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SEPARATION OF FLAVIVIRUS MEMBRANE AND CAPSID PROTEINS BY MULTISTEP HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OPTIMIZED BY IMMUNOLOGICAL MONITORING

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SUMMARY

Complete separation of the three structural proteins, E, C and M, of an enveloped virus (tick-borne encephalitis virus) was achieved by means of a two-step high-performance liquid chromatography (HPLC) technique in less than 1 h. The hydrophobically associated membrane proteins E and M were successfully separated by high-performance gel permeation chromatography (TSK-3000 SW column) in the presence of sodium dodecyl sulphate (SDS), whereas the separation of M and C as well as desalting and removal of SDS was achieved by subsequent reversed-phase chromatography on a C_3 column. With regard to further characterization by peptide mapping, analysis of the amino acid composition and aminoterminal sequencing, the second step was performed with volatile buffer systems. Quality control of the separation was achieved by a combination of HPLC with a highly sensitive dot immunoassay by the use of polyclonal as well as monoclonal antibodies. This method proved extremely sensitive and revealed strong tailing effects and cross-contaminations of peaks well-separated in reversed-phase chromatography, which were neither apparent in the absorbance curve at 214 nm nor in the analysis by SDS-polyacrylamide gel electrophoresis. By visualization of the peak-tailing effect, the chromatographic conditions could be modified in order to achieve an optimum separation of proteins.

INTRODUCTION

The wide range of column materials with different properties, *e.g.*, gel permeation, ion-exchange, reversed-phase, has rendered high-performance liquid chromatography (HPLC) one of the most powerful and versatile tools for the rapid preparative and analytical separation of proteins and peptides[1,2]. Depending on the characteristics of the proteins to be separated, specific strategies must be developed to exploit fully the potential of available HPLC methods. This is especially true for membrane proteins, which are prone to solubility problems and have a strong tendency to aggregate. Therefore, for the complete dissociation of membrane-associated protein complexes, strong denaturating agents must also be present during the separation procedure. This can be achieved by high-performance gel permeation chro-

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matography in the presence of sodium dodecyl sulphate (SDS)[3], which can yield good separations provided that the molecular weight differences are sufficiently large[4,5]. On the other hand, reversed-phase chromatography represents a completely different and powerful separation tool, which is independent of molecular weight. Especially when applied to hydrophobic proteins, however, tailing[6] and memory effects[7] may seriously impair the separations.

We have investigated these problems by using the separation of the structural proteins of an enveloped RNA virus, tick-borne encephalitis (TBE) virus, as a model system. Belonging to the family of flaviviridae, TBE virus contains three structural proteins, termed E, C and M, with estimated molecular weights of 55 000, 15 000 and 7500, respectively; C is the only protein constituent of the nucleocapsid, whereas E and M are found in the viral membrane[8]. The glycoprotein E represents an amphiphilic protein, which remains associated with the hydrophobic membrane protein, even in the presence of high concentrations of certain non-ionic detergents like Triton X-100[8].

The immunological properties of the structural proteins have been defined by monospecific polyclonal as well as monoclonal antibodies[9,10]. These highly specific reagents represent extremely sensitive tools for analyzing the quality of the separation, which could not be performed with conventional optical methods. The system described can therefore serve as a model for designing efficient separation conditions, especially in the case of membrane proteins, by the combination of HPLC with sensitive immunoassay methods.

MATERIALS AND METHODS

HPLC

The HPLC system (Beckman, Berkeley, CA, U.S.A.) consisted of two Model 112 pumps, a Model 421 system controller, a Model 210 automatic sample-injection valve and a Model 460 detector (fixed wavelength). Chromatograms were recorded by a Shimadzu Chromatopac C-R1B recorder.

Gel permeation chromatography was performed on two 300 × 7.5 mm I.D. Spherogel TSK-G 3000 SW columns (Altex, Berkeley, CA, U.S.A.) connected in series. The peak fractions obtained were subjected to further purification on a 75 × 4.6 mm I.D. Ultrapore RPSC (C_3, pore diameter 300 Å) column (Beckman).

Acetonitrile and 2-propanol were of HPLC grade (J. T. Baker, Deventer, The Netherlands). Buffers were made up with Analar-grade reagents (Merck, Darmstadt, F.R.G.) in water doubly distilled from glass apparatus. "Research-grade" trifluoroacetic acid (TFA) was purchased from Serva (Heidelberg, F.R.G.) and SDS ("electrophoresis-grade") from Bio-Rad (Richmond, CA, U.S.A.). All solvents were filtered through a 0.2-μm Millipore GVWP filter (Millipore, Bedford, MA, U.S.A.) and degassed before use. All chemicals described without further specification were of Analar grade (Merck).

Separation of the TBE virus structural proteins

Purified TBE virus was precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 12% (w/v). The precipitate was washed twice with acetone at −20°C, and the pellet was dissolved in 50 m*M* phosphate buffer (pH 6.5),

containing 2% (w/v) SDS, by heating in a bath of boiling water for 3 min. The final protein concentration was *ca.* 0.4 mg/ml. Gel permeation chromatography was performed in the same buffer in the presence of 0.1% SDS. Detailed chromatographic data are given in Fig. 1. The peak fractions thus obtained were directly applied to the C_3 column for reversed-phase chromatography. Usually, the sample load was 1 ml. The elution conditions are described in Fig. 1.

Immune sera and monoclonal antibodies. Two different rabbit immune sera were used: (a) anti-TBE virus serum, obtained by immunization with sucrose gradient-purified whole virus, and (b) monospecific anti-core protein(C) serum, obtained by immunization with cores isolated from Triton X-100 solubilized virus and purified as described by Heinz and Kunz[8]. The preparation and characterization of monoclonal antibodies, directed to the glycoprotein E, have been described[10].

Immunoblotting. TCA-precipitated and acetone-washed viral proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12% gel, according to Laemmli and Favre[11]). Using a Trans-Blot apparatus from Bio-Rad, the proteins were subsequently transferred electrophoretically to nitrocellulose sheets, which were then used as solid-phase antigen for radioimmunoassay with the antisera described above. The blotting and immunoassay conditions were as described in detail by Heinz *et al.*[12].

Nitrocellulose dot immunoassay

Eluents from reversed-phase columns were fractionated at intervals of 0.5 min. The samples were dried in a Savant Speed Vac concentrator (Savant, Hicksville, NY, U.S.A.) and resolubilized in an appropriate volume of phosphate-buffered saline (PBS) (pH 7.4), containing 0.1% (w/v) SDS. Usually, 1 μl of each fraction (1% of the fraction volume) was dotted onto a nitrocellulose membrane (Bio-Rad), and the sheets were incubated in PBS (pH 7.4), containing 1% (w/v) bovine serum albumin (BSA, Serva) to saturate non-specific protein-binding sites on the nitrocellulose. Next, individual sheets were incubated for 1 h at room temperature with each of the three antisera described above, diluted 1:100 in PBS, containing 1% (w/v) BSA and 2% (w/v) Tween-20 (Serva). After repeated washing with the above buffer, the sheets were incubated for another hour with a peroxidase-conjugated rabbit anti-mouse immunoglobulin (IgG) (Nordic Immunology, Tilburg, The Netherlands) or a goat anti-rabbit IgG (Nordic Immunology), respectively, in the same buffer, washed as above and stained in a saturated solution of 3-amino-9-ethylcarbazole (Sigma, Louis, MO, U.S.A.) in 0.2 *M* sodium acetate, 0.2 *M* acetic acid containing 0.1% (w/v) hydrogen peroxide. The reaction was stopped by washing with the water.

RESULTS AND DISCUSSION

High-performance gel permeation chromatography

As described in detail in a previous paper[13], gel permeation chromatography in the presence of SDS (Fig. 1a) allows the separation of the two membrane proteins, E and M, which usually tend to form hydrophobically associated complexes in detergent-free solvents. When TSK-G 3000 SW columns are used, the small membrane protein is eluted as a shoulder to the core protein peak, due to the small difference in their molecular weights. Even with G 2000 SW columns and a flow-rate of 0.2

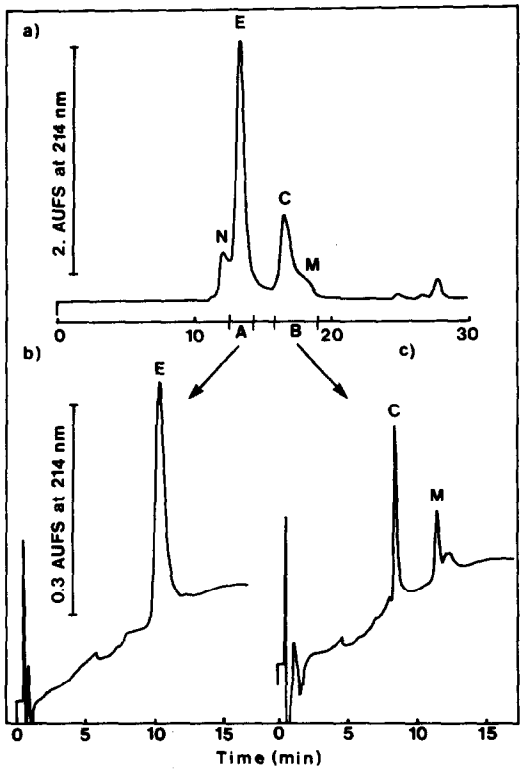

Fig. 1. Separation of the three structural proteins of TBE virus by multistep HPLC. a, High-performance gel permeation chromatography on Spherogel TSK-G 3000 SW (two columns, 300 × 7.5 mm I.D., connected in series). Mobile phase: 50 m*M* phosphate buffer (pH 6.5) containing 0.1% SDS; flow-rate, 1 ml/min. Sample load: 0.5 ml, containing 180 μg of proteins. b and c, Reversed-phase HPLC of the peak fractions A and B, obtained as above. Column: Ultrapore RPSC (C_3), 75 × 4.6 mm I.D. Solvents: 1, 0.01 *M* TFA; 2, acetonitrile–2-propanol (1:1, v/v). Flow-rate: 2 ml/min. Gradient: 0–90% solvent 1 (6% per min) in 15 min, linear. Temperature: ambient. Sample load: 1 ml, containing 30 μg of E (b), 8.5 μg of C and 3.2 μg of M (c). N = Nucleic acid; E = glycoprotein (MW 55 000); C = core protein (MW 15 000); M = small membrane protein (MW 7500).

TABLE I

RECOVERY OF THE TBE VIRUS STRUCTURAL PROTEINS AFTER MULTISTEP HPLC SEPARATION

Protein	*% of specified injected viral protein**		*% of SDS removed by reversed-phase chromatography***
	Gel chromatography	*Reversed-phase chromatography*	
E	67.0 ± 1.9	43.4 ± 2.1	93.8
C	101.2 ± 1.8	96.9 ± 1.4	93.1
M		77.6 ± 10.6	90.6

* Determined from the proportion of the three structural proteins, found by quantitative analysis of ^{14}C-labelled proteins generated in SDS-PAGE, namely 74.1 ± 1.9% E, 18.8 ± 0.9% C and 7.1 ± 1.1% M.

** Determined by the use of [^{35}S]SDS.

ml/min, no baseline separation could be achieved and, therefore, G 3000 SW columns were used in the experiments described, because they have a better ratio of performance and speed. As shown in Table I, the recovery of the proteins is highly satisfactory, nearly quantitative for C plus M, and about 70% for the glycoprotein.

Reversed-phase HPLC

The potential of combining gel permeation chromatography and reversed-phase chromatography was evaluated by directly applying the peak fractions A and B (Fig. 1a) to a reversed-phase column (C_3), with the aim of obtaining complete resolution of insufficiently separated proteins as well as of removing SDS and recovering salt-free proteins for further analysis. For this purpose we chose a C_3 column material with large pore diameter since this seems to represent the best compromise between high resolution and reasonable recovery of hydrophobic membrane proteins.

To optimize recovery and separation, different elution conditions were investigated. One of the critical parameters was the content of the organic modifier. The use of acetonitrile alone resulted in very low recoveries of the M protein. Much better elution was obtained with 2-propanol, which however is not appropriate for sensitive detection at 214 nm. A mixture of 50% (v/v) 2-propanol in acetonitrile was therefore used, which did not impair recovery, and by keeping baseline drift to an acceptable level, detection was feasible even in the range of 0.2 a.u.f.s.

The quality of separation was also extremely dependent on the steepness of the gradient. An increase of less than 6% per minute (flow-rate 2 ml/min) resulted in strong peak broadening, and a steeper gradient caused fusion of the two peaks rep-

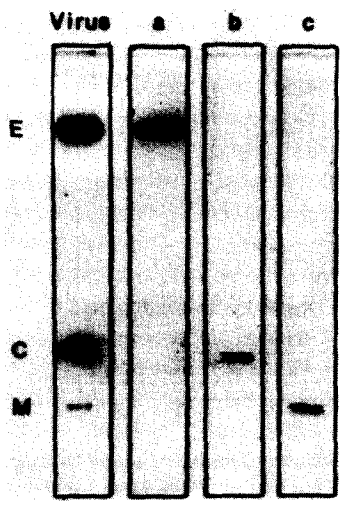

Fig. 2. SDS-PAGE analysis (15% gel, according to Laemmli and Favre[11]) of the three TBE virus structural proteins, obtained by multistep HPLC separation. a, Peak E from Fig. 1b; b, peak C from Fig. 1c; c, peak M from Fig. 1c.

Fig. 3. Immunoblotting of the TBE virus structural proteins, characterizing the specificity of three different antisera: A, polyclonal rabbit anti-TBE virus; B, monoclonal anti-glycoprotein (E); C, monospecific rabbit anti-core protein (C).

resenting C and M (Fig. 1c). It is interesting that sharp peaks and optimum recoveries were obtained for each of the different proteins under identical conditions; the corresponding chromatograms are shown in Fig. 1b and 1c. The identity and purity of each peak were verified by SDS-PAGE (Fig. 2). By this means the small peak following M was also found to represent M. This may be explained by solvation effects, which have been shown to cause multiple-peak formation during RP chromatography of polypeptides[14].

As is seen in Table I, considerable losses had to be accepted for the glycoprotein E and to a certain extent also for M, whereas C was recovered almost quantitatively. A substantial amount of the glycoprotein E apparently remained adsorbed on the column, leading to memory effects, as shown by successive blank experiment. Covering the surface of the stationary phase by increasing the SDS concentration in the sample loading buffer had no influence on retention times and recoveries. Despite the losses of certain proteins, reversed-phase HPLC has the advantage of yielding salt- and detergent-free proteins, which can easily be processed for amino acid and sequence analysis.

Optimization of reversed-phase separation by immunological monitoring

Tailing effects are one of the inherent problems associated with reversed-phase HPLC of proteins, leading to cross-contaminations of otherwise well separated peaks. In the chromatograms shown in Fig. 1b and c, no tailing effects were apparent

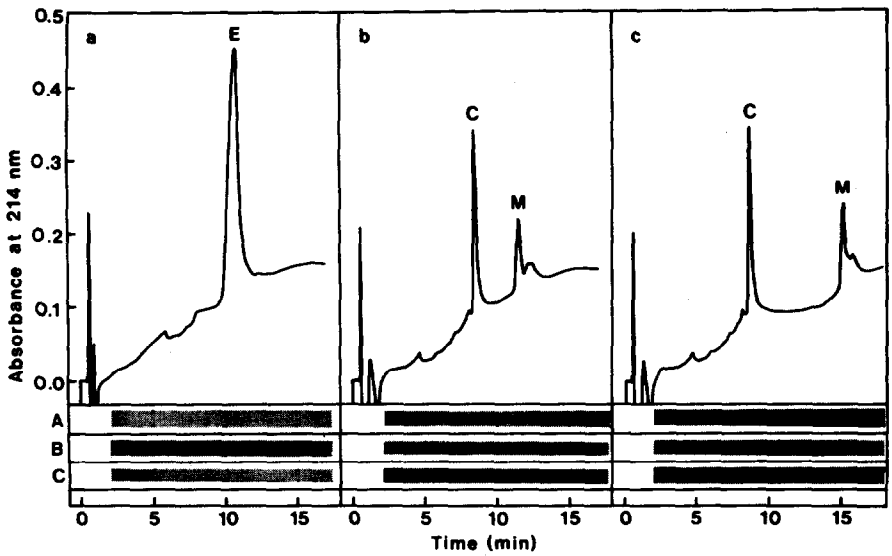

Fig. 4. Purity control of the HPLC-separated TBE virus structural proteins by immunological monitoring. Reversed-phase gradients were fractionated at intervals of 0.5 min (1 ml) and subjected to nitrocellulose dot immunoassay by using three well defined antisera (Fig. 3): a, polyclonal rabbit anti-TBE virus; b, monoclonal anti-glycoprotein; c, monospecific rabbit anti-core protein. The separation conditions for chromatograms a and b were as described in the legend to Fig. 1. There are no cross-contaminations between E and the two other proteins (panel a, line C and panel b, line B); elution of the core protein C leads to extensive peak tailing, thus contaminating the small membrane protein M (panel b, lines A and C). Improved resolution of C and M (panel c) was achieved by altering the gradient: 0–45% solvent 2 in 7.5 min (6% per min), 45–55% in 5 min (2% per min) and 55–91% in 6 min (6% per min).

by detection at 214 nm nor by SDS-PAGE analysis. To assess the purity of the protein preparations obtained, we designed highly sensitive immunological monitoring methods by using monospecific polyclonal and monoclonal antibodies. These antibodies were characterized with respect to their protein specificity by immunoblotting as shown in Fig. 3, reacting either with all three structural proteins (A), exclusively with E (B) or exclusively with the core protein C (C). The selection of the monoclonal antibody (Fig. 3b) was based on the fact that its corresponding epitope in the glycoprotein is resistant to denaturation under non-reducing conditions.

Eluted fractions from the reversed-phase separations shown in Fig. 1 were collected at intervals of 0.5 min, evaporated, resuspended in an appropriate volume of PBS, containing 0.1% SDS, and subjected to dot immunoassays, as described in Materials and Methods. The analysis of the chromatogram in Fig. 4a with the polyclonal and the monoclonal glycoprotein-specific antibodies revealed that elution of the glycoprotein is associated —as expected for a hydrophobic membrane protein— with considerable peak tailing, which is not apparent from UV-monitoring. The purity of E, estimated by SDS-PAGE, is again confirmed by the lack of reactivity with the core protein-specific immune serum. Astonishingly, an even stronger tailing effect was observed with the non-membrane protein, C (Fig. 4b). Despite an optically recorded baseline separation, it is evident from Fig. 4b that M is contaminated by C, due to retarded elution. Based on the results on monitoring these cross-contaminations by the present method, we have altered the gradient to allow complete elution of C before M is desorbed from the column. This was achieved by inserting a flat gradient section after elution of the C peak, and this, as shown in Fig. 4c by immunological monitoring, resulted in a complete separation of C and M. The absence of contamination by E is evident from the lack of reactivity with the monoclonal antibody.

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