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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

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SUMMARY

Virus envelope proteins were isolated from Triton X-100 extracts of purified Sendai virions by gel-filtration, ion-exchange and reversed-phase high-performance liquid chromatography (HPLC). The fusion protein F, the matrix protein M and the tetrameric and dimeric form of the HN protein were isolated by gel-filtration HPLC with a solvent containing 0.1% sodium dodecyl sulphate. HN and F were also isolated by ion-exchange HPLC with 0.1% Triton X-100 in the eluent. Reversed-phase HPLC was performed on a C_1 column with acetonitrile as the organic solvent. Especially the F_1 and F_2 component of the fusion protein F were obtained in pure form.

The immunological activity of the proteins after HPLC was determined with an enzyme-linked immunosorbent assay (ELISA). After gel-filtration and ion-exchange HPLC, proteins still reacted with antiserum to the intact virus while proteins purified by reversed-phase HPLC did not react.

INTRODUCTION

Membrane proteins of viruses which are embedded to a variable extent in the lipid bilayer or are associated to its inner surface show a strong tendency to aggregate and therefore are difficult to purify by conventional chromatographic methods. Before any chromatographic procedure can be applied, the virion or the viral membrane has to be solubilized. Biomembranes can be solubilized in three ways: (a) by mechanical means, (b) by organic solvents and (c) by detergents[1]. Of these, solubilization by detergents is most widely used[2]. Selective extraction of purified virions with mild non-ionic detergents like Triton X-100 and the similar detergent NP-40 is often used to solubilize and to isolate viral proteins. More drastic solubilization is achieved with an ionic detergent like sodium dodecyl sulphate (SDS), which results in denaturation of the protein molecule. After treatment with detergent the purification problem is then reduced to the separation of a mixture of viral proteins to which detergent

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molecules are attached. Detergent has to be added to the solvents commonly used for chromatography to overcome aggregation during chromatography. Only when using organic solvents is the addition of detergent unnecessary, since in that case the protein can be solubilized by the solvent.

Sendai virus, a paramyxovirus and a natural infectious virus of mice, which is not pathogenic for man[3], was used as a model virus. The envelope of Sendai virus consists of a lipid bilayer from which two glycoproteins protrude as spike-like projections[4]. These glycoproteins are the hemagglutinin-neuraminidase protein (HN) and the fusion protein F with molecular weights of 66,000 and 65,000 kilodaltons, respectively. The F protein consists of F_1 (M_r = 50,000) and F_2 (M_r = 13,000–15,000), which are connected by disulfide bridges[5]. A non-glycosylated protein, M (M_r = 38,000), is associated with the inner surface of the envelope[4]. HN and F can be selectively extracted from purified virions by treatment with Triton X-100, and addition of 1 *M* salt to the extraction buffer results in solubilization of HN, F, and M[6].

Three chromatographic methods (gel-filtration, ion-exchange and reversed-phase chromatography) currently in use for high-performance liquid chromatography (HPLC) of proteins[7] were applied to purify the proteins present in a detergent extract of purified Sendai virions.

Also of interest is the extent to which the original structure of the proteins remains intact or is regained after high-performance liquid chromatography (HPLC). An enzyme-linked immunosorbent assay (ELISA) was used to measure the immunological activity of the eluted fractions.

EXPERIMENTAL

Virus

Sendai virus was grown in 10-day-old embryonated chicken eggs. Allantoic fluid was harvested after 48 h of incubation at 36°C. Debris was pelleted at 2000 *g* for 30 min. Virions were purified by sucrose-gradient centrifugation.

Detergent extraction and removal of detergent

Purified virions were disrupted by treatment with Triton X-100 (BDH, Poole, U.K.) at a final concentration of 2% (v/v) for 15 min at room temperature. The detergent: viral protein ratio was 0.9 (w/w). After centrifugation at 100,000 *g* for 90 min, the resulting supernatant was stored at −80°C. To extract the M protein in addition to HN and F, 1 *M* sodium chloride was added to the extraction medium[6]. Prior to reversed-phase HPLC, Triton X-100 was removed with Amberlite XAD-2 (Serva, Heidelberg, F.R.G.) and the proteins were reduced with 20 m*M* dithiothreitol (Sigma, St. Louis, MO, U.S.A.) as described earlier[8,9].

HPLC (gel-filtration, ion-exchange and reversed-phase)

Chromatography was performed with a system consisting of one Waters M 6000A pump, a low-pressure mixing system, a Rheodyne 7125 injector and a Pye Unicam LC-UV detector. The gradient was generated by an Acorn computer, interfaced with a three-way solenoid valve (Lee, Westbrook, CT, U.S.A.)[10].

Gel-filtration HPLC was performed on a 600 × 7.5 mm I.D. column, TSK

4000 SW (Toyo Soda, Tokyo, Japan), by isocratic elution with 50 m*M* sodium phosphate, pH 6.5, containing 0.1% SDS[11]. Samples were boiled for 2 min in 4% SDS prior to chromatography. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm.

Anion-exchange HPLC was carried out by chromatography of a detergent extract 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 20 m*M* Tris · HCl, pH 7.8, containing 0.1% Triton X-100 to 0.5 *M* sodium chloride in the same buffer. The flow-rate was 1 ml/min and the absorbance was monitored at 275 nm.

After gel-filtration and ion-exchange HPLC, fractions were collected in low-protein absorption tubes (Nunc, Roskilde, Denmark). The tubes were covered with dialysis-membrane tubing and dialyzed overnight against water with Biobeads SM2 (Bio-Rad Labs., Richmond, CA, U.S.A.)[12] when the samples contained Triton and against water when they contained SDS.

Reversed-phase HPLC was performed by chromatography of detergent extracts from which the detergent was removed as described above on a C_1 column (75 × 4.6 mm I.D.) with pores of 25 nm and a particle size of 10 μm (TMS-250, Toyo Soda, Tokyo, Japan). The column was eluted with a 25-min gradient consisting of 25% acetonitrile (E. Merck, Darmstadt, F.R.G.) in water with 0.05% trifluoroacetic acid (TFA) to 75% acetonitrile in water with 0.05% TFA. The flow-rate was 1 ml/min and the absorbance was monitored at 205 nm or, when 100 μg amounts were chromatographed, at 225 nm. The organic solvent was removed by evaporation in a Speed Vac centrifuge (Savant Instr., Hicksville, NY, U.S.A.). The remaining aqueous solution was lyophilized.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The column eluates were analyzed on 10 or 12.5% SDS–polyacrylamide gels[13]. Polypeptide bands were visualized with a silver-staining method[14].

Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiterplates (Dynatech, Denkendorf, F.R.G.) were coated with 1–10 μg Sendai virus protein in 100 μl 50 m*M* sodium carbonate, pH 9.6, overnight at 4°C or for 2 h at 37°C. The coated plates were washed three times with phosphate-buffered saline, pH 7.2, containing 0.2 *M* sodium chloride, 0.3% Tween 20, and 1 mg SDS/l. Antisera from Sendai virus-infected mice and peroxidase conjugates (ShaM/IgG/PO-Pasteur) were diluted in the same buffer, supplemented with 0.5% bovine serum albumin. After addition of the serum dilution (100 μl/well), incubation for 1 h at room temperature and washing, the peroxidase conjugate was added and the plates were incubated for 1 h at 37°C. Peroxidase activity was visualized with *o*-phenylenediamine–hydrogen peroxide (30 min, room temperature). The reaction was terminated by adding 2 *M* sulphuric acid, and the absorbance at 492 nm was measured in a microplate photometer (Titertek-Multiskan).

RESULTS AND DISCUSSION

The detergent-extracted Sendai virus proteins were subjected to three modes of HPLC: (a) gel filtration; (b) ion-exchange and (c) reversed-phase HPLC.

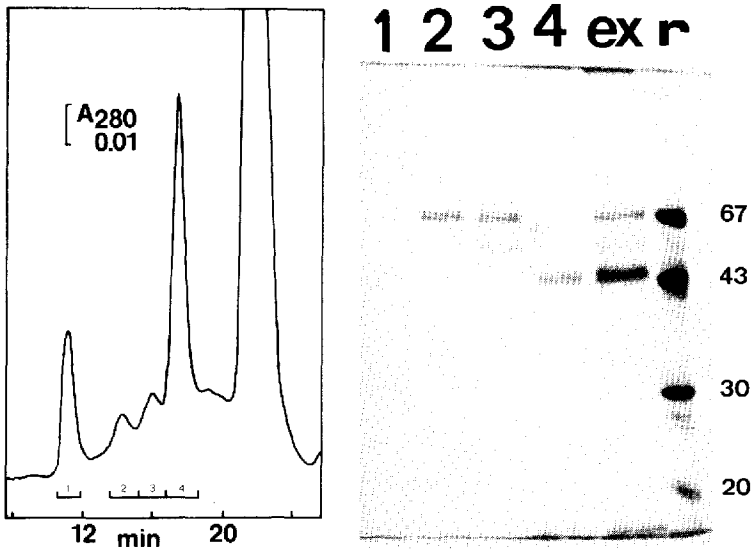

Fig. 1. Gel-filtration HPLC of a Triton X-100 extract of purified Sendai virions. The TSK 4000 SW column was eluted with 50 m*M* sodium phosphate, pH 6.5, containing 0.1% SDS. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm. Fractions (1–4) were analyzed by SDS–PAGE (10% gels). The molecular weight of reference proteins (r) is given in kilodaltons. ex = the Triton extract.

Gel-filtration HPLC

A detergent extract which contained predominantly HN and F was applied to a TSK 4000 SW column. The solvent, 50 m*M* sodium phosphate, pH 6.5, contained 0.1% SDS. The elution pattern and the analysis by polyacrylamide gel electrophoresis (SDS–PAGE) is shown in Fig. 1. The peak which eluted in 20 24 min contained Triton X-100. SDS–PAGE showed that peak 1 did not contain protein. Peaks 2 and 3 contained HN and peak 4 contained F_1 protein. Since the samples were reduced prior to SDS–PAGE, peak 2 contained the tetrameric form of HN, peak 3 the dimeric form of HN and peak 4 the fusion protein F. Markwell and Fox[15] always found the tetrameric and dimeric forms in a ratio of 1:1.42 We calculated from the peak heights of the elution patterns from five different extracts an average ratio of 1:1.40. Minor bands are probably the result of proteolytic cleavage of virus protein or cellular contaminants. For example, peak 3 also contains a dimer of a fragment of HN of molecular weight 55,000[16].

When 1 *M* sodium chloride is added to the extraction medium, the extract also contains, in addition to HN and F, the matrix protein M. When such an extract is subjected to gel-filtration HPLC, separation of F and M is not obtained and M is even present in the HN peaks. This suggests that the M protein easily aggregates with other virus proteins to form heteropolymers. M can be purified by first dialyzing the extract against water. M precipitates since it only dissolves at salt concentrations of 1 *M* or more. The precipitate was boiled in a buffer containing 4% SDS and the resulting solution was then subjected to chromatography (not shown). Reduction of purified F protein results in F_1 and F_2 which can easily be separated on a TSK 3000 SW column (not shown). In all cases the yield was high (90–100%). Table I shows

TABLE I

PRESENCE (+) OR ABSENCE (−) OF PROTEINS IN DETERGENT EXTRACTS OF EGG-GROWN SENDAI VIRUS, DETECTED BY SDS–TSK HPLC

*Molecular weight (kilodaltons)**	*Prior to chromatography*		*Identity*
	Non-reduced	*Reduced*	
260	+	−	$(HN)_4$
130	+	−	$(HN)_2$
66	−	+	HN
65	+	−	F
50	−	+	F_1
38	+	+	M
13–15	−	+	F_2

* Molecular weights were determined from molecular weight standards, chromatographed under identical conditions.

the Sendai virus proteins which can be isolated by gel-filtration HPLC and their molecular weights.

Ion-exchange HPLC

It has been shown[17] that the F protein can be purified from Triton X-100 extracts of Sendai virions. The HN protein was not adsorbed on the column material when 0.15 *M* sodium chloride in 0.02 *M* sodium phosphate, pH 7.2, containing 0.1% Triton X-100 was used as eluent. In the present study we used a 20 m*M* Tris · HCl buffer, pH 7.8, with 0.1% Triton X-100 as the starting buffer. These conditions also allow chromatography of the HN protein. A gradient to 0.5 *M* sodium chloride was used. The elution pattern is shown in Fig. 2. Many peaks are visible which might be

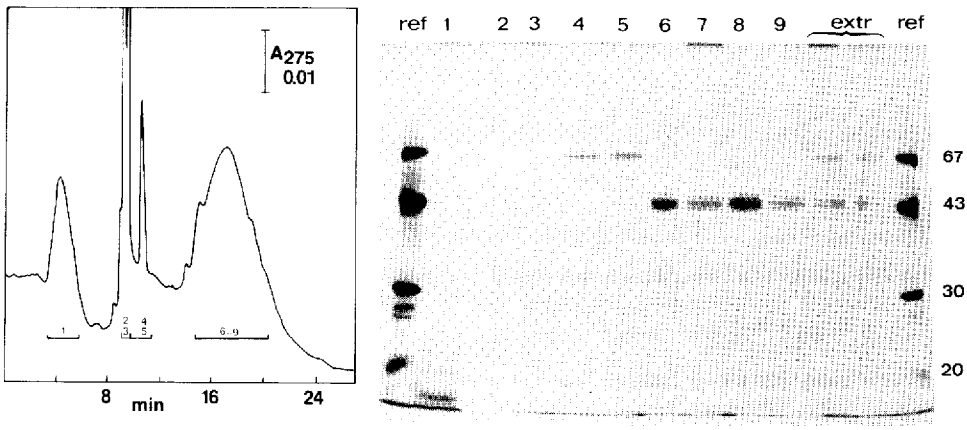

Fig. 2. Anion-exchange HPLC of a Triton X-100 extract of purified Sendai virions. The Mono Q column was eluted with a 24-min gradient from 20 m*M* Tris · HCl, pH 7.8, containing 0.1% Triton X-100, to 0.5 *M* sodium chloride in the same buffer. The flow-rate was 1 ml/min and the absorbance was monitored at 275 nm. Fractions (1 9) were analyzed by SDS PAGE (10% gels). The molecular weight of reference proteins (ref) is given in kilodaltons. extr = the Triton extract.

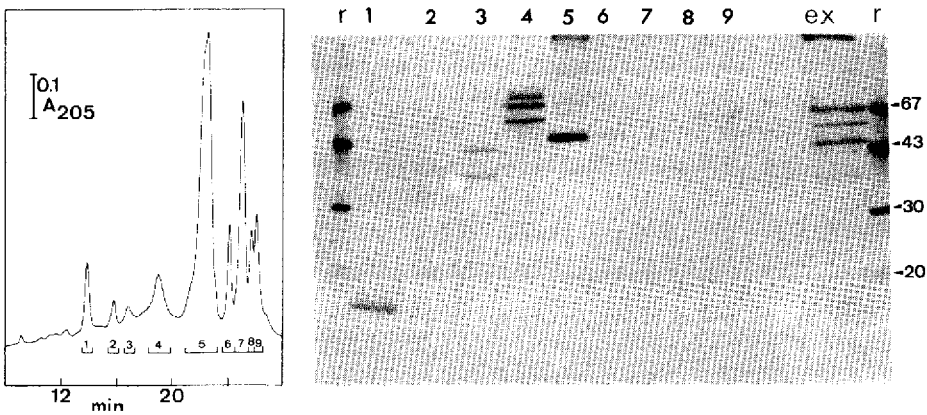

Fig. 3. Reversed-phase HPLC of a Triton X-100 extract of purified Sendai virions which was reduced with DTT. The column (TMS-250) was eluted with a 25-min gradient, consisting of 25–75% acetonitrile in water containing 0.05% TFA. The flow-rate was 1 ml/min and the absorbance was monitored at 205 nm. Fractions (1–9) were analyzed by SDS–PAGE (12.5% gels). The molecular weight of reference proteins is indicated in kilodaltons. ex = the Triton extract.

due to different aggregates or polymeric forms present in the extract but probably also to differences in charge, caused by acidic carbohydrate moieties. More than 75% of the oligosaccharides from F protein are acidic while 18% of the oligosaccharides from HN are acidic[18]. SDS–PAGE (Fig. 2) shows that a distinct separation between HN and F was obtained. HN is present in fractions 4 and 5. In addition, small amounts of the 55,000-molecular-weight fragment of HN[16] are also present in these fractions.

Several ion-exchange columns were used for this separation, in which the Mono Q column (Pharmacia) and the IEX 645 DEAE (Toyo Soda) gave similar results, although a slower gradient has to be used for elution of the latter column. The total yield of HN and F was difficult to calculate but was estimated to be at least 50%.

Reversed-phase HPLC

Reversed-phase HPLC is becoming exceedingly popular for the isolation of

TABLE II

COMPARISON OF TWO RP-HPLC SYSTEMS

System 1: C_{18}, ethanol–butanol–HCl (see ref. 9); system 2: C_1, acetonitrile–TFA (this study).

System		*Protein*			
		F_2	*M*	*HN*	*F_1*
1	% Ethanol + butanol	32	40–57	45–57	50–57
	Yield*	++	−	−	±
2	% Acetonitrile	36	40	44	50
	Yield*	++	+	+	+

* ++, 90–100%; +, 60–90%; ±, 30–60%; −, <30%.

proteins. In some way, the detergent is substituted by a hydrophilic solid support (silica) with the apolar tail (very often C_{18}) attached to it. To elute the relatively hydrophobic virus proteins from this support, high concentrations of organic solvent and a low pH are necessary[7]. This may result in irreversible denaturation of the protein, but this is no problem if the protein is going to be used for amino acid sequence studies. In an earlier study[9] a detergent extract, containing HN, F_1, F_2 and M proteins, was chromatographed on C_{18} columns with a gradient of ethanol–1-butanol (4:1, v/v) in 12 m*M* hydrochloric acid. In the present study we used a newly developed C_1 column with pores of 25 nm, which was eluted with a gradient of acetonitrile in water containing 0.05% TFA. A detergent extract, containing predominantly F_1, F_2, HN, the 55,000-molecular-weight fragment of HN[16] and a small amount of M protein, was chromatographed on this column. The results are shown in Fig. 3. Compared with the C_{18}–ethanol–butanol system[9], resolution is better and peaks are eluted at about the same organic solvent concentration. Peak 1 contained the F_2 protein; peak 4 contained HN, the 55,000-molecular-weight fragment of HN and some unknown proteins. Peaks 5–7 contained predominantly the F_1 protein. Most of it was found in peak 5. The matrix protein M was present in peak 3. Yields were 60% or higher. A comparison of the yields and the organic solvent concentrations at which the proteins are eluted is given in Table II.

Immunological activity

An important aspect of chromatographic studies involving detergent extraction and chromatography in denaturing solvents is how much of the original structure or activity is left. For virus proteins, the immunological activity is of particular interest. In the present study, we applied three different modes of HPLC for the purification of viral membrane proteins which might affect their immunological reactivity with antibodies to the intact virus.

For gel-filtration HPLC, proteins were boiled for 2 min in 4% SDS and eluted with a phosphate buffer containing 0.1% SDS. These conditions will affect the structure of the proteins. SDS molecules will attach themselves to the surface of the hydrophobic protein molecule and also penetrate its interior, thus affecting the native structure. To investigate to what extent the original structure is left or regained after chromatography, purified Sendai virus proteins or eluate fractions were used to coat the polystyrene surface of ELISA microtiterplates. If these proteins react with an antiserum obtained from mice after a Sendai virus infection, and if the IgG molecules in the latter antiserum react with anti-IgG to which peroxidase is conjugated, then the intensity of the color obtained after addition of the substrate *o*-phenylenediamine–hydrogen peroxide will be a measure of the residual immunological activity. Fig. 4 shows the elution pattern obtained after gel-filtration HPLC of a Sendai virus extract containing $(HN)_4$, $(HN)_2$ and F. Every 2 min, fractions were collected and then dialyzed against water, lyophilized and subsequently used for coating in the ELISA. The absorbance at 492 nm reflects the immunological activity. All Sendai virus proteins present in the extract were still immunologically active. This was further investigated by determination of the immunological activity of purified $(HN)_4$, $(HN)_2$, M and F. In that case 1–10 μg of purified protein were used to coat one well of an ELISA tray. Again, all Sendai virus proteins were immunologically active, although M also showed a considerable amount of reactivity

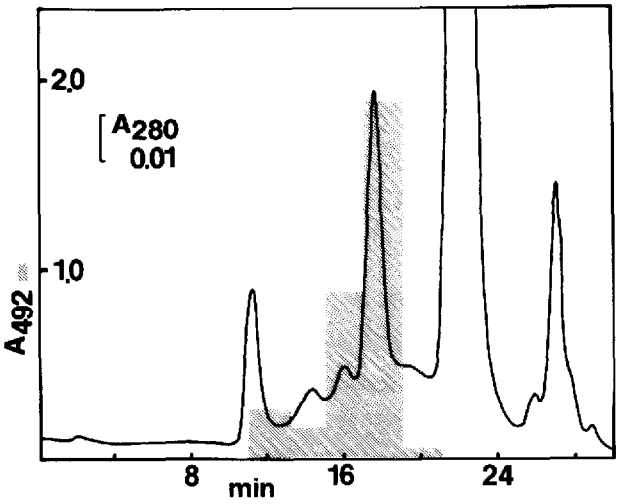

Fig. 4. Gel-filtration HPLC of detergent-extracted Sendai virions. Elution conditions as mentioned in Fig. 1. Fractions (2 ml) were analyzed with an ELISA. The immunological activity is indicated by the absorbance at 492 nm (shaded area).

with a Sendai-negative antiserum. It was not unexpected that Sendai virus proteins (HN and F) isolated under the relatively mild conditions of ion-exchange HPLC would be immunologically active. The activity was similar to that found after gel-filtration HPLC. This is in contrast to the conditions used for reversed-phase HPLC.

In an earlier study[19], we investigated the structure and activity of four reference proteins after reversed-phase HPLC. These proteins were eluted by 17–41% organic solvent, and their reaction with antibodies to the native protein decreased with increasing organic solvent concentration. No reaction was found between ovalbumin, which was eluted at 41% organic solvent concentration, and antibodies to ovalbumin. Peak fractions, containing up to 200 μg of Sendai virus proteins (F, F_1, F_2, M, HN), were used to coat the ELISA microtiterplates. No reaction was found with antibodies to Sendai virus, suggesting that the low pH and the high concentration of organic solvent (36–50%, see Table II) had an adverse effect on the native structure of the molecule.

From the results we conclude that, after reversed-phase HPLC, Sendai virus proteins are unable to react with antiserum to the intact virus. A contributory factor to this is the relatively high organic solvent concentrations needed for elution of membrane proteins. The mild conditions of ion-exchange HPLC will not affect the structure of the proteins to any significant extent, and it is interesting to note that boiling in SDS and elution in 0.1% SDS leaves a considerable part of the native protein structure intact. Protein blotting techniques, in which, after SDS–PAGE, proteins are transferred to nitrocellulose and are then allowed to react with antibodies to the native protein, show similar results[20,21]. An advantage of the HPLC technique is the ease of recovery of purified protein for further studies.

From studies by Yonath *et al.*[22] with crystals of SDS-denatured lysozyme it was concluded that SDS molecules penetrate the interior of the molecule and affect the overall structure of the molecule. However, they found that several regions in the

difference map were clean, indicating that parts of the protein were in their native conformation. A similar consideration might be applicable to proteins in solution. After treatment with SDS, a number of antigenic determinants may still be in their native conformation or will have enough flexibility to react with antibodies to the native protein.

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