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COMBINED SIZE-EXCLUSION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF A DETERGENT EXTRACT OF SENDAI VIRUS

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SUMMARY

Virus envelope proteins obtained by Triton X-100 extraction of Sendai virions were purified to a high degree by a combination of high-performance liquid chromatography (HPLC) methods. Size-exclusion HPLC on a TSK 4000 PW column with several concentrations of acetonitrile or ethanol–1-butanol in 0.1% hydrochloric acid as eluent was used as the first chromatographic step. Peak fractions were diluted in water and further fractionated on reversed-phase columns (TMS-250 or Vydac 218 TP). Size-exclusion HPLC with 45% acetonitrile in 0.1% hydrochloric acid, combined with reversed-phase HPLC on either column, was most suitable for obtaining highly purified F_2 protein. Antibodies obtained after injection of this protein were reactive with the intact virus.

INTRODUCTION

There is growing interest in peptides or protein fragments that can be used to elicit the formation of antibodies to the intact protein[1,2]. This approach can be used to localize proteins in tissues, to isolate the intact protein by immunoaffinity chromatography or to develop synthetic vaccines. A similar approach is applicable to proteins purified by reversed-phase high-performance liquid chromatography (HPLC).

Sendai virus proteins are denatured by the low pH and the high concentration of organic solvent normally used for chromatographic elution. Antibodies against the intact virus do not react with proteins exposed to these conditions. If, however, antibodies are produced against denatured peptides or protein fragments, they may well react with the intact protein. Therefore it was argued that antibodies against Sendai virus proteins purified by reversed-phase HPLC might react with the intact virus. In order to obtain highly purified proteins for immunization studies, a combination of size-exclusion HPLC and reversed-phase HPLC[3] was used to isolate Sendai virus proteins from a detergent extract of a crude virion preparation.

EXPERIMENTAL

Virus; detergent extraction

Sendai virus was grown in 10-day-old embryonated chicken eggs. Allantoic fluid was harvested after 48 h of infection at 36°C. The debris was pelleted at 2000 *g* for 20 min, and virions were pelleted by centrifugation at 22000 rpm (70000 *g*) for 1 h. A crude virion preparation (40–50 mg protein per ml) was extracted by treatment with Triton X-100 (ref. 4). Prior to size-exclusion HPLC, Triton X-100 was removed with Amberlite XAD-2 and the proteins were reduced with 20 m*M* dithiothreitol as described earlier[5,6]. Volumes of 200 μl of extract, containing 200–400 μg of protein, were subjected to chromatography.

Size-exclusion and reversed-phase HPLC

Chromatography was performed with one HPLC pump (Waters M 6000A), a low-pressure mixing system[7], a Rheodyne 7125 injector and a Pye Unicam LC-UV detector.

Size-exclusion HPLC was performed on 600 × 7.5 mm I.D. columns of TSK 4000PW or TSK 3000SW (Toyo Soda, Tokyo, Japan) by elution with 35 and 45% (v/v) acetonitrile in 0.1% hydrochloric acid or 25 and 40% ethanol–1-butanol (4:1) in 0.1% hydrochloric acid. The flow-rate was 1 ml/min, and the absorbance was monitored at 220 nm. Prior to subsequent reversed-phase HPLC, column eluates were diluted 1:1 in water.

Reversed-phase HPLC was performed on a 75 × 4.6 mm I.D. column of TMS-250 (C_1, silica-based, 25-nm pores) from Toyo Soda or a 50 × 4.6 mm I.D. column of Vydac 218 TP (C_{18}, silica-based, 30-nm pores) from The Separations Group (Hesperia, CA, U.S.A.). The TMS-250 column was eluted with a 25–75% gradient of acetonitrile in 0.05% trifluoroacetic acid (TFA) in 25 min. The Vydac column was eluted with a 20–65% ethanol–butanol (4:1) gradient in 0.1% hydrochloric acid in 25 min. The flow-rate was 1 ml/min and the absorbance was monitored at 210 nm.

Organic solvents were from Merck (Darmstadt, F.R.G.). 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. Chromatographic yields were estimated by comparing the intensities of polypeptide bands by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) prior to and after HPLC. The relative yields of the F_2 protein were based on peak heights.

SDS-PAGE and enzyme-linked immunosorbent assay (ELISA)

The column eluates were analyzed by SDS-PAGE[8]. Polypeptides were visualized by a silver-staining method[9]. For ELISA, polystyrene micro titerplates (Dynatech, Denkendorf, F.R.G.) were coated with 10 μg purified Sendai virions and allowed to react with antiserum obtained after immunization of C57B1 mice with 5 μg HPLC-purified F_2 protein. ELISA was carried out as described earlier[4].

RESULTS AND DISCUSSION

Size-exclusion HPLC of a number of reference proteins with 45 and 36% ace-

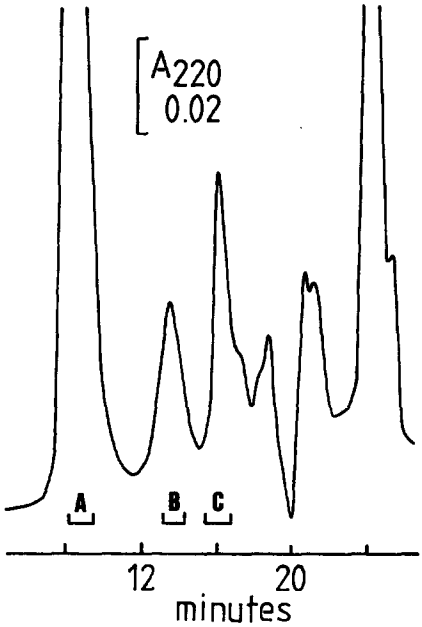

Fig. 1. Size-exclusion HPLC of a Triton X-100 extract of Sendai virions, which was reduced with dithiothreitol. The TSK 4000PW column was eluted with 45% acetonitrile in 0.1% hydrochloric acid. The flow-rate was 1 ml/min, and the absorbance was monitored at 220 nm. Fractions A–C were further purified by reversed-phase HPLC (see Fig. 2).

tonitrile in 0.1% TFA was described by Swergold and Rubin[3]. These conditions but with hydrochloric acid instead of TFA were used to fractionate a detergent extract of purified Sendai virions. In addition, it was investigated whether 25 or 40% ethanol–1-butanol (4:1) in 0.1% hydrochloric acid was suitable as solvent for size-exclusion HPLC. Prior to size-exclusion HPLC, Triton X-100 was removed, since this detergent would result in a large peak with a molecular weight of 20000 and would interfere with the detection of proteins in that molecular weight range.

We reduced the proteins in the detergent extract, since the multimeric forms of the Sendai virus proteins tend to precipitate at the above-mentioned organic solvent concentrations. Reduction with dithiothreitol converts the tetramer and dimer of the haemagglutinin-neuraminidase protein (HN) into a monomer (molecular weight, M_r = 66000), and the fusion protein (F) into its two components, F_1 (M_r = 50000) and F_2 (M_r = 13000–15000). The F_2 protein is particularly suitable for isolation by combined size-exclusion and reversed-phase HPLC. Its molecular weight is sufficiently different from those of the other Sendai virus proteins to obtain a relatively pure preparation by size-exclusion chromatography, and the isolation of F_2 by reversed-phase HPLC was described earlier[6]. Size-exclusion HPLC with 45% acetonitrile was most suitable for the separation of F_2 protein from the other Sendai virus proteins.

Fig. 1 shows the separation obtained by size-exclusion HPLC on a TSK 4000PW column. Fractions A, B, C were diluted in water and fractionated further by reversed-phase HPLC on a TMS-250 column. We only observed peaks after chro-

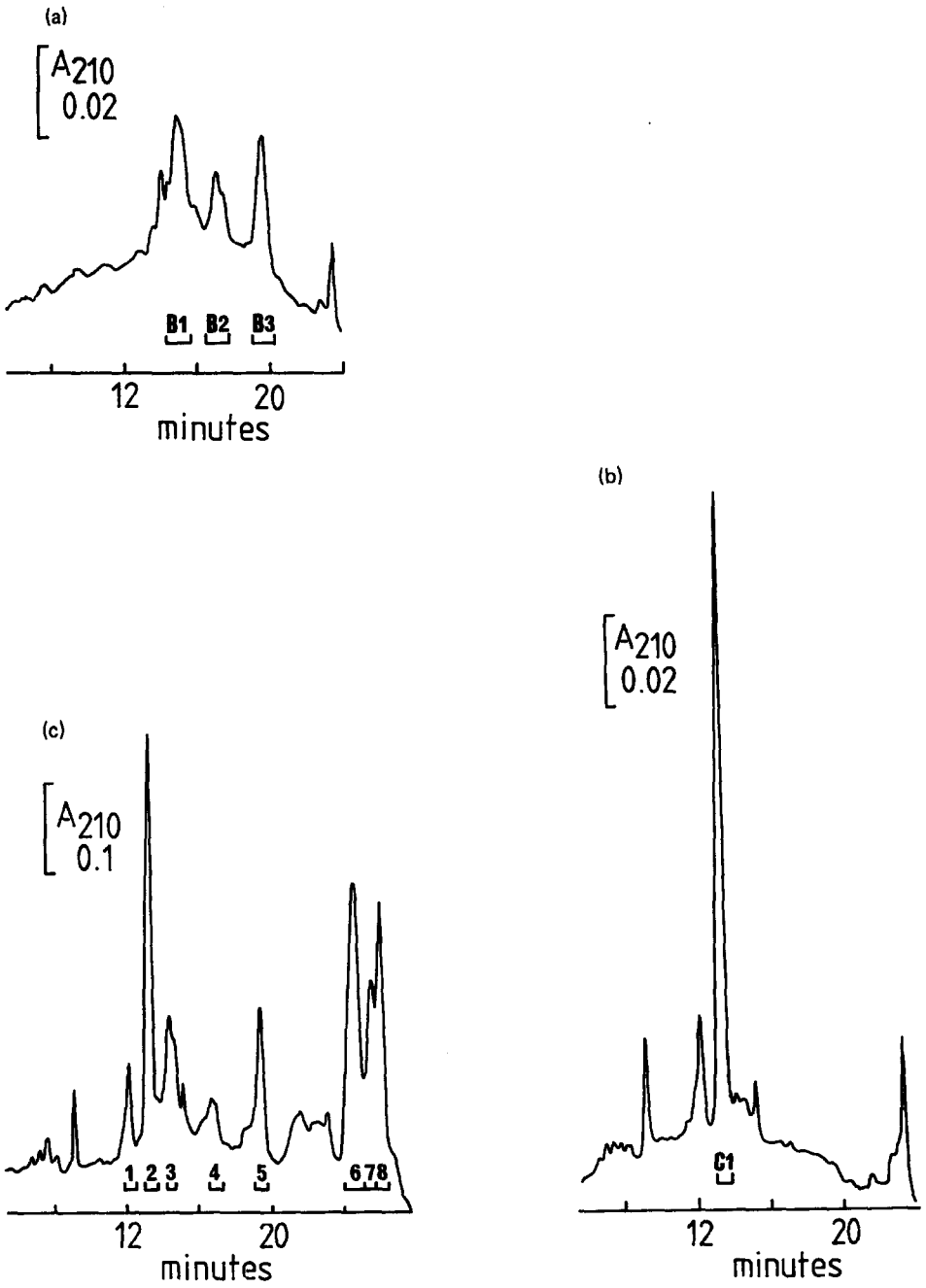

Fig. 2. Reversed-phase HPLC of fractions B and C (a and b, respectively) and of a detergent extract of Sendai virus which was not subjected to size-exclusion HPLC prior to reversed-phase HPLC. The TMS-250 column was eluted with a 25-min gradient, consisting of 25–75% acetonitrile in 0.05% TFA. The flow-rate was 1 ml/min and the absorbance was monitored at 210 nm. Fractions were analyzed by SDS-PAGE (Fig. 3).

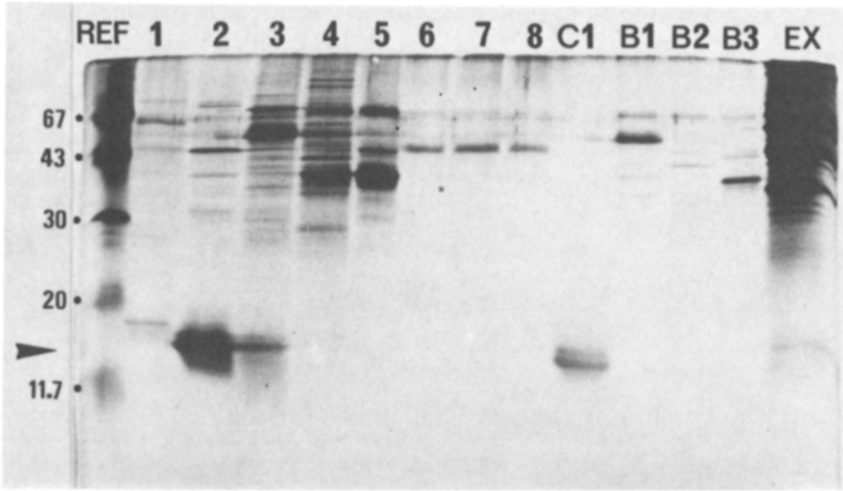

Fig. 3. SDS-PAGE (12.5% gels) of fractions 1–8, Cl and B1–B3 from Fig. 2. The molecular weights of reference proteins (REF) are indicated in kilodaltons. EX = Triton extract. The F_2 protein is indicated by an arrow.

matography of fractions B and C (see Fig. 2a and b, respectively). In fraction A, aggregated protein material may have been present that could not be eluted from the reversed-phase column. The detergent extract was also analyzed by reversed-phase HPLC without being first subjected to size-exclusion HPLC (Fig. 2c). The peaks were analyzed by SDS-PAGE, Fig. 3. Although a detergent extract from a relatively crude Sendai virus preparation was analyzed (Fig. 3, EX), the F_2 protein was obtained in pure form (Fig. 3, C1) after the combined size-exclusion and reversed-phase HPLC. The only other bands are artifacts, associated with the silver-staining procedure[10]. The F_1 protein and the matrix protein M (M_r = 38000) present in fractions B1 and B3, respectively, were purified to a considerable degree. The HN protein (M_r = 66000) was not eluted as one peak and was present in B1–B3. A comparison of lanes 1–8 (reversed-phase HPLC only) with lanes C1, B1–B3 illustrates the potential of combining the two HPLC modes. Considering the small amounts of protein (200–400 μg) that were subjected to chromatography the yields were generally high. For example, a single reversed-phase HPLC step resulted in an almost 100% yield of the F_2 protein (17.5 μg). With the combined HPLC procedure the recovery of F_2 was about 77% (13.5 μg).

The 40% ethanol–1-butanol (4:1) solvent was not suitable for size-exclusion HPLC. Most of the proteins precipitated at this organic solvent concentration. The precipitate was redissolved at 20–25% organic solvent concentration; by subsequent reversed-phase HPLC on a Vydac 218 TP column, 90% of the F_2 protein could be recovered. A concentration of 25% of this solvent was suitable for gel chromatography but broader peaks were obtained than with acetonitrile.

In addition a TSK 3000SW column was also used for the size-exclusion step with 45% acetonitrile in 0.1% hydrochloric acid as the eluent. Peaks were narrower, but this was not essential for the final purification of the F_2 protein by reversed-phase HPLC.

Mice were immunized with F_2 protein, purified as described above. Preliminary ELISA results showed that the antibodies against HPLC-purified F_2 protein reacted with purified virions.

Thus combinations of different HPLC modes are particularly suitable for the rapid purification of small amounts of protein which can be used as immunogens. Despite the fact that these proteins generally are denatured by the solvents used for chromatography, antibodies against HPLC-purified proteins may react with the native proteins. Such antibodies can subsequently be used for the isolation of the native protein, which allows investigations of the rôle of various viral components as antigens contributing to immunity or immunopathology[11].

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