient programs were used: In the first program, a glycine
gradient was used to elute the proteins, and in the second a gradient from sodium
chloride to ammonium chloride. In both programs, 0.1% decyl-PEG was present in
the buffers. Both programs gave essentially the same results. An example obtained
with the second program is shown in Fig. 3. The F protein was hardly retarded and
was eluted in several peaks. The HN protein was retarded and was eluted in peaks
4 and 5.

Reversed-phase HPLC

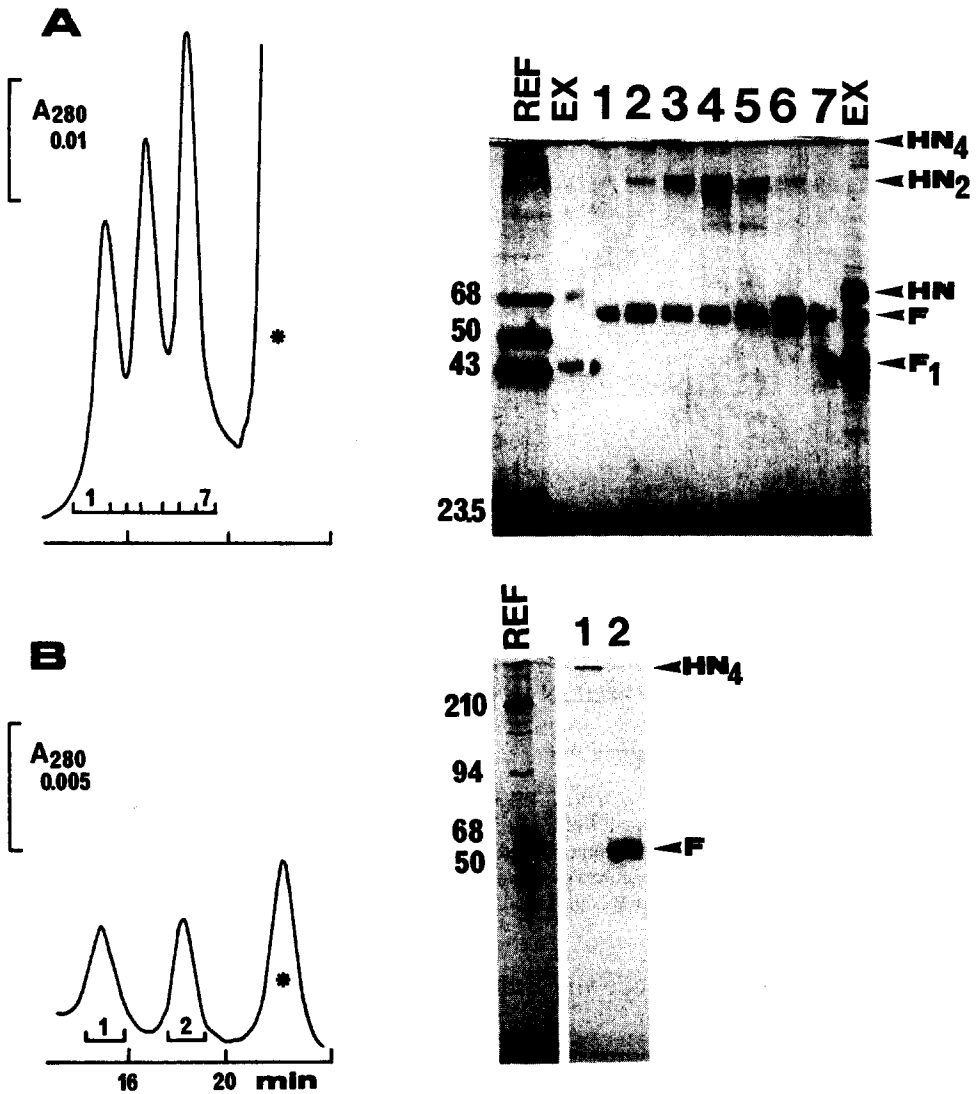A non-reduced Sendai virus extract was applied to a 100 nm reversed-phase

Fig. 2. Size-exclusion HPLC under the same elution conditions as in Fig. 1. Fractions (1–7) were analyzed by SDS-PAGE on 10% gels. The reference protein sample (REF) and the extract (EX) were reduced before electrophoresis. (A) A Triton X-100 (200 μl) extract was subjected to chromatography; (B) fraction 1 from Fig. 2A was dialyzed against water, freeze-dried, and subjected to size-exclusion HPLC under the same elution conditions as above. The molecular weight of reference proteins is indicated in kilodaltons. * = Triton X-100.

column without removal of the decyl-PEG. Two relatively broad peaks were obtained, both of which contained the tetramer and the dimer of HN and the F protein (see Fig. 4). In addition, the same procedure was applied to a reduced Sendai virus extract. Results were similar to those described earlier[2]. The Sendai virus membrane
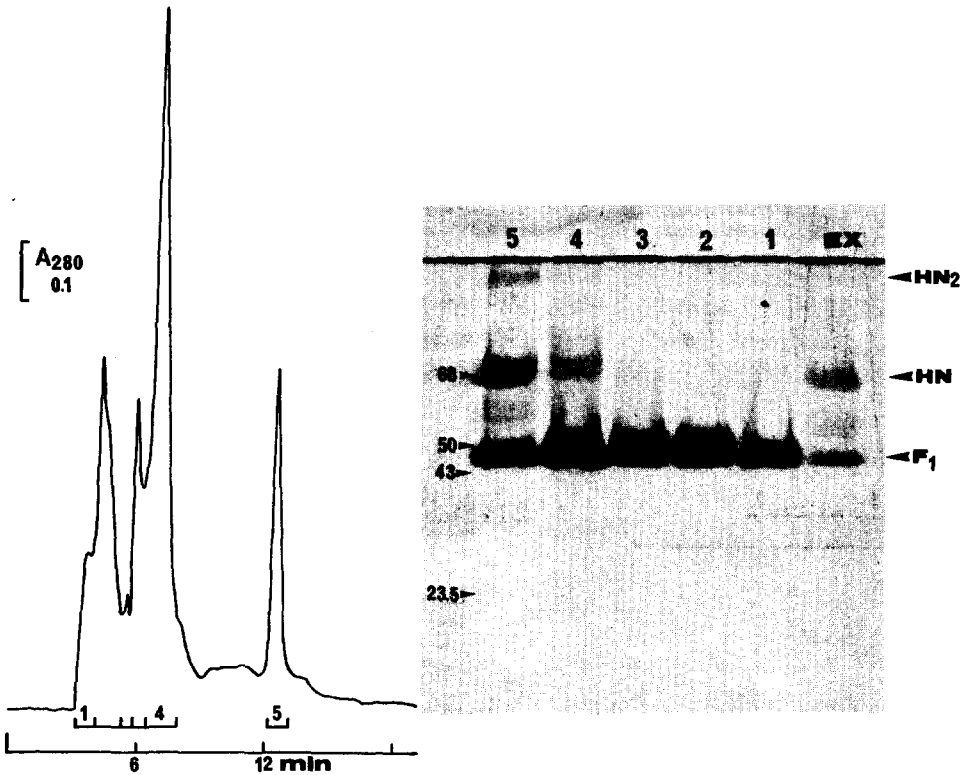
Fig. 3. Metal chelate affinity HPLC of a decyl-PEG extract of purified Sendai virions. TSK Chelate-5PW column loaded with Zn^{2+} and eluted with a 30-min gradient from 0.5 M sodium chloride to 0.5 M ammonium chloride, both in 0.2 M sodium acetate (pH 7.0), containing 0.1% decyl-PEG; flow-rate, 0.5 ml/min; UV detection at 280 nm. Fractions (1–5) were analyzed by SDS-PAGE (12.5% gel). The molecular weight of reference proteins (left lane) is indicated in kilodaltons. EX = the decyl-PEG extract.

proteins were eluted in the following order: F_2, HN, and then F_1. The detergent did not interfere with the elution.

Yields

Yields for most proteins varied from 50–90% independent of the HPLC method. Quantitative recoveries were obtained after reversed-phase HPLC of the F_2 protein.

DISCUSSION

The non-ionic detergent decyl-PEG, which contains an average of 7 oxyethy-lene units, was shown to be more efficient than Triton X-100 in extracting the HN and F proteins from purified Sendai virions. More than twice as much protein was extracted from the lipid bilayer[2]. Decyl-PEG also has the convenient property that it is transparent at 280 nm, and, therefore, we have used it as an additive in the gradient programs for ion-exchange and metal chelate affinity HPLC. Moreover,
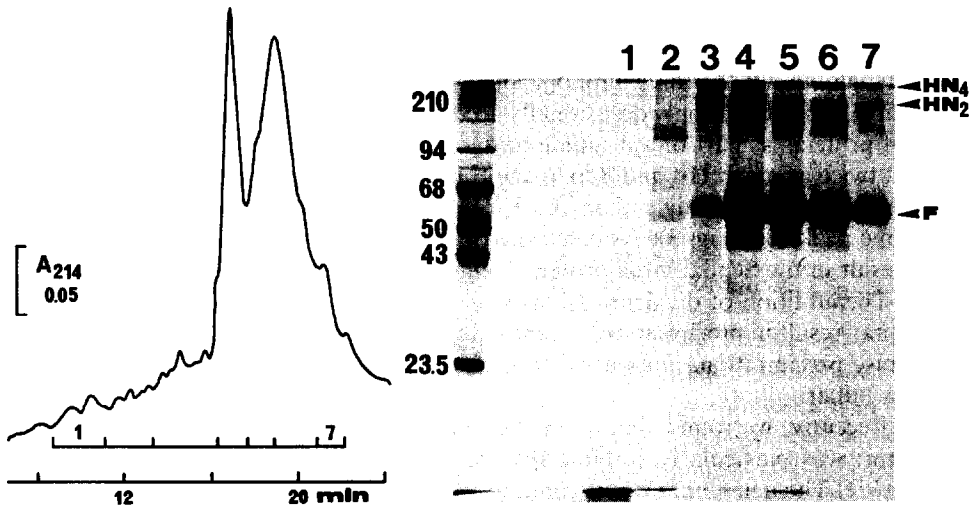
Fig. 4. Reversed-phase HPLC of a decyl-PEG extract of purified Sendai virions on a Phenyl 5PW-RP column, which was eluted with a 24-min gradient of 15–75% acetonitrile in water, containing 0.05% TFA. Flow-rate, 1 ml/min; UV detection at 214 nm. Fractions (1–7) were analyzed by SDS-PAGE on 8% gels. The molecular weight of reference proteins (left lane) is indicated in kilodaltons.

decyl-PEG does not interfere with detection at 214 nm in reversed-phase HPLC, and, as a consequence, it was not necessary to remove the detergent prior to chromatography.

Size-exclusion HPLC of a boiled Sendai virus extract yields relatively pure proteins because of the large difference in molecular weight between the tetramer and the dimer of HN and the F protein (272, 136 and 65 kilodaltons, respectively). Nevertheless, the F peak still contains the HN dimer. In an earlier paper[10], we used decyl-PEG in the elution buffer for size-exclusion HPLC. This resulted in a number of broad peaks, all of which contained the F protein. It was concluded that micelles of different size were separated. In the presence of SDS, a similar phenomenon is observed when the protein sample is not boiled prior to chromatography. The F protein is eluted in multimeric forms from the size-exclusion column. Dialysis against water is sufficient to break up the large micelles, which, in addition to the F protein, contain the HN multimers. These effects were utilized to achieve the separation shown in Fig. 2B, which yielded a pure preparation of F protein and the tetramer of HN.

In the elution buffers for ion-exchange HPLC we have used Triton X-100[1], which has the disadvantage that it strongly absorbs at 280 nm. In another study[11] we also used Brij 35, which does not absorb at 280 nm. This resulted in a similar separation, but tailing of peaks was observed. The addition of 0.1% decyl-PEG did not give rise to these problems and it does not interfere with protein detection at 280 nm.

Metal chelate affinity chromatography was introduced by Porath et al.[12] Separation is based on differences in affinity of the column matrix, which contains iminodiacetic acid with a metal ion, for particular amino acid side chains in a protein. The most important amino acids in this respect are His, Trp, and Cys[12,13]. However,

these particular amino acids in a protein will not always have access to the column ligand. Lee and Richards[14] studied the side chain accessibility in RNase S, lysozyme and myoglobin. Of the three amino acids, His, Trp, and Cys, His and Trp are often accessible, while Cys is only occasionally accessible. Cys is often located in the interior part of proteins as part of a disulfide bridge. The Sendai virus HN protein contains almost twice as much His and Trp as the F protein, which may explain why the HN protein was retained longer than the F protein. The proteins were eluted in more than one peak (See Fig. 3). As mentioned before, mild chromatographic conditions may result in the Sendai virus proteins being contained in different micelles.

The addition of divalent cations (e.g. Fe^{2+}, Ca^{2+}, Co^{2+}) to non-ionic detergents may result in precipitation[15]. Zinc chloride in combination with decyl-PEG did not cause precipitation, in contrast to Cu^{2+}. Therefore Zn^{2+}, could be used in the elution buffer.

Recently, we found that a reversed-phase packing material with large pores (100 nm) was particularly suitable for the purification of a reduced Sendai virus extract[2,16]. The detergent extract contained the 13–15 kilodaltons F_2 protein, the 50 kilodaltons F_1 protein and the monomer of the HN protein (68 kilodaltons). When the extract was not reduced, it contained proteins with much higher molecular weights (up to 272 kilodaltons). In the present study, the unreduced extract was chromatographed on the RPLC column. The results (Fig. 4) show that the large proteins could be eluted but no separation between the tetramer and the dimer of

TABLE I

HYDROPHOBICITY, EXPRESSED AS THE PERCENTAGE OF LEUCINE, ISOLEUCINE, VALINE AND METHIONINE (LIVM), AND MOLECULAR WEIGHTS OF PROTEINS WITH AT LEAST ONE TRANSMEMBRANE SPANNING REGION

Reference proteins (ribonuclease, lysozyme, ovalbumin) and the "average protein" are entered for comparison.

Protein	Mol. wt. (kilodaltons)	% LIVM	Ref.
Sendai virus F2	13–15	29.7	3
F1	50	29.6	3
HN	67	25.7	4
Hepatitis B surface antigen	25	29.2	17
Protein E1 from Corona virus	26	33.3	18
H-Subunit R. viridis	28	26.7	19
Cytochrome P-450	56	26.3	20
Bacteriorhodopsin	27	32.0	21
ω-Subunit energ. complex	8	33.3	22
Cardiac membrane proteolipids	11	28.2	23
sn-1,2-Diacylglycerol kinase	13	36.9	24
Moloney MuLV p15 E	20	32.2	25
Bovine ribonuclease	14	14.5	26
Lysozyme	14	17.8	27
Ovalbumin	43	27.0	28
Average protein	–	20.2	29

HN and the F protein was obtained. The broad peaks may be caused by repeated precipitation and dissolution of these large membrane proteins.

In view of·these results, the strategy for the purification of a particular membrane protein will primarily depend 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, either alone or in combination. When the structure of the protein must remain intact for further studies, buffer systems of physiological pH, in which a mild detergent is included, are recommended. Secondary factors which may play a role, especially in reversed-phase HPLC of membrane proteins, are the overall hydrophobicity and the size of the protein. Membrane proteins are generally more hydrophobic than an average protein (see Table I). An average membrane protein contains 30% large hydrophobic amino acids and the average percentage of such amino acids in 314 families of proteins[29] is 20.2%. As a consequence, a membrane protein will have more sites available for interaction with hydrophobic column ligands, and relatively high concentrations of organic solvent will be needed for elution. A similar effect is caused by the size of the protein. A larger protein will have more interaction sites with a column matrix than a small protein. This is reflected in the results obtained with reversed-phase HPLC of Sendai virus proteins. The smallest protein, F_2 (13–15 kilodaltons), can be purified and is recovered quantitatively, whereas the other proteins, F_1, HN, F, and the dimer and tetramer of HN, can only be purified partially[2] or not at all by reversed-phase HPLC.

In conclusion, the results of this study indicate that the integral membrane proteins of Sendai virus, the hydrophobicity of which is similar to that of an average membrane protein, and which cover a broad molecular weight range (from 13 to 272 kilodaltons), can be used as reference membrane proteins to evaluate chromatographic purification methods.

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