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PURIFICATION OF DETERGENT-EXTRACTED SENDAI VIRUS PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Sendai virus envelope proteins were isolated by reversed-phase high-performance liquid chromatography. The F (F_1 and F_2, connected by disulphide bonds), M and HN proteins were extracted from purified Sendai virions with Triton X-100. After removal of the detergent from the extract with Amberlite XAD-2 and reduction of the proteins, separation was performed on a small (10-nm) and on a larger (30-nm) pore size C_{18} column. Proteins were eluted with a gradient of an ethanol–1-butanol mixture in 12 m*M* hydrochloric acid. On the 10-nm pore size column, F_2 was completely recovered in pure form, whereas the recoveries of the other proteins were low (5–25%). Similar results were obtained with the 30-nm pore size column, except for protein F_1 of which the yield was higher (50%).

INTRODUCTION

Sendai virus contains only a few structural proteins[1,2]. Besides the internal nucleoprotein (NP) and the polymerase protein (P) there are three envelope-associated proteins (HN, F and M). The HN protein (molecular weight, M_r = 66,000) is responsible for the haemagglutination and neuraminidase activities[3]. The F or fusion protein is involved in hemolysis, cell fusion and virus penetration[3]. The F protein is composed of two disulphide-linked polypeptides F_1 (M_r = 52,000) and F_2 (M_r = 13,500), generated by proteolytic cleavage of the inactive precursor F_0 (refs. 1 and 2). The M or matrix protein (M_r = 38,000) plays a central rôle in virus assembly and viral budding from the cell membrane[3].

The use of several detergents for the solubilization of the envelope proteins has been described. Selective extraction of the two glycoproteins HN and F can be achieved with Triton X-100[4–7], NP-40[8,9] and other detergents[10–12]. Proteins HN, F and M can be solubilized with Triton X-100 in the presence of 1 *M* salt as was shown by Scheid and Choppin[1]. The individual proteins have been isolated from extracts by various chromatographic methods in the presence of detergents[1,6–13].

During the last few years, reversed-phase high-performance liquid chromatography (RP-HPLC) has become exceedingly important for larger proteins[14–19]. The importance of column materials with a pore size of 30 nm instead of 10 nm for this

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purpose has been stressed[20–22]. Although RP-HPLC has been used for the purification of some proteins[16,23,24], reports concerning the purification of viral membrane proteins are scarce[25].

Here we report the RP-HPLC separation of the envelope-associated proteins of Sendai virus on two different C_{18} columns with pore sizes of 10 and 30 nm, respectively. The amino acid composition of one of the purified proteins, F_2, was determined.

EXPERIMENTAL

Virus

Sendai virus was grown in 10-day-old embryonated eggs. Allantoic fluid was harvested 72 h after infection and clarified by centrifugation at 3000 rpm (Beckman, Model TJ-6) for 15 min. The virus was concentrated by ultracentrifugation at 5°C for 2 h at 22,000 rpm (Christ Ultracentrifuge, Model Omega 70.000, W 23/534 rotor). The viral pellet was resuspended in a few ml of Hanks buffer[26], and purification was performed by density gradient centrifugation [20–60% (w/v) sucrose in 10 m*M* N-(2-hydroxyethyl)-1-piperazine-N′-ethanesulphonic acid (HEPES) buffer] at 5°C for 1 h at 22,000 rpm (S 27/245 rotor). The broad virus bands were collected and after dilution with Hanks buffer, pelleted by ultracentrifugation. The resuspended virus (5–10 mg/ml in Hanks buffer, containing 10% sucrose) was stored at −80°C.

Extraction of the envelope proteins

The envelope proteins were solubilized according to the procedure of Scheid and Choppin[1] with some minor modifications. The virus storage medium was removed by ultracentrifugation, and the viral pellet was resuspended in 0.01 *M* sodium phosphate buffer (pH 7.2), containing 1 *M* potassium chloride (or sodium chloride). The virions were disrupted by the addition of 2% (final concentration, w/v) Triton X-100 for 30 min at room temperature. The detergent/viral protein ratio was 3.3 (w/w). The extract was obtained as the supernatant after ultracentrifugation at 145,000 *g* for 60 min.

Removal of detergent and reduction

Triton X-100 was removed with Amberlite XAD-2 (Serva, Heidelberg, G.F.R.), as described by Kruse *et al.*[4] with minor modifications. Briefly, the extract was incubated with 500 mg of resin per ml for 20 min at 37°C. After sedimentation, dithiothreitol (Sigma, St. Louis, MO, U.S.A.) was added to the recovered supernatant in a final concentration of 20 m*M*. Finally, a second Amberlite incubation under the same conditions was performed in the presence of the reducing agent. Prior to chromatography, the turbid supernatant was passed through a 2-μm filter to remove Amberlite fines.

Amino acid analysis

F_2-containing peak fractions were lyophilized after partial evaporation. Protein was hydrolyzed with 6 *M* hydrochloric acid for 20 h at 110°C. Amino acid analysis was performed on a Kontron Liquimat III analyzer.

RP-HPLC

Chromatography was performed with a system consisting of a Waters M6000 A pump, an LKB 11300 Ultrograd gradient mixer, a Rheodyne 7125 injector and a Pye Unicam LC-UV detector. Two types of C_{18} packings were employed: one column (300 × 4.6 mm) was slurry-packed with Nucleosil 10 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) and another column (40 × 3.2 mm) with Supelcosil LC-318 (Supelco, Bellefonte, PA, U.S.A.), which have pore sizes of 10 and 30 nm, respectively. Extracts were chromatographed at a flow-rate of 1 ml/min with a linear gradient from 10 to 60% of solvent B in A, during 24 min. Solvent A was 12 m*M* hydrochloric acid in triple-distilled water; B was 12 m*M* hydrochloric acid in ethanol–1-butanol (4:1, v/v). Both organic solvents (LiChrosolv) were from E. Merck (Darmstadt, G.F.R.).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Viral proteins were analyzed on 12.5% polyacrylamide gel slabs[27]. Samples, reduced with dithiothreitol (DTT), were prepared as described by Lamb *et al.*[28]. Silver-staining of the protein bands was performed according to Wray *et al.*[29]. The protein bands were designated as described by Scheid and Choppin[1] and Gething *et al.*[10]. The P protein was not visible on the silver-stained gels, but its presence was established by staining with Coomassie brilliant blue.

RESULTS AND DISCUSSION

The proteins were eluted with gradients of an ethanol–1-butanol mixture in 12 m*M* hydrochloric acid instead of the more frequently used trifluoroacetic acid–propanol system[17,21,22] because (a) it allows detection down to 200 nm, (b) it is less viscous, (c) proteins are eluted at a lower organic solvent concentration, (d) the mixture is cheaper than propanol and (e) proteins are equally well resolved in both systems.

The purity of the Sendai virions and the detergent-solubilized envelope protein extract was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The molecular weights of the proteins corresponded well with those described by others[1,5,10,12,28]. Preliminary chromatography of the extracted proteins on the Nucleosil column separated only protein M and, after reduction with DTT, protein F_2 from the strongly absorbing Triton X-100 peak. To avoid interference from the detergent in the elution of F_1, Triton was removed with Amberlite XAD-2 in all further experiments. Analysis by SDS-PAGE before and after Amberlite treatment showed that the viral proteins were not adsorbed on the resin.

Since the amounts of protein to be analyzed were very small (10–50 μg), recoveries after chromatography were determined semiquantitatively by comparing the relative intensities of polypeptide bands after silver-staining of polyacrylamide gels. As F_1 and F_2 originate, in equimolar amounts, from the precursor protein F_0[1,2], amino acid analysis was also used to determine their relative recoveries.

Chromatography of the detergent-free extract, after reduction with DTT, on the 10-nm pore size Nucleosil 10 C_{18} column resulted in the elution of only two distinct peaks (Fig. 2A). SDS-PAGE showed that the first peak contained pure F_2 (*ca.* 100% recovery) and that the second peak contained protein M (20% recovery), which was slightly contaminated with HN. However, when the original analysis was

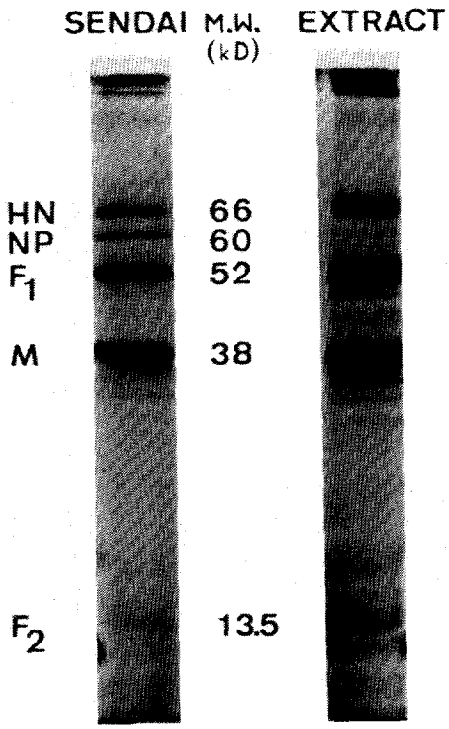

Fig. 1. SDS-PAGE of Sendai virions (SENDAI) and the extracted envelope proteins (EXTRACT). Protein bands were silver-stained as in ref. 29 and designated as in refs. 1 and 10. Molecular weights (M.W.) were determined by simultaneous electrophoresis of reference proteins as shown in Fig. 4.

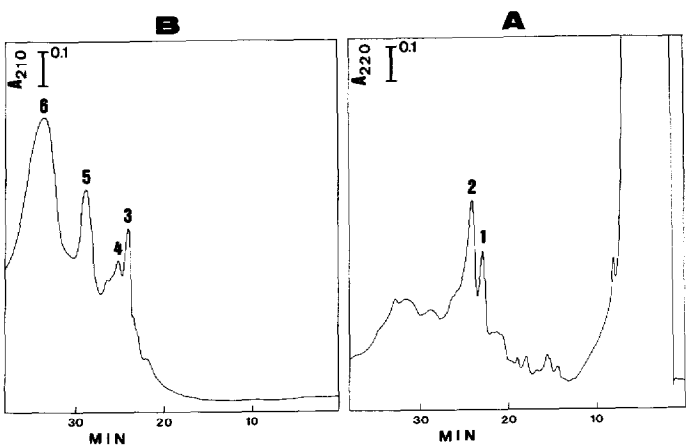

Fig. 2. A, RP-HPLC of detergent-extracted proteins of Sendai virus on a column of Nucleosil 10 C_{18} (300 × 4.6 mm). B, Chromatogram obtained during the blank run which was performed directly after the separation shown in A.

followed by a blank analysis, peaks were again obtained (Fig. 2B). Analysis on polyacrylamide gel showed that peaks 3, 4 and 5 could be assigned to M (30% of the amount which was eluted during the original analysis), HN in low yield (about 5%) and F_1 (20% recovery), respectively. Peak 6 was not always observed and, aside from a very small amount of F_1 (less than 5%), no other proteins were found in this fraction. Thus, a considerable memory effect occurred using the Nucleosil column, with a selective preference for M, HN and F_1. The latter proteins, except for M, had an even higher recovery than during the original analysis. Still, major amounts of M, HN and F_1 were not recovered and probably remained irreversibly bound to the column.

This memory effect was less prominent (between 5 and 10%) when the extract was separated on the Supelcosil LC-318 column, which has a pore size of 30 nm. The separation on this column is shown in Fig. 3. SDS-PAGE showed that on this larger-pore-size column material F_2 was also totally recovered in pure form (Fig. 4).

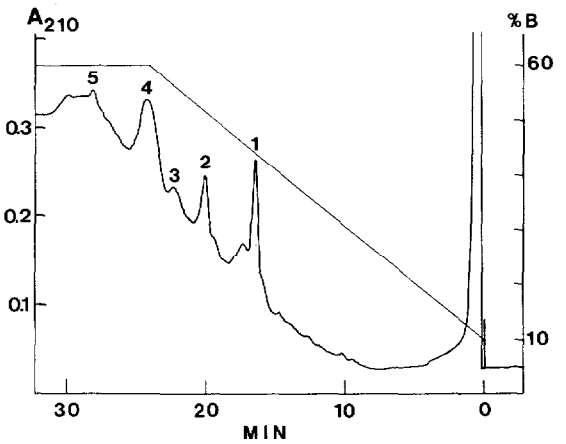

Fig. 3. RP-HPLC of detergent-extracted proteins of Sendai virus on a column of Supelcosil LC-318 (40 × 3.2 mm).

The heterogeneity in the molecular weight of this protein could be due to differences in the carbohydrate content. Peak 2 contained protein M (about 10% yield), which was slightly contaminated with HN. Highly enriched F_1 protein was eluted in peak 4 (25% recovery) and about an equal amount of this protein was found in peak fraction 5, although this was not reflected by the peak height. This, and the fact that F_1 was eluted in more than one peak might be due to residual detergent, firmly attached to the protein, or to low solubility of the protein in the mobile phase. Such a phenomenon probably also results in the presence of small residual amounts of M and HN in the F_1 peaks (see lanes 4 and 5, Fig. 4). No distinct peak of protein HN was observed during chromatography on the Supelcosil column. This protein, with a total recovery of less than 5%, was found only as a contaminant in peaks 3–5. Peak 3 always contained all Sendai proteins and additional bands with a molecular weight of about 40,000 daltons the origin of which is unknown. The shoulder on peak 1

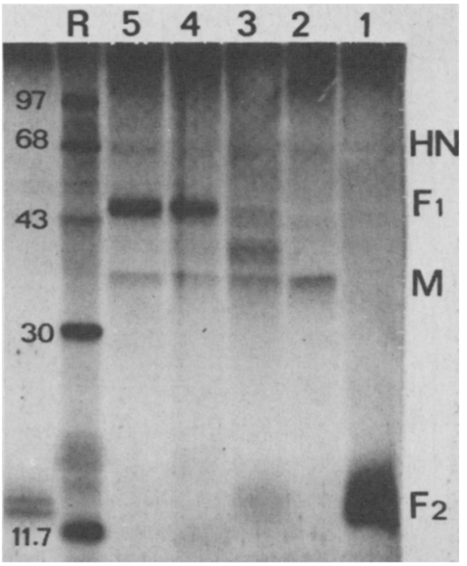

Fig. 4. SDS-polyacrylamide gel electrophoresis of the peak fractions 1–5 from RP-HPLC on Supelcosil LC-318 as shown in Fig. 3. Molecular weights are given in kilodaltons (first column). Reference proteins (R) were cytochrome *c*, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase *b*.

TABLE I

AMINO ACID COMPOSITION OF THE F_2 PROTEIN

The relative composition, based on a total of 90 residues as in ref. 10, was calculated from the average for six analyzed F_2 protein samples (2–35 μg each). F_2 was purified on the Supelcosil LC-318 column as described in the Experimental section. I = Present study; II = the F_2 composition cited[10].

*Residue**	*I*	*II*	*Diff. > 0.5*
Asp	10.4	10.6	
Thr	5.0	5.4	
Ser	6.9	6.8	
Glu	10.8	10.7	
Pro	3.9	4.7	−0.8
Gly	7.4	7.9	
Ala	5.4	5.7	
Cys	1.3	0.9	
Val	5.6	7.1	−1.5
Met	0.0	0.0	
Ile	7.1	6.5	+0.6
Leu	11.8	11.8	
Tyr	2.3	2.3	
Phe	1.1	0.8	
Lys	4.5	3.1	+1.4
His	1.2	1.1	
Arg	4.9	4.5	

* Trp was not determined.

(Fig. 3) results from a small amount of residual detergent. In comparison with the 10-μm pore size Nucleosil column, recoveries obtained on the 30-nm pore size Supelcosil column were better in the case of F_1, while the amounts of HN and M which were not recovered remained the same. Resolution, however, between the F_2 and the M proteins was better on the Supelcosil column (see Figs. 2A and 3). The smaller difference in retention time of these two proteins on the much longer Nucleosil column (30 cm) is probably due to the molecular sieving effect, which was found during an earlier study[19].

Because of the better resolution on the Supelcosil column, amino acid analyses were performed with F_2, purified on this column. The results of these analyses are shown in Table I, together with the F_2 composition, as published by Gething *et al.*[10]. Comparison of both compositions shows that F_2, purified in this study, is almost identical with the F_2 protein obtained by Gething *et al.* after affinity chromatography and two gelchromatography steps. In conclusion, RP-HPLC is an excellent tool for the purification of Sendai virus envelope proteins. Especially the F_2 protein was isolated in high yield (100%) and with a high degree of purification. Recoveries of the other three proteins were low (from less than 5% to 50%), but μg quantities of relatively pure protein were obtained.

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