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SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SENDAI VIRUS MEMBRANE PROTEINS IN DIFFERENT DETERGENTS

A COMPARISON OF DIFFERENT COLUMNS

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SUMMARY

Four column packings for size-exclusion high-performance liquid chromatography (HPLC) of proteins with particle sizes from 3 to 13 μm were compared, using 0.1% sodium dodecyl sulphate in the solvent. Their suitability for the purification of hydrophobic membrane proteins was studied with Sendai virus proteins as a model. The calibration curves of the two 13-μm column packings were linear up to high molecular weights. In contrast to this, large proteins (> 100–150 kD) were eluted later than expected from the 3- and 6-μm packings. Peak capacities for proteins larger than 20 kD ranged from 4.7 to 5.5. Therefore, purification of complex mixtures of membrane proteins will often require rechromatography by a different mode of HPLC. Non-ionic detergents are suitable for further ion-exchange chromatography. The effect of addition of 0.1% of five non-ionic detergents (three gluco-methylalkanamide detergents, octylglucoside, and decyl-polyethyleneglycol-300) to the solvent was investigated and decyl-polyethyleneglycol-300 was found to be most suitable. Size-exclusion HPLC with this detergent resulted in the separation of micelles of three different sizes, of which the larger two contained exclusively the Sendai virus F protein.

INTRODUCTION

Sendai virus is a paramyxovirus of mice. It consists of RNA, associated with a nucleoprotein (NP) and the polymerase protein (P), surrounded by a lipid bilayer or envelope. Three proteins are associated with the envelope. The matrix protein M, with molecular weight (M_r) = 38 000, is associated with the inner surface of the envelope. The haemagglutinin-neuraminidase protein (HN) and the fusion protein (F), with molecular weights of 68 and 65 kD, respectively, are partly embedded in the lipid bilayer and appear as spikes on the outside of the surface of the virion[1]. The amino acid sequence of the F protein contains regions that are homologous with the fusion region of the influenza virus haemagglutinin (HA) protein; the neuramin-

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idase region and the haemagglutinin region of the Sendai virus HN protein show homology with regions having the same functions in the influenza neuraminidase (NA) protein and HA protein, respectively[2].

The F and HN proteins of Sendai virus are hydrophobic membrane proteins, which have been used as model proteins to study the suitability of different modes of high-performance liquid chromatography (HPLC) and various column packings for the purification of membrane proteins[3,4]. Membrane proteins show a strong tendency to aggregate, which renders them insoluble in many solvents used for HPLC. With detergents, the integral membrane proteins can be extracted from their lipid bilayer. The resulting mixture, containing protein molecules with detergent attached, is soluble in aqueous solvents and can be subjected to chromatography. Extraction with an ionic detergent, *e.g.* sodium dodecyl sulphate (SDS), often results in denaturation of the proteins. The soluble protein–SDS complexes, which are more than twice as large as the original protein, are not suitable for purification by ion-exchange HPLC, since they do not differ in charge but only in size. In contrast to this, non-ionic detergents, like Triton X-100, are mild detergents. The biological activity of the proteins is generally conserved after extraction, and the resulting complexes may be subjected to both size-exclusion and ion-exchange HPLC. Reversed-phase HPLC of such extracts is possible after removal of detergent[4] or by using a relatively long column[5].

In our study, four commercial columns for size-exclusion HPLC with particle sizes ranging from 3 to 13 μm were compared, using the tetramer $(HN)_4$ and dimer $(HN)_2$ of Sendai virus (M_r = 272 000 and 136 000, respectively), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000) and trypsin-inhibitor (M_r = 20 000) as reference proteins for the construction of a calibration curve. Proteins were boiled prior to chromatography and eluted with a buffer contaning 0.1% SDS. Two of the columns were studied further, using a detergent extract of Sendai virus as sample and, instead of 0.1% SDS, 0.1% of a non-ionic detergent in the elution buffer. In this case, the detergent extract containing the Sendai virus membrane proteins was not boiled prior to chromatography.

EXPERIMENTAL

Virus: detergent extraction

Sendai virus was grown in 10-day-old embryonated chicken eggs. Allantoic fluid was collected after 48 h incubation at 36°C. The debris was pelleted at 2000 *g* for 20 min, and virions were pelleted at 22 000 rpm (70 000 *g*) for 1 h. Virions were extracted with Triton X-100 (ref. 3). Volumes of 200 μl of extract were subjected to chromatography. The detergent extract was boiled in 4% SDS prior to chromatography, when 0.1% SDS was included in the solvent.

Detergents

Detergents were added to the solvent used for size-exclusion HPLC, 50 m*M* sodium phosphate (pH 6.5), in a concentration of 0.1% (w/v). The ionic detergent used was SDS, electrophoresis grade (Biorad, Richmond, CA, U.S.A.). The non-ionic detergents were: three N-D-gluco-N-methylalkanamide detergents, which were synthesized as described by Hildreth[6], octanoyl-N-methylglucamide, nonanoyl-N-

methylglucamide and decanoyl-N-methylglucamide; octylglucoside (practical, Pfanstiehl Laboratories, Waukegan, IL, U.S.A.); decyl-polyethyleneglycol-300 (Janssen Chimica, Beerse, Belgium or Kwant-Hoog vacolie Recycling and Synthese, Bedum, The Netherlands).

Size-exclusion HPLC

Chromatography was performed with an LKB 2150 pump (LKB, Bromma, Sweden), a Rheodyne 7125 injector (Inacom, Veenendaal, The Netherlands) and a Pye Unicam LC-UV detector (Philips, Eindhoven, The Netherlands). Unless otherwise mentioned, the flow-rate was 1 ml/min, and the absorbance was monitored at 280 nm.

Size-exclusion column packings

The following column packings were investigated:

Superose 6 HR 10/30 (300 × 10 mm I.D.) (Pharmacia, Uppsala, Sweden), which is 6% agarose, cross-linked with a mixture of long-chain di- and polyfunctional epoxides, followed by further cross-linking with short-chain bifunctional cross-linkers[7]. The separation range for proteins is reported to be 5–5000 kD[7]. The particle size 13 μm and the pore size is *ca.* 900 Å[8].

TSK 4000 SW (600 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan), which consists of spherical silica particles with bonded hydrophilic polar groups. The separation range is 5–1000 kD. The particle size and the pore size are 13 μm and 450 Å, respectively.

Zorbax Bio Series GF-450 (250 × 9.4 mm I.D.) (Du Pont Company, Wilmington, DE, U.S.A.). The material consists of spherical diol-bonded silica particles with a metal (zirconium) oxide-stabilized surface. The separation range is 25–900 kD. The particle size and the pore size are 6 μm and 300 Å, respectively.

Si300 Polyol (500 × 9.5 mm I.D.) (Serva, Heidelberg, F.R.G.), which consists of polyhydroxylated, almost spherical silica particles. Proteins can be separated up to a molecular weight of 900 kD. The particle size and the pore size are 3 μm and 300 Å, respectively.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The column eluates were analysed on 8% SDS-polyacrylamide gels[9]. The tetramer and dimer of the Sendai HN protein remained intact during electrophoresis, because the samples were not reduced prior to chromatography. Polypeptide bands were visualized with a silver-staining method[10].

RESULTS AND DISCUSSION

Fig. 1a–d shows the separation of the Sendai virus proteins on the Superose 6, TSK 4000 SW, Zorbax GF-450 and Si300 Polyol columns, respectively. Peaks 1, 2 and 3 in the elution patterns contain proteins with a molecular weight of 272, 136 and 65 kD, respectively. A similar result with the TSK 4000 SW column only and subsequent SDS-PAGE has been reported earlier[3]. A smaller particle size results in narrower peaks (Fig. 1c and d). This is also illustrated in Fig. 2, in which the elution patterns obtained after chromatography of bovine serum albumin, ovalbumin and

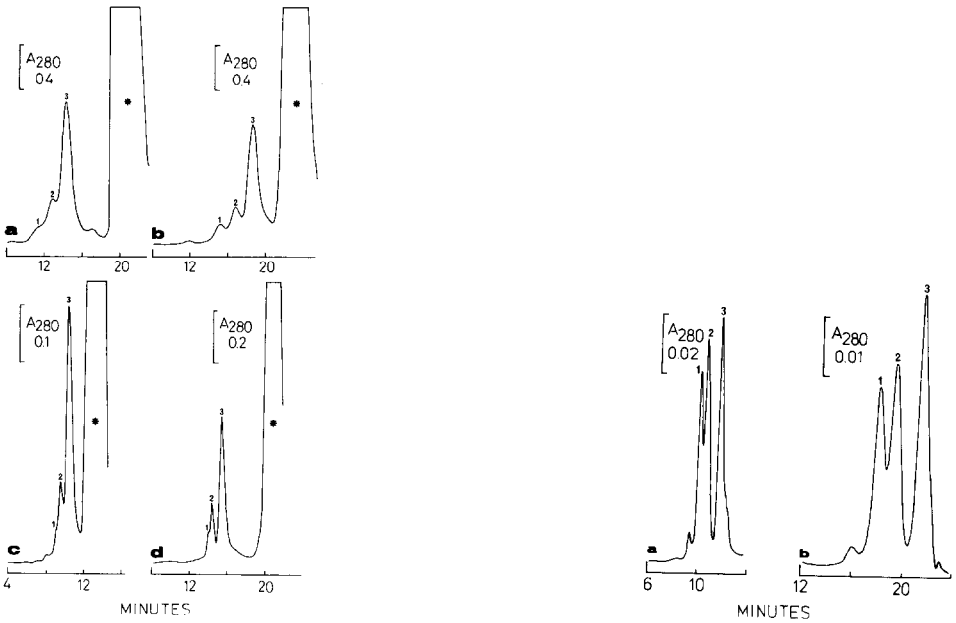
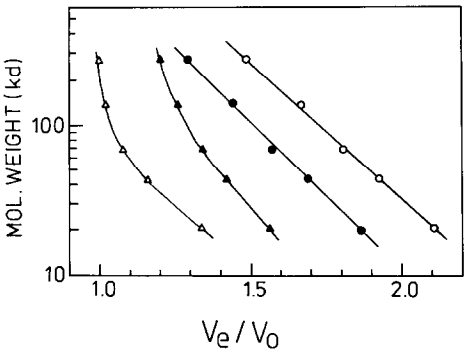


Fig. 1. Size-exclusion HPLC of a Triton X-100 extract of purified Sendai virions. Different columns were eluted with 50 m*M* sodium phosphate (pH 6.5) containing 0.1% SDS. (a) Superose; (b) TSK 4000 SW; (c) Zorbax GF-450; (d) Si300 Polyol. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm. Peaks: 1 = the tetramer; 2 = the dimer of the Sendai virus haemagglutinin-neuraminidase protein; 3 = the Sendai virus fusion protein; * = Triton X-100.

Fig. 2. Size-exclusion HPLC of a mixture of reference proteins (1 = serum albumin; 2 = ovalbumin; 3 = trypsin-inhibitor) on a column of Zorbax GF-450 (a) and TSK 4000 SW (b). Experimental conditions, see Fig. 1.

trypsin inhibitor on the Zorbax GF-450 column and the TSK 4000 SW column are shown. However, this does not necessarily result in better resolution in the higher molecular weight range (peaks 1 and 2 in Fig. 1). The calibration curves, *i.e.* ratio of elution volume (V_e) to void volume (V_0) *versus* molecular weight, are shown in Fig. 3. The slopes are rather similar, except in the higher molecular weight range. However, the peak volume is also of importance, and it could be that a column with a steeper calibration curve but a smaller peak volume would show a better resolution (see below).

The columns with smaller particles, especially the Si300 Polyol column, tend to deviate from the linear relationship between molecular weight and V_e/V_0. Considering the fact that the denatured SDS-protein complexes are 2.4 times as large as the original protein[11], the tetrameric HN–SDS complex would have a molecular weight of 653 kD. For adequate size-exclusion HPLC of a denatured protein of that size, the minimum particle size should be between 2.23 and 4.08 μm, as calculated by Guiochon and Martin[12]. This covers the size of the Si300 Polyol particles (3 μm) and is slightly less than the 6 μm of the Zorbax GF-450 particles. It is possible that these larger complexes are obstructed to some extent in the interstitial space, resulting in retarded elution. With regard to this, the pore size is also of importance and it is not



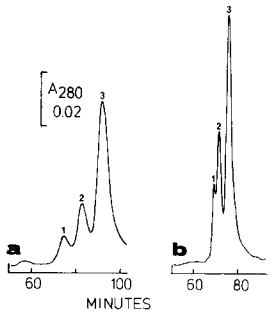


Fig. 3. Calibration curves of Zorbax GF-450 (▲), Superose 6 (○), Si300 Polyol (△), TSK 4000 SW (●) for random coil protein–SDS complexes.

Fig. 4. Size-exclusion HPLC of a Triton X-100 extract of purified Sendai virions. The TSK 4000 SW (a) and the Si300 Polyol (b) columns were eluted with 50 m*M* sodium phosphate (pH 6.5) containing 0.1% SDS. The flow-rate was 0.2 ml/min and the absorbance was monitored at 280 nm. 1 = $(HN)_4$; 2 = $(HN)_2$; 3 = F.

surprising that the TSK 4000 SW and Superose 6 columns showing a linear relationship between molecular weight and V_e/V_0, have larger pore sizes, 450 and 900 Å, respectively.

The specific resolution (R_s) of pairs of proteins was determined using the equation (adapted from ref. 13):

$$R_s = 2(V_2 - V_1)/[(V_{p2} + V_{p1}) \times (\log M_1/M_2)]$$

where V, V_p and M are the elution volumes, peak volumes and the molecular weights, respectively. The peak volume was determined as 1.7 × peak-width at half-height[4]. The results are shown in Table I. The best resolution with the high molecular weight pair (272 and 136 kD) is obtained with the TSK 4000 SW column and can be further increased by lowering the flow-rate from 1.0 to 0.2 ml/min (*cf.* Fig. 4a with Fig. 1b). For comparison, the increase in resolution obtained with the Si300 Polyol column is also shown (*cf.* Fig. 4b and Fig. 1d). To some extent this is also true for the

TABLE I

SPECIFIC RESOLUTION

	Superose 6	*TSK 4000 SW*	*Zorbax GF-45*	*Si300 Polyol*
Sendai $(HN)_4$	<2.0	2.25 (3.26)*	<2.0	<2.0
Sendai $(HN)_2$	<2.0	2.64 (6.71)*	2.25	2.71
Sendai F				
Bovine serum albumin	2.66 (3.11)**	4.06	3.94	7.57 (2.59)***
Ovalbumin	2.75 (3.14)**	3.85	3.93	9.84 (4.87)***
Trypsin inhibitor				

* Flow-rate 0.2 ml/min.
** Flow-rate 0.4 ml/min.
*** After 40 runs.

Superose column, which after a few runs had to be used at a flow-rate of 0.4 ml/min or less (see Table I). In the lower molecular weight range (20–68 kD) the highest specific resolution was obtained with the Si300 Polyol column, although after 40 runs, resolution decreased considerably (see Table I). Taking into account the small size of the Zorbax GF-450 column (250 × 9.4 mm I.D.) compared to the other columns (600 × 7.5, 500 × 9.5 and 300 × 10 mm I.D.), the specific resolution is remarkably good. The peak-width is proportional to the square root of the column length[14]. Therefore, coupling two of these columns would result in a higher specific resolution of 2.73 instead of 1.95 for the high molecular weight pair.

In Table II the properties of the columns are summarized. A practical size-exclusion peak capacity for proteins larger than 20 kD was determined by dividing the volume that is covered from V_0 to $V_{20\ kD}$ by the peak volume. The Si300 Polyol column shows the highest peak capacity (5.5) but it was much lower after 40 runs. Together with the better specific resolution in the higher molecular weight range (see Table I), the TSK 4000 SW column is most suitable for separating protein–SDS complexes. However, we expect that the combination of two Zorbax GF-450 columns will show equal or even better results. The peak capacity of the Superose column as well as the resolution is lower than that of the other columns, but this column has the advantage of a wider molecular weight range (up to 5000 kD) and of high chemical stability. As a consequence of the latter, it can be used under harsh conditions over the whole pH range (1–14) with a large variety of solvents.

These results show that in only a limited number of cases can a pure protein be obtained by a single size-exclusion HPLC step. Recycling of selected fractions or a second purification step by, for example, ion-exchange HPLC[3] will often be necessary. In contrast to SDS, a non-ionic detergent is suitable for further ion-exchange chromatography. Therefore, we selected two column packings, TSK 4000 SW and Zorbax GF-450, to investigate the effect of addition of five non-ionic detergents to the elution buffer on column performance. Again, we used a Triton X-100 extract of Sendai virus as a model mixture of membrane proteins. As detergents, we used three glucomethylalkanamides[3], octylglucoside and decyl-polyethyleneglycol-300 (decyl-PEG). Fig. 5a and b shows the elution pattern obtained after chromatography on the TSK 4000 SW column with 0.1% decyl-PEG and 0.1% decanoylmethylglucamide, respectively, as additives to the solvent. Similar results were obtained with the GF-450 column (not shown). Addition of the three remaining detergents resulted in elution patterns similar to that obtained with decanoylmethylglucamide (Fig. 5b).

TABLE II

COMPARISON OF COLUMN PACKINGS FOR SIZE-EXCLUSION HPLC

Packing	*Pore size (Å)*	*Particle size (μm)*	*Column size (mm × mm I.D.)*	$V_0 - V_{20\ kD}$ *(ml)*	*Peak volume* (ml)*	*Size exclusion peak capacity***
Superose 6	900	13	300 × 10	8.6	2.16	4.0
TSK 4000 SW	450	13	600 × 7.5	10.1	1.99	5.1
Zorbax GF-450	300	6	250 × 9.4	4.0	0.85	4.7
Si300 Polyol	300	3	500 × 9.5	4.8	0.87	5.5

* Mean peak volume; peak volume is 1.7× peak-width at half-height.

** The peak capacity for proteins larger than 20 kD.

SDS-PAGE of the column eluates (Fig. 5) without prior boiling and reduction of the samples, shows that the results obtained with decyl-PEG are quite different from those obtained with the other detergents. The first peak, fraction 1 in Fig. 5a, presumably contains a micelle of decyl-PEG and exclusively F protein with a molecular weight of > 1000 kD. Fractions 2–5 also contain micelles with F protein. SDS-PAGE shows the presence of monomeric F and a small amount of an aggregate of F, which could be converted to the monomeric form after boiling and reduction of the sample

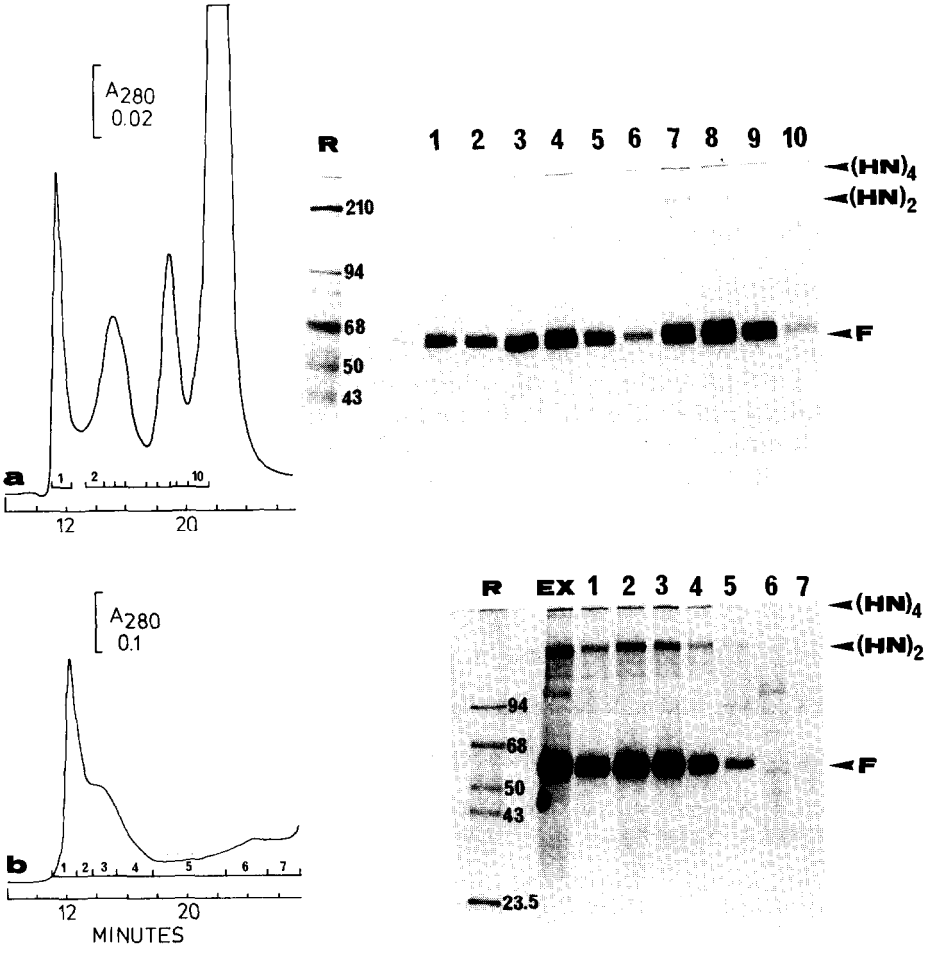


Fig. 5. Size-exclusion HPLC of a Trion X-100 extract of purified Sendai virions on a TSK 4000 SW column eluted with 50 m*M* sodium phosphate (pH 6.5) containing 0.1% decyl-polyethyleneglycol-300 (a) or 0.1% decanoylmethylglucamide (b). The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm. Fractions indicated by numbers were analysed by SDS-polyacrylamide gel electrophoresis (8% gels). The molecular weight of reference proteins (R) is given in kD. Reference proteins were, myosin (210), phosphorylase *b* (94), bovine serum albumin (68), immunoglobulin heavy chain (50), ovalbumin (43) and chymotrypsinogen (23.5). The tetramer and the dimer of the haemagglutinin-neuraminidase protein of Sendai virus, $(HN)_4$ and $(HN)_2$, respectively, as well as the fusion protein F are also indicated. EX = Triton extract.

prior to electrophoresis (not shown). Fractions 6–9 contain complexes of decyl-PEG with F, $(HN)_2$ and $(HN)_4$, with a molecular weight of 360 kD. The last peak probably contains Triton X-100. SDS-PAGE of fractions obtained after size-exclusion HPLC using 0.1% decanoylmethylglucamide in the solvent showed (Fig. 5b) that the Sendai virus proteins $(HN)_4$, $(HN)_2$ and F remained associated during chromatography and were eluted in fractions 1–4 as a complex with a molecular weight larger than 1000 kD.

This study shows that the addition of SDS or decyl-PEG to the solvents used for size-exclusion HPLC of hydrophobic membrane proteins results in entirely different elution patterns and in purified proteins with different features. Membrane proteins separated by size-exclusion HPLC in 0.1% SDS cannot be purified further by ion-exchange chromatography, but they are still immunologically active[3]. Size-exclusion HPLC of membrane proteins in 0.1% decyl-PEG results in either a mixture of proteins complexed with decyl-PEG molecules, which may be further purified by ion-exchange chromatography[3], or in a high-molecular-weight complex of a single protein species and detergent molecules (fraction 1 in Fig. 5a). Such a complex might be used to elicit antibodies to that particular protein.

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