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COMPARISON OF ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS FOR PURIFICATION OF SENDAI VIRUS INTEGRAL MEMBRANE PROTEINS

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SUMMARY

The recovery and separation of the integral membrane proteins, the haemagglutinin-neuraminidase (HN) and the fusion protein (F), from a Sendai virus detergent extract were compared on three different ion-exchange high-performance liquid chromatography (IE-HPLC) columns: Mono Q, TSK DEAE-NPR and Zorbax BioSeries SAX. The detergent, either 1-O-*n*-octyl-β-glucopyranoside (octylglucoside) or decyl polyethylene glycol-300 (decyl PEG-300), used for extraction of HN and F proteins from the virions, was also present in the elution buffers at a concentration of 0.1%. Recovery of HN and F proteins was primarily dependent on the detergent present in the eluent, resulting in yields of HN varying from 18 to 28 and 56 to 67%, when octylglucoside and decyl PEG-300, respectively, were used. The highest yield for HN protein was obtained by separation on either a Mono Q or a TSK DEAE-NPR column with decyl PEG-300 as the additive. Yields of F protein were lower, and the highest recovery of 46% was found in the presence of decyl PEG-300 by separation on the Mono Q column. Analysis of the fractions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and by size-exclusion HPLC indicated that the HN protein eluted in the presence of decyl PEG-300 from the Mono Q and the TSK DEAE-NPR columns was obtained in pure form, while the F protein was slightly contaminated with HN. Analysis of the fractions with monoclonal antibodies directed against conformational epitopes of HN and F proteins indicated that after IE-HPLC the conformation of the proteins is largely retained.

INTRODUCTION

Ion-exchange high-performance liquid chromatography (IE-HPLC) has been applied to the isolation of viral proteins, either alone or in combination with other

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modes of HPLC[1–10]. Differences in the electrostatic interaction between the column ligands and charged groups on the proteins are the basis for the separation. Many types of IE columns are commercially available[11]. They consist of porous or non-porous silica or polymeric particles with different charged ligands, *e.g.*, diethyl aminoethyl or trimethyl aminomethyl groups, resulting in a medium anion exchanger and a strong anion exchanger, respectively. Proteins are generally eluted by a salt gradient under physiological conditions (pH near 7). Therefore, IE-HPLC is particularly suitable for purification procedures where it is important to retain the biological activity of a protein.

Prior to purification, viral proteins must be solubilized. Detergents (surfactants) are widely used for this purpose[12–14]. To avoid aggregation of membrane proteins during purification, detergents are added to eluents as well. Non-ionic detergents generally do not affect the native conformation and electrostatic properties of proteins, and therefore they are preferentially used as additives in elution buffers for IE-HPLC. Some non-ionic detergents (Berol, Nonidet, Triton) absorb UV light and interfere with the spectrophotometric determination of proteins at 280 nm, while others, like octylglucoside and alkyl polyoxyethylene ethers, do not.

We studied the separation of integral membrane protein present in a detergent extract of Sendai virus particles on three different columns: two porous columns, Zorbax BioSeries Sax (Du Pont) and Mono Q (Pharmacia), and a non-porous column, TSK DEAE-NPR (Toyo Soda) with either 1-O-*n*-octyl-β-glucopyranoside (octylglucoside) or decyl polyethylene glycol-300 (decyl PEG-300) in the eluent. Sendai virus is a paramyxovirus of mice and belongs to the same family as the human parainfluenza, measles and mumps viruses. These virus particles are enclosed in a loose and fragile envelope, a lipid bilayer in which two integral membrane proteins are embedded. These proteins are the haemagglutinin-neuraminidase protein HN (M_r = 68 000) and the fusion protein F (M_r = 65 000). In the mature virus particles the HN protein is probably present only as the dimer and tetramer (HN_2 and HN_4, respectively) and can be converted into the monomeric form (HN) by treatment with a reducing agent. Occasionally, truncated forms of the HN protein (HN_4- and HN_2-), without the membrane-spanning region, are observed, due to degradation during the extraction procedure. 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 disulphide bridges. Multimeric forms of the F protein have been described[15], depending on the medium used for solubilization. The HN and F proteins are present as spikes on the outside of the virus particle.

In this study, the separation, recovery and immunological activity of Sendai virus HN and F proteins were compared after IE-HPLC on the three different columns.

EXPERIMENTAL

Detergent extracts of Sendai virus particles

Sendai virus was grown in 10-day-old embryonated eggs. The allantoic fluid was harvested 72 h after infection. Cell debris was removed by low-speed centrifugation (30 min at 2000 *g*, 5°C), and virus particles were pelleted from the supernatant by ultracentrifugation (1 h at 70 000 *g* at 5°C). The virus pellet was resuspended in 10

m*M* Tris-HCl (pH 7.2), supplemented with 10% sucrose and stored at −80°C. The protein concentration of the virus pellet was determined according to Lowry *et al.*[16].

Extraction of Sendai virus glycoproteins was performed with the detergents octylglucoside (Boehringer, Mannheim, F.R.G.) and decyl PEG-300 (Kwant-Hoog Vacolie Recycling and Synthese, Bedum, The Netherlands). Briefly, to a Sendai virus pellet suspension, containing 40 mg protein per ml buffer (10 m*M* Tris–HCl, pH 7.2), the same volume of buffer, containing 4% detergent, was added. The final detergent concentration was 2% and there were 40 mg viral proteins in 2 ml buffer. After incubation for 20 min at room temperature, the extraction procedure was terminated by ultracentrifugation for 1 h at 70 000 *g* at 5°C. The extracted HN and F proteins are present in the supernatant, which was stored in 200-μl portions at −80°C. The decyl PEG-300 extract of Sendai virus contained 298 μg HN protein and 400 μg F protein per 200 μl, and the octylglucoside extract 232 μg HN protein and 396 μg F protein per 200 μl.

Ion-exchange and size-exclusion HPLC

Chromatography was performed with a system consisting of a M 6000A pump (Waters, Etten-Leur, The Netherlands) or a 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).

Anion-exchange HPLC was performed with a Mono Q HR 5/5 (50 mm × 5 mm I.D.) column (Pharmacia, Uppsala, Sweden), with trimethyl aminomethyl groups and consisting of hydrophilic polymer beads with a particle size of 10 μm and pores of 80 nm; a TSK DEAE-NPR (35 mm × 4.6 mm I.D.) column (Toyo Soda, Tokyo, Japan), with diethyl aminomethyl groups and consisting of a non-porous hydrophilic resin with a particle size of 2.5 μm (ref. 17) and a Zorbax BioSeries SAX (80 mm × 6.2 mm I.D.) column (Du Pont, Wilmington, DE, U.S.A.), with trimethyl aminomethyl groups and consisting of zirconium oxide-stabilized silica with a particle size of 6 μm and a pore size of 30 nm. The protein capacities of the Zorbax BioSeries SAX and Mono Q columns, as reported by the supplier, are 42 and 26 mg per ml column volume, respectively. The capacity of the TSK DEAE-NPR column is 8.6 mg per ml column volume[17].

After injection of an octylglucoside extract of Sendai virus (439 and 900 μg of protein) or a decyl PEG-300 extract (342 and 698 μg of protein), the column was eluted isocratically for 5 min. The proteins retained were eluted with a linear 12-min gradient from 20 m*M* Tris–HCl (pH 7.8), containing 0.1% detergent, to 0.5 *M* sodium chloride in the same buffer. The detergent (either oxtylglucoside or decyl PEG-300) used for the extraction of HN and F proteins from the virions was also present in the elution buffers. The gradient was generated by a low-pressure mixing system[18]. The flow-rate was 1 ml/min, and the absorbance was monitored at 280 nm. Fractions were collected manually in Minisorp tubes (Nunc, Roskilde, Denmark). Aliquots of each fraction were taken for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The remaining part of the fraction was dialyzed overnight against water by covering the tubes with a square piece of dialysis membrane tubing, and the tubes were closed by fitting a slice of silicone rubber tubing over the dialysis membrane. After dialysis, the fractions were freeze-dried in the

tubes. The freeze-dried fractions were used to determine the recovery of proteins HN and F, and they were analyzed in an enzyme-linked immunosorbent assay (ELISA) for reaction with conformation-dependent monoclonal antibodies against HN and F proteins.

Size-exclusion (SE) HPLC was performed on two Zorbax GF 450 (250 mm × 9.4 mm I.D.) columns (Du Pont) in tandem, to determine the amounts of HN and F proteins present in the fractions collected during IE-HPLC. The IE-HPLC fractions (freeze-dried after dialysis) were dissolved in 100 μl water. Of the dissolved fraction, 20 μl were taken (the remainder was used in the ELISA), and SDS was added to a final concentration of 4%. The fractions were heated for 3 min in a bath of boiling water and subjected to analysis by SE-HPLC. The proteins were eluted with 50 m*M* sodium phosphate (pH 6.5), containing 0.1% SDS, at a flow-rate of 1 ml/min. The absorbance was monitored at 280 nm. The recovery of HN and F proteins was calculated from the peak height, using the elution pattern of a mixture of 50 μg bovine serum albumin (BSA), 50 μg ovalbumin and 50 μg trypsin inhibitor as a standard.

SDS-PAGE

Samples of the eluate fractions (50 μl) were prepared for SDS-PAGE[19] on 8% gels under non-reducing conditions by the addition of ten-fold concentrated sample buffer (15 μl) without reducing agent. After electrophoresis, the gels were fixed and silver-stained as described[20].

ELISA

The IE-HPLC fractions were analyzed for the presence of structurally intact HN and F proteins by determination of the reaction with conformation-dependent monoclonal antibodies HN 851 and F 1.216. The production of these monoclonal antibodies, directed against HN and F proteins, and their characterization as conformation-dependent has been described[21,22]. For coating of the ELISA trays, the remaining part (80 μl) of the IE-HPLC fractions, dissolved in water, was diluted in coating buffer (50 m*M* sodium carbonate, pH 9.6) to concentrations of 10, 2 and 0.5 μg protein per ml. Plates were coated with 100 μl per well of these three concentrations. After coating overnight at 4°C (or 2 h at 37°C), plates were washed three times for 5 min with phosphate-buffered saline (pH 7.2), containing 0.2 *M* sodium chloride, 0.3% Tween 20 and 1 mg SDS/l (washing buffer). The coated proteins were allowed to react with monoclonal antibodies HN 851 and F 1.216, diluted 1:1000 in washing buffer supplemented with 0.5% BSA (dilution buffer). After incubation at room temperature for 1 h and washing (three times for 5 min), peroxidase-labelled anti-mouse immunoglobulin G (IgG) (conjugate), diluted 1:1000, was added and the plates were incubated for 1 h at 37°C. After washing, the peroxidase activity was visualized by adding 100 μl substrate, consisting of 0.2 mg *o*-phenylenediamine dihydrochloride (Eastman Kodak, Rochester, NY, U.S.A.)–0.006% (v/v) H_2O_2 in 2% methanol–50 m*M* sodium phosphate (pH 5.6) per well. The reaction was terminated by adding 50 μl 2 *M* sulphuric acid per well. The optical density at 492 nm was measured in a microplate photometer. Optical density values below 0.2 were considered as negative.

RESULTS AND DISCUSSION

In Fig. 1 the elution profiles of a decyl PEG-300 extract of Sendai virus, separated on the Mono Q, TSK DEAE-NPR and Zorbax Bio Series SAX columns (Fig. 1a, b and c, respectively), are shown. The elution profiles after IE-HPLC of decyl PEG-300 and octylglucoside extracts for the Mono Q and the Zorbax BioSeries SAX columns showed many sharp peaks and one broad peak, while the peaks from the TSK DEAE-NPR column were generally broader. Analysis by SDS-PAGE of the IE-HPLC fractions showed that, despite differences in elution profiles, the separation of the Sendai virus proteins was rather similar (data not shown). In Fig. 2, the elution pattern of a decyl PEG-300 extract, separated on the Mono Q colum, together with the analysis by SDS-PAGE, is shown as an example. The first peak fractions that were eluted did not contain protein material. Then the multimeric forms of the HN protein (first HN_2-, and thereafter a mixture of HN_4, HN_2 and HN_2-) were eluted, followed by a broad peak of the F protein. These peaks do not entirely reflect the different multimeric forms of HN (HN_4, HN_2 and HN_2-, respectively) and F protein, but may be due to different aggregate forms in the extract and probably also to differences in charge, caused by partial glycosylation. Eighteen percent of the oligosaccharides from the HN protein are acidic, while more than 75% of the oligosaccharides from the F protein are acidic[23]. In addition to this general separation pattern, IE-HPLC of octylglucoside extracts of Sendai virus with the same detergent added to the eluent often showed HN_2- protein in one of the first peaks. The data obtained by SDS-PAGE of the fractions from the different IE-HPLC columns show that the time

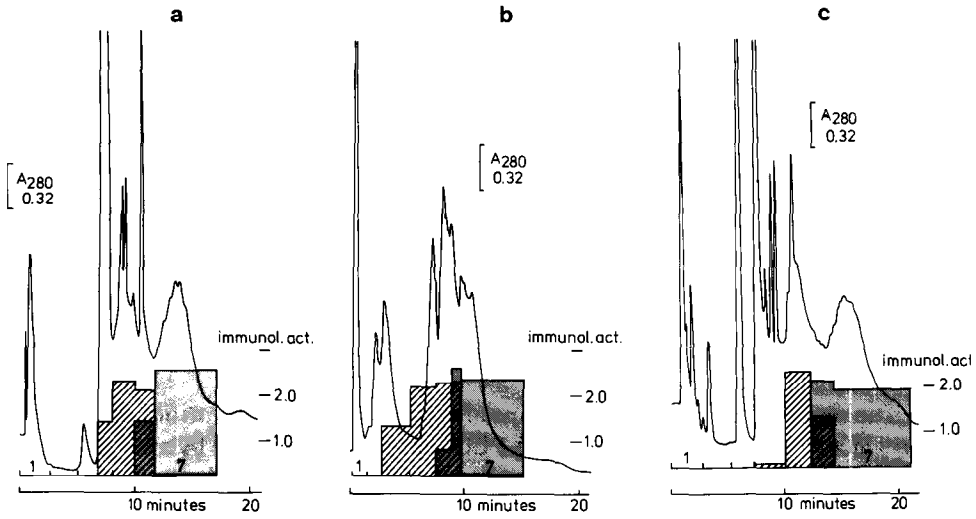

Fig. 1. IE-HPLC of a decyl PEG-300 extract of Sendai virions, containing 298 μg HN and 400 μg F protein, separated on Mono Q (a), TSK DEAE-NPR (b) and Zorbax BioSeries SAX (c) columns. Elution was performed with a 12-min gradient from 20 m*M* Tris-HCl (pH 7.8), containing 0.1% decyl PEG-300, to 0.5 *M* sodium chloride in the same buffer. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm. The fractions were collected as indicated. Amounts of 0.2 μg of each fraction were investigated for reactivity with monoclonal antibodies HN 851 (hatched area left), directed against intact HN protein, and with monoclonal antibodies F 1.216 (hatched area right), directed against intact F protein.

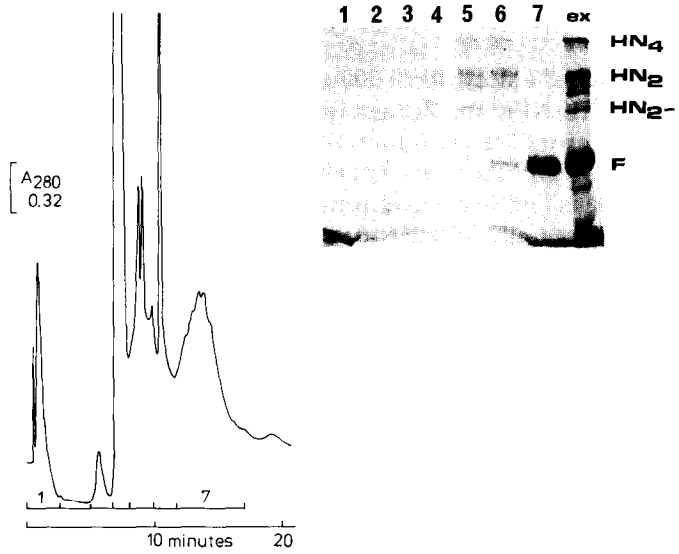

Fig. 2. IE-HPLC of a decyl PEG-300 extract of Sendai virus membrane proteins on a Mono Q column. Elution was performed as in Fig. 1. The flow-rate was 1 ml/min; the absorbance was monitored at 280 nm. Fractions were collected as indicated, and samples were analyzed by SDS-PAGE (8% gel) under non-reducing conditions. Lane numbers of the gel refer to IE-HPLC fractions; ex = the decyl PEG-300 extract; HN_4, HN_2, HN_2- and F are the tetramer of HN, dimer of HN, truncated form of the HN dimer and the F protein, respectively.

at which the multimeric forms of HN and F proteins were eluted is different. HN and F proteins were eluted from the Mono Q and Zorbax BioSeries SAX column after starting the salt gradient. In contrast, HN was partially eluted from the non-porous TSK DEAE-NPR column before the salt gradient was started. This can be explained by the medium-strong anion-exchange properties of this diethylaminoethyl-functionalized support, compared to the strong anion-exchange properties of the trimethyl aminomethyl groups in the Mono Q and Zorbax BioSeries SAX columns. The HN and F proteins were eluted later from the Zorbax BioSeries SAX column than from the Mono Q column. The anion-exchange properties of both types of columns are rather similar. The retarded elution of the proteins may be caused by the slower diffusion of the proteins HN and F from the smaller pores of the Zorbax BioSeries SAX packing.

The recovery of HN and F proteins after IE-HPLC on the various columns in the presence of octylglucoside and decyl PEG-300 was quantitated by analysis of the IE-HPLC fractions using SE-HPLC. In Fig. 3 this is illustrated by the analysis of fractions 4-7, obtained after IE-HPLC on the Mono Q column in the presence of decyl PEG-300. Peak 1 is an aggregate peak, which contains no proteins, peaks 2 and 3 contain the tetramer and dimer of HN protein, respectively, peak 4 contains the F protein. Fraction 4 contains mainly HN_2 forms (12 μg) and small amounts of HN_4 and F proteins (3 μg of each). In fraction 5 the amounts of HN_2 and HN_4 are increased to 36 and 42 μg, respectively, while the amount of F protein is still small (5 μg). Fraction 6 contains 33, 26 and 16 μg of HN_4, HN_2 and F proteins, respectively,

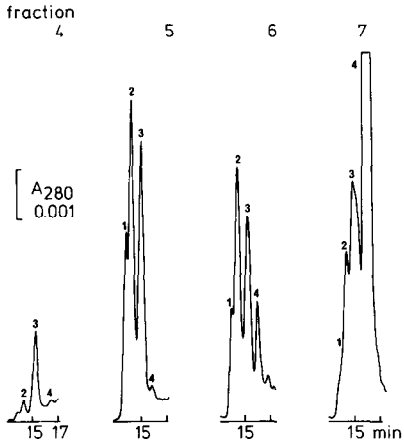

Fig. 3. Size-exclusion HPLC of fractions 4–7 of the IE-HPLC shown in Fig. 2. Samples of the fractions were made 4% in SDS and heated for 3 min in a bath of boiling water prior to analysis on two tandem-linked Zorbax GF-450 columns. The elution was performed with 50 m*M* sodium phosphate buffer (pH 6.5), containing 0.1% SDS. The flow-rate was 1 ml/min, and absorbance was monitored at 280 nm. Peaks: 1 = nonproteinaceous material; 2 = tetramer of HN; 3 = dimer of HN; 4 = F protein.

while in fraction 7 predominantly F protein (193 μg) is eluted. Table I summarizes the recoveries for HN and F proteins obtained after IE-HPLC on the various columns. The recovery is mainly determined by which detergent is present in the elution buffer. Values for HN and F proteins are approximately three times higher with decyl PEG-300 in the buffer than with octylglucoside in the eluents and are independent of the amount of protein injected. The highest recovery (28%) of HN in the presence of octylglucoside is obtained by separation on the TSK DEAE-NPR column, while with decyl PEG-300 in the eluents the recoveries of HN (67%) are similar for the Mono Q and the TSK DEAE-NPR columns. The recoveries of F protein are lower than those

TABLE I

PERCENT RECOVERY OF HN AND F PROTEINS FROM DIFFERENT IE-HPLC COLUMNS WITH OCTYLGLUCOSIDE AND DECYL PEG-300 AS DETERGENTS IN THE ELUTION BUFFERS

Column type	*HN protein*		*F protein*	
	Octylglucoside[a]	*Decyl PEG-300*[b]	*Octylglucoside*[a]	*Decyl PEG-300*[b]
Mono Q HR 5/5	20	67	12	46
TSK DEAE-NPR	28	67	12	27
Zorbax BioSeries SAX	18	56	<10	34

[a] Two different amounts of an octylglucoside extract of Sendai virus (containing 162 and 332 μg of HN protein, and 277 and 568 μg F protein, respectively) were separated with a linear salt gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* Tris–HCl (pH 7.8) with 0.1% octylglucoside.

[b] Two different amounts of a decyl PEG-300 extract of Sendai virus (containing 146 and 298 μg HN protein, and 196 and 400 μg F protein, respectively) were separated with a linear salt gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* Tris-HCl (pH 7.8) with 0.1% decyl PEG-300.

of the HN protein for all three columns investigated. IE-HPLC in the presence of 0.1% octylglucoside results in a recovery of 12% F protein from the Mono Q and TSK DEAE-NPR columns; for the Zorbax BioSeries SAX column the recovery was less than 10%. With 0.1% decyl PEG-300 as additive the recoveries are higher, 46, 34 and 27% for the Mono Q, Zorbax BioSeries SAX and TSK DEAE-NPR, respectively. Relatively low recoveries with oxtylglucoside in the eluent have also been described by others[24,25]. Various viral membrane proteins have been successfully isolated by IE-HPLC from detergent extracts, *e.g.*, polypeptides of 84 000, 90 000 and 105 000 daltons from bovine viral diarrhea virus (BVDV)[5,7], a membrane protein gp 340 from Epstein-Barr virus (EBV)[8] and the haemagglutinin (H protein) protein from measles virus[10]. The BVDV proteins were separated on a TSK DEAE-5PW column in the presence of 0.5% Berol 185 with a recovery of 60–70%. The recovery of gp 340 from EBV was 77% after IE-HPLC on a Mono Q column with 0.5% MEGA-9 in the eluent. The H protein of measles virus, related to the HN protein of Sendai virus, was purified on a Mono Q column in the presence of 0.03% Triton X-100, with a recovery of 55%. The recoveries of the above-mentioned viral membrane proteins range from 55 to 77%. These values are comparable to the recoveries of 56–67% of the HN protein obtained in the presence of decyl PEG-300. The recoveries (46–27%) of F protein in the presence of decyl PEG-300 are slightly lower than those reported for other viral membrane proteins. Results from SE-HPLC in combination with SDS-PAGE analysis of the IE-HPLC fractions indicated that only small quantities of HN protein (fractions 3 in Fig. 1a and b) that were eluted in the presence of decyl PEG-300 from the Mono Q and the TSK DEAE-NPR columns were obtained in pure form. F Protein was always slightly contaminated with HN protein. A second IE-HPLC of appropriate fractions probably may result in pure HN and F proteins.

The structural alterations possibly resulting from IE-HPLC were measured by determination of the immunological activity of the HN and F proteins in the eluate fractions. In Fig. 1 the immunological activity of the fractions is shown together with the elution profiles in the presence of 0.1% decyl PEG, for the Mono Q, TSK DEAE-NPR and Zorbax BioSeries SAX columns. Amounts of 1, 0.2 and 0.05 μg of each fraction were used to investigate the reaction with two conformation-dependent monoclonal antibodies: HN 851, directed against protein HN, and F 1.216, directed against F protein. Both monoclonal antibodies reacted with the fractions collected during IE-HPLC (Fig. 1), indicating that the native structure of a considerable number of the HN and F molecules is preserved during the chromatographic procedure. A high reactivity of the monoclonal antibodies with either HN or F protein does not imply that there may not be small, local changes, distantly located from the epitopes to which the monoclonal antibodies are directed. However, large structural changes in any part of the protein molecule are expected to have long-range effects and will therefore affect the reactivity of the monoclonal antibodies. In agreement with this is the observation that the reaction of a mixture of conformation-dependent monoclonal antibodies, directed against different epitopes, is identical with the reaction of one conformation-dependent monoclonal antibody HN 851 (unpublished results). In an earlier study[22] we have described the immunological activity of proteins HN and F after SE-HPLC with 0.05% sarkosyl in the eluent. After SE-HPLC, amounts of 1 mg of the eluate fractions were necessary to obtain the same level of immunological activity as is present in 0.2 μg of the IE-HPLC fractions described here. This indicates

that, in this particular study, after IE-HPLC more protein molecules have the native conformation than after SE-HPLC.

CONCLUSIONS

The results show that IE-HPLC is a relatively mild separation method yielding structurally intact viral membrane proteins. Recovery is largely determined by the detergent present in the eluent, and to a lesser extent by the type of IE-HPLC column used. The highest recovery of HN protein was obtained by using either a Mono Q column of a TSK DEAE-NPR column, while the highest recovery of F protein was obtained after chromatography on the Mono Q column. Differences observed in the elution profiles of the IE-HPLC columns (Mono Q, TSK DEAE-NPR and Zorbax BioSeries SAX) were more likely due to the anion-exchange properties and pore sizes of the supports than to their porous or non-porous character.

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