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EXCLUSIVE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES FOR THE ISOLATION, 4-DIMETHYLAMINOAZOBENZENE-4′-SULPHONYL CHLORIDE AMINO ACID ANALYSIS AND 4-N,N-DIMETHYLAMINOAZOBENZENE-4′-ISOTHIOCYANATE–PHENYL ISOTHIOCYANATE SEQUENCING OF A VIRAL MEMBRANE PROTEIN

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SUMMARY

Protein chemical analysis of the membrane glycoprotein of a flavivirus (tick-borne encephalitis virus) was performed in the subnanomole range by the use of high-performance liquid chromatographic techniques. Complete separation of the amphiphilic glycoprotein from the other structural proteins was achieved by gel-permeation chromatography in the presence of sodium dodecyl sulphate using TSK-3000 SW columns. Peptides generated by digestion with various proteases were separated by reversed-phase chromatography on a large pore diameter C_3 support in a volatile buffer system using detection at 214 nm. Antigenically reactive peptides were identified by immunoassays using monoclonal antibodies as determinant-specific probes. For quantitative amino acid analysis, proteins and peptides were pre-column derivatised with 4-dimethylaminoazobenzene-4′-sulphonyl chloride and conditions were established for the complete separation of all amino acids by a single gradient using a C_{18} column. Manual amino-terminal sequencing was performed using the 4-N,N-dimethylaminoazobenzene-4′-isothiocyanate phenyl isothiocyanate double-coupling method. Highly sensitive identification of each 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin amino acid, including leucine and isoleucine, was possible by reversed-phase chromatography.

INTRODUCTION

One problem in the structural analysis of certain animal and human pathogenic viruses is their relatively poor growth in cell culture, which means that only limited amounts of purified virus can be prepared. The protein–chemical analysis of such low-abundance viruses and their proteins therefore necessitates a set of methodologies that yields reliable results from nanomole or subnanomole amounts of protein. An additional problem is that strong protein–protein interactions leading to stable viral capsid structures, many of which are surrounded by lipid envelopes carrying functionally important viral membrane proteins. Therefore, for the isolation of pure single viral structural proteins, dissociating agents such as urea or membrane-solu-

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bilising detergents have to be used before and during separation procedures. We have faced all these problems in our work on tick-borne encephalitis (TBE) virus, which is a small, enveloped RNA virus, belonging to the flavivirus genus of togaviridae (for reviews see ref. 1). TBE virus contains only 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[2]. Immunisation experiments with defined viral subunits have shown that the glycoprotein E represents the most important component of the virus with respect to the induction of a protective immune response[3].

The antigenic structure of this protein and the biological function of antigenic determinants have been elucidated by the use of monoclonal antibodies. These studies revealed several distinct non-overlapping conformation-dependent as well as conformation-independent antigenic sites[4,5]. For an extension of our work by a more detailed protein-chemical analysis of this protein and its biologically active determinants, we had to establish highly sensitive protein and peptide separation as well as amino acid analysis and sequencing techniques. In recent years, high-performance liquid chromatography (HPLC) has considerably enhanced the possibilities of protein analysis, especially with the introduction of reversed-phase supports for the separation of protein peptides, amino acids, and their derivatives[6,7]. In the present paper we describe the successful adaptation of different HPLC techniques to our project, including preparative isolation of the amphiphilic viral membrane glycoprotein by gel filtration in the presence of sodium dodecyl sulphate (SDS), separation of proteolytic fragments by reversed-phase chromatography, combined with immunoassay for the location of antigenic determinants on single peptides, quantitative amino acid analysis, annd amino-terminal sequencing.

MATERIALS AND METHODS

HPLC

The HPLC system (Beckman) 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). Peak areas were integrated by a Shimadzu chromatopac C-R1B.

Gel-permeation HPLC was performed on two 300 × 7.5 mm I.D. Spherogel TSK-3000 SW columns (Altex) in series. Peptides derived from enzymatic digests were separated using a 75 × 4.6 mm I.D. Ultrapore RPSC (C_3) column (Beckman). 4-Dimethylaminoazobenzene-4′-sulphonyl chloride (DABS-Cl) amino acid analysis was performed on a 250 × 4.6 mm I.D. Ultrasphere ODS 5-μm column (Beckman), and 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin amino acids were identified on a 75 × 4.6 mm I.D. Ultrasphere ODS 3-μm column (Beckman).

Acetonitrile and 2-propanol were of HPLC grade (J. T. Baker). Buffers were made up with G.R. (Analar grade) reagents (Merck) using double-glass-distilled water. "Research grade" trifluoroacetic acid (TFA) was purchased from Serva, triethylamine from J. T. Baker, and SDS ("electrophoresis grade") from Bio Rad.

All solvents were aspirated through a 0.2-μm Millipore GVWP filter and degassed by a continuous flow of helium during the chromatographic process. All chemicals described in the following paragraphs without further specification are of Analar grade (Merck).

Methods

Preparation of the glycoprotein. 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% SDS, by heating in a boiling water bath for 3 min. The final protein concentration was 1 mg/ml. Gel-permeation chromatography was performed with the same buffer in the presence of 0.1% SDS. Detailed chromatographic data are given in Fig. 1. Before amino acid analysis and amino-terminal sequencing, SDS was removed from the TCA-precipitated isolated glycoprotein by the method of Henderson *et al.*[8].

Enzymatic digestion and peptide separation. Digestion of native glycoprotein in the form of "rosettes"[2] by TPCK-trypsin (Worthington), TLCK-α-chymotrypsin (Sigma), and thermolysin (Serva) was performed in TAN buffer (0.05 *M* triethanolamine, 0.1 *M* sodium chloride), pH 8, at 37°C for 24 h. The protein concentration varied between 29 and 51 μg/ml and the protein/enzyme weight ratio was 50:1. For the separation of the resulting peptides, 1 ml of the digest was applied to a RPSC-column and chromatographed as detailed in the legend to Fig. 2. The collected peptides were dried *in vacuo*, dissolved in 30 μl of 70% (v/v) formic acid, and subjected to rechromatography under altered conditions (Fig. 2 insert). Such purified peptides were the starting material for amino acid analysis and amino-terminal sequence determination.

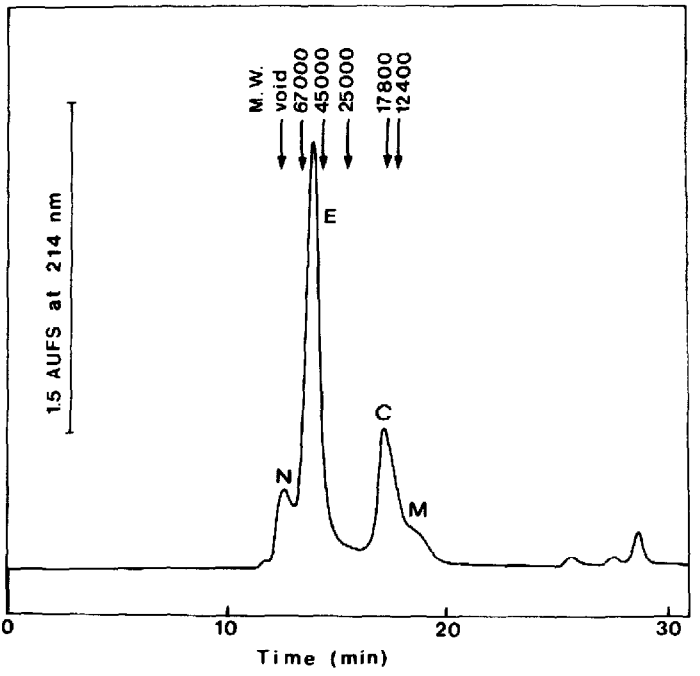

Fig. 1. SDS gel-permeation HPLC of TBE virus proteins (175 μg) on Spherogel TSK 3000 SW (two columns, 300 × 7.5 mm I.D. in series). Mobile phase: 50 m*M* phosphate buffer, pH 6.5, containing 0.1% SDS; flow-rate: 1 ml/min. N = Nucleic acid; E = glycoprotein; C = core protein; M = membrane protein. Calibration of the columns was performed using SDS-denaturated protein standards under identical conditions.

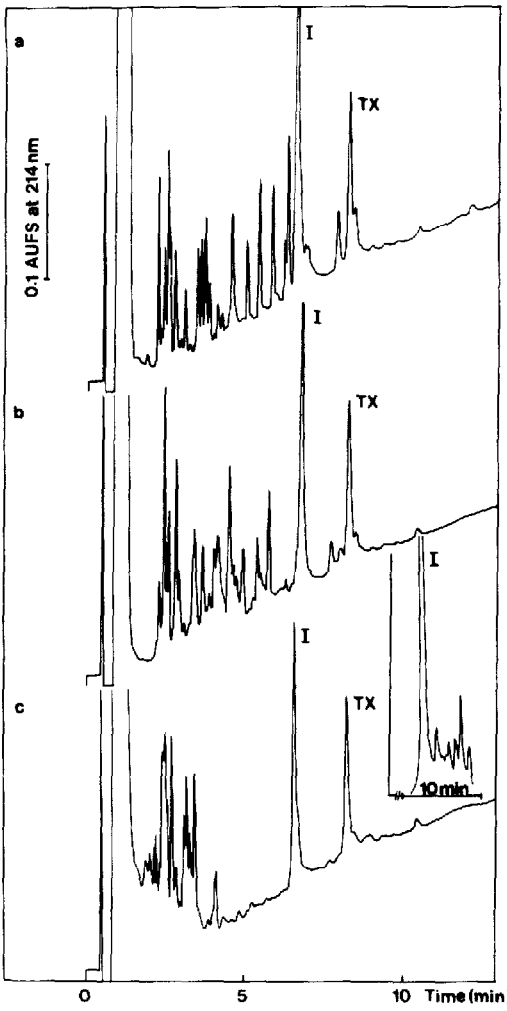

Fig. 2. Reversed-phase HPLC of glycoprotein fragments obtained after proteolysis of the native glycoprotein with trypsin (a), α-chymotrypsin (b), and thermolysin (c). Column: Ultrapore RPSC, 75 × 4.6 mm I.D. Samples (51 μg digest in 1 ml of TAN buffer, pH 8.0) were injected without further treatment. Elution conditions: Solvent A, 0.01 *M* TFA; Solvent B, 50% (v/v) acetonitrile, 50% (v/v) 2-propanol, 0.01 *M* TFA. The gradient (linear) was 0–90% B in 15 min at a flow-rate of 2 ml/min and ambient temperature. Peaks: I = immunoreactive peptide; TX = Triton X-100. Insert: Rechromatography of the immunoreactive peptide dissolved in 100 μg of 70% formic acid under the following conditions: Solvent A, 0.01 *M* TFA; Solvent B, acetonitrile, 0.01 *M* TFA. The gradient was 0–90% B in 20 min at a flow-rate of 0.25 ml/min at ambient temperature. Retention time of I, 22.3 min.

Assay for immunoreactivity of peptides. Dried peptide samples were solubilised in an appropriate volume of phosphate-buffered saline (PBS) containing 0.1% SDS. A 1-μl volume of each resuspended fraction was dotted onto a nitrocellulose membrane (Bio Rad), which was then used as a solid-phase antigen for radioimmunoassay using polyclonal as well as monoclonal antibodies[5].

Amino acid analysis. Samples of a lyophilised glycoprotein or peptides were hydrolysed with 6 *N* HCl (constant-boiling, Pierce) under reduced pressure at 110°C. The reaction was stopped at different time intervals by freezing. Derivatisation of free amino acids was performed with the coloured reagent DABS-Cl[9], which was purchased from Fluka and recrystallised from boiling acetone. The hydrolysate of 0.1–1 μg polypeptide was transferred to a silicone-treated glass tube with a conical bottom and a screw cap, dried, and redissolved in 0.1 *M* sodium bicarbonate buffer, pH 9.0. The pH was controlled and, if necessary, readjusted with 0.1 *M* sodium hydroxide before the addition of 20 μl of DABS-Cl solution (4 nmol/μl in acetone). The reaction was allowed to proceed at 70°C for 15 min, interrupted by brief shaking after the first minute. Then the sample was diluted with 100 μl of 70% (v/v) ethanol and analysed by reversed-phase chromatography. Standard amino acids were prepared following the same ijrocedure, using an amino acid kit from Sigma as well as a standard protein hydrolysate from Pierce.

Amino-terminal sequence analysis. For the stepwise manual Edman degradation the 4-N,N-dimethylaminoazobenzene-4′-isothiocyanate–phenyl isothiocyanate double-coupling method (DABITC-PITC) of Chang[10–12] was used with slight modifications. Pyridine, TFA, PITC, *n*-butyl acetate and ethyl acetate, all sequanal grade, were supplied by Pierce and stored under nitrogen. DABITC from Fluka was recrystallised from boiling acetone, dissolved in acetone (1000 nmol per 500 μl) and portioned into useful aliquots. For storage (−20°C) the reagent was vacuum-dried and dissolved in pyridine (10 nmol/μl) before use. *n*-Heptane "sequencing-grade" was purchased from Merck. Acetic acid saturated with hydrochloric acid was prepared by bubbling hydrochloric acid gas through "buffer-grade" acetic acid (Pierce). Reactions were performed in glass tubes (25 × 7 mm I.D., conical bottom) with acetic acid fitted with a glass stopper.

For the first coupling, the polypeptide (0.5–3 nmol) was dissolved in 40 μl of 67% (v/v) pyridine, and 20 μl of the DABITC pyridine solution were added. The reaction was performed under nitrogen at 50°C for 50 min. For the second coupling, 5 μl of pure PITC were pipetted into the reaction mixture, and the tube was flushed with nitrogen and heated at 50°C for another 30 min. Excess reagent was extracted four times with 400-μl portions of *n*-heptane–ethyl acetate (2:1, v/v). After vigorous mixing, complete phase separation was achieved by brief centrifugation. The organic phase was removed with a Pasteur pipette. The dried 4-N,N-dimethylaminoazobenzene-4′-thiocarbamyl (DABTC) peptide was then dissolved in 50 μl of anhydrous TFA and heated under nitrogen at 50°C for 10 min. The TFA evaporated and the cleaved 4-N,N-dimethylaminoazobenzene-4′-thiazolinone (DABTZ) amino acid was extracted twice with 150 μl of butyl acetate. The first butyl acetate extract was dried *in vacuo* for further processing, whereas the second extract was discarded. After lyophilisation, the peptide was ready for the next degradation cycle. The DABTZ amino acid was converted into the DABTH derivative by heating it with a mixture of 15 μl of water and 30 μl of acetic acid saturated with hydrochloric acid at 50°C for 50 min. For identification the dried DABTH amino acid was dissolved in 50 μl of 70% (v/v) ethanol and subjected to reversed-phase chromatography on a C_{18} support, as described in the legend to Fig. 4.

Standard DABTH amino acids were prepared as described by Chang[12].

RESULTS AND DISCUSSION

Preparative isolation of the virus glycoprotein (E)

Owing to the lipid envelope of TBE virus, detergents must be used for the solubilisation and isolation of the membrane proteins E and M. The glycoprotein E represents an amphiphilic protein and tends to remain associated with the hydrophobic membrane protein M in the presence of mild non-ionic detergents, such as Triton X-100[2]. Strongly denaturating detergents have therefore to be used for the complete separation of these proteins, which can readily be achieved by PAGE in the presence of SDS. Despite its strong resolving power and potential use for preparative purposes, SDS PAGE has the inherent disadvantage that proteins must be eluted from the gel after electrophoresis, and this may involve considerable losses of protein. Gel-permeation HPLC of proteins on porous gel columns (TSK-Gel SW)[13] is available as an alternative, which can also be performed in the presence of SDS[14]. We have exploited the speed and resolving power of this method for the preparative isolation of the TBE virus glycoprotein (Fig. 1). Purified virus was solubilised with 2% SDS and then applied to a TSK 3000 SW column, as detailed in the legend to Fig. 1: two major protein peaks (E and C + M) were obtained. Viral RNA is eluted in the void volume under the conditions used. Although the resolution of SDS–gel-permeation HPLC is inferior to that obtained with SDS–PAGE, it was adequate in this instance for our problem, and separation of the 55,000 MW glycoprotein from the 15,000 and 7500 MW proteins was easily achieved. As shown by SDS–PAGE using high sample loads, the glycoprotein peak was completely free of the other two viral structural proteins, which, however, were not sufficiently separated under the conditions used to allow their individual isolation. Using molecular weight standards for calibration of the column, we estimated the molecular weight of the glycoprotein to be 55,000, which is in excellent agreement with the value obtained by SDS–PAGE[15]. The glycoprotein represents 75% of the total viral protein, and the recovery after SDS–gel-permeation HPLC was calculated to be *ca.* 65%. The buffer system employed permits detection at 214 nm, which proved to be *ca.* 10 times more sensitive than detection at 280 nm and also extends the potential of this method to analytical purposes. For further analysis (amino acid analysis, amino-terminal sequencing, proteolytic and chemical cleavage), the glycoprotein was precipitated with TCA, and SDS was extracted with acetone in the presence of triethylamine and acetic acid by the method of Henderson[8].

In summary, SDS–gel-permeation HPLC proved to be a powerful and rapid means for preparing the amphiphilic membrane glycoprotein for further protein–chemical analysis.

Separation of proteolytic peptides

After digestion of the native glycoprotein with trypsin, α-chymotrypsin, and thermolysin, several monoclonal antibody-defined denaturation-resistant epitopes could be located on a 9000 MW fragment by immunoblotting of the proteolytic fragments separated by SDS–PAGE[5]. For further analysis of the peptides carrying biologically important sites an efficient preparative peptide separation procedure had to be designed. The method of choice was reversed-phase HPLC, which has been developed into one of the most powerful peptide separation techniques available. As

buffer components must be removed before further analysis of the separated peptides by micromethods, such as sequencing by the manual DABITC–PITC double-coupling method[12], all separations were performed in the presence of 0.1% TFA[16], which can easily be removed because of its volatility and, in addition, allows sensitive detection at 214 nm. It is known that with small hydrophilic peptides recovery from C_{18} columns is *ca.* 80%, although this decreases with increasing chain length and hydrophobicity. Some peptides may not be eluted from the column at all[17]. In our hands, preliminary experiments for the separation of proteolytic fragments of the native glycoprotein with C_{18} solid-phase supports were not satisfactory owing to low recoveries and strong peak broadening at high concentrations of organic solvents. Since we were especially interested in the isolation of immunoreactive proteolytic fragments shown to have molecular weights of *ca.* 9000[5], a large pore size (300 Å) column, composed of short (C_3) alkyl-bonded silica was used as an alternative (RPSC; Beckman). The suitability of this type of column for hydrophobic peptides, large peptides, and even proteins has been demonstrated[18,19]. The chromatograms in Fig. 2 show the separation of tryptic, α-chymotryptic, and thermolytic peptides, obtained after digestion of the native glycoprotein in the form of polymeric water-soluble complexes ("rosettes"). These "rosettes" were prepared after solubilization of the virus with TX-100 by density-gradient centrifugation into detergent-free sucrose gradients[2]. As can be seen in the figure, a characteristic peptide pattern was obtained for each protease tested. However, in each case a major peak was eluted at *ca.* 38% acetonitrile–propanol with slightly but significantly different retention times: 6.6 min after trypsin, 6.75 min after α-chymotrypsin, and 6.58 min after thermolysin digestion. Fractions were taken from the gradients shown in Fig. 2, dried in a Speed Vac Concentrator and resuspended in PBS containing 0.1% SDS. These samples were then tested for immunoreactivity as described in the "Experimental" section with defined monoclonal as well as polyclonal antibodies. By this means the peaks with retention times of *ca.* 6.6 min were identified as the 9000 MW peptides previously shown by immunoblotting to carry several conformation-independent antigenic determinants.

Two methods could be used for the further resolution of peaks obtained after RPSC chromatography. Those eluted at lower solvent concentrations and thus representing hydrophilic and small peptides could be readily rechromatographed on RP-18 columns, where they were eluted at a higher solvent concentration and either proved to be homogeneous or split into two or more peaks (data not shown). However, peptides eluted at higher solvent concentrations from the RPSC column could not be successfully purified on RP-18 columns. For subsequent amino acid and sequence analysis the immunoreactive peaks were therefore rechromatographed on the same RPSC column but using different chromatographic conditions (as an example see insert in Fig. 2c). At a reduced flow-rate, from 2 ml/min to 0.25 ml/min, the size exclusion properties of the large-pore silica could be exploited for the further purification of these peptides.

An interesting by-product of this work is that chromatography on RPSC columns under the conditions used evidently allows the precise quantification of even trace amounts of the non-ionic detergent TX-100, which is widely used in membrane protein work. TX-100 was eluted as a sharp peak with a retention time of 8.23 min, and its quantification, based on standards of known TX-100 concentration, revealed

that 0.0004% (w/v) residual TX-100 was present in the glycoprotein preparation used for proteolysis.

Quantitative amino acid analysis

Several methods involving HPLC have been developed for the quantitative analysis of amino acids. These include separation of free amino acids and their detection by post-column derivatisation using *o*-phthalaldehyde (OPA)[20] or pre-column derivatisation using OPA[21,22] or dansyl chloride[23]. We have adopted another pre-column derivatisation procedure involving DABS-Cl[9] because it does not necessitate a fluorescence monitor, and very sensitive detection can be obtained in the visible range at 436 nm. A disadvantage of this method so far has been that not all derivatised amino acids can be resolved at room temperature by a single solvent and gradient system. Although Chang has recently reported the complete separation of all amino acids at elevated temperature (50°C)[24], we have attempted to optimise the chromatographic conditions with the aim of obtaining complete separation at room temperature. This work involved the analysis of different types of organic anion in the eluent (acetate, formate, trifluoroacetate), ion-pairing agents (dodecylamine, dodecyl sulphate), organic solvents (acetonitrile, ethanol, methanol), pH, flow-rate, gradient forms, and columns (alkyl phenyl μBondapak-Phenyl; 5 μm and 3 μm Ultrasphere ODS). Best results were obtained by the use of a 5 μm ODS column using 50 m*M* sodium-acetate buffer at pH 4.11 and acetonitrile as organic solvent. Fig. 3 shows the successful separation of all amino acids, including Leu and Ile, by a single gradient. The most critical parameter proved to be pH, and even slight changes cause more or less peak fusion of NH_3 and Phe, Ser and Asp, and Glu and Arg. Apparently, optimal Ph conditions have to be established for each special column, and this also

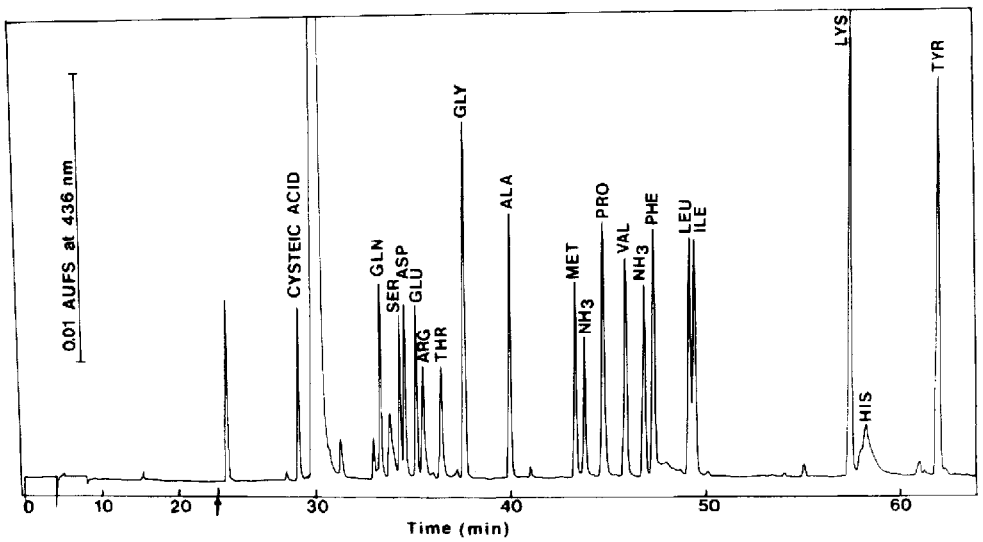

Fig. 3. Separation of DABS amino acids (50 pmol each) by reversed-phase HPLC on a Beckman Ultrasphere ODS 5-μm column (250 × 4.6 mm I.D.). Solvent A, 50 m*M* sodium acetate buffer, pH 4.11; Solvent B, acetonitrile; gradient (linear), 0 70% B in 58 min, 70–90% B in 10 min; flow-rate 0.8 ml/min; temperature, ambient. Arrow indicates change of chart speed from 2 mm/min to 5 mm/min.

TABLE I

AMINO ACID COMPOSITION OF THE STRUCTURAL GLYCOPROTEIN E AND THE IMMUNOREACTIVE PEPTIDES OBTAINED BY DIGESTION OF THE NATIVE GLYCOPROTEIN WITH VARIOUS PROTEASES

All data are expressed as mol/100 mol.

	*E protein**	*I peptide*** *from tryptic digest (Fig. 2a)*	*I peptide*** *from chymotryptic digest (Fig. 2b)*	*I peptide*** *from thermolytic digest (Fig. 2c)*
Ser	5.4	9.3	9.4	8.7
Asp	6.4	8.7	9.1	9.2
Glu	8.2	8.0	7.7	7.8
Arg	4.3	4.6	5.8	4.1
Thr	7.8	9.0	9.2	9.0
Gly	12.3	11.4	10.9	10.6
Ala	7.9	5.0	4.7	4.7
Met	2.5	2.3	2.4	3.3
Pro	4.4	6.5	7.1	7.7
Val	8.9	6.8	7.2	7.6
Phe	3.9	6.4	5.9	6.5
Leu	9.3	4.4	3.7	4.3
Ile	4.1	5.0	5.0	5.1
Lys	6.6	4.3	4.2	4.3
His	4.1	3.7	4.3	3.2
Tyr	1.9	2.6	1.4	2.0
Cys***	1.5	2.0	2.0	1.9

* Data are obtained after hydrolysis for 20 h, 26 h, 48 h, 72 h and 96 h.
** Data are obtained by a single hydrolysis for 22 h.
*** Cysteine was determined as cysteic acid using oxidised protein.

holds true for each new column from the same manufacturer. For all analyses cysteic acid was used as internal standard to compensate for variations that might occur during sample derivatisation with DABS-Cl. This allowed us to calculate the absolute amount of amino acid present in the hydrolysate. The suitability of the final method was verified by the analysis of standard peptides with known amino acid composition.

Table I shows the amino acid analysis of the viral glycoprotein (E) isolated by SDS-gel-permeation chromatography on a TSK 3000 SW column as well as the immunoreactive fragments isolated by reversed-phase chromatography on a RPSC column. For the analysis of the glycoprotein only 1.9 pmol have been used per chromatogram. The values obtained agree well with those obtained by conventional amino acid analysis (Durrum D 500 analyser)[25].

The amino acid composition of the immunoreactive fragments obtained after trypsin, α-chymotrypsin, and thermolysin digestion of the native glycoprotein revealed many additional potential cleavage sites for these proteases which are apparently inaccessible under the digestion conditions used. These results confirm the assumption that this fragment represents a stabilised compact core structure which is not easily hydrolysed by proteolytic enzymes without prior denaturation[5].

Amino-terminal sequence analysis

We have adopted the DABITC–PITC double-coupling method described by

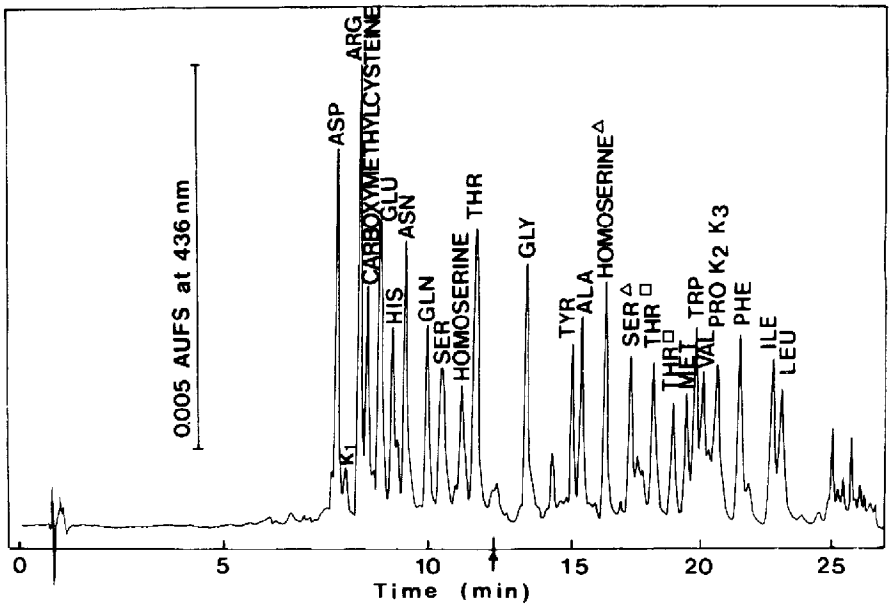

Fig. 4. Separation of DABTH amino acids (0.1 μg each corresponding to 0.5–1.3 nmol) by reversed-phase HPLC on a Beckman Ultrasphere ODS 3-μm column (75 × 4.6 mm I.D.). Solvent A, 50 m*M* sodium acetate buffer, pH 5.0, containing 2% (v/v) triethylamine; Solvent B, acetonitrile; gradient (linear), 20–40% B in 6 min, 40–60% B in 16 min, 60 90% B in 1 min; flow-rate, 1 ml/min; temperature, ambient. The symbols △ and □ mark the dehydro products of DABTH-Ser and DABTH-Thr[12]. K_1 = α-DABTH-ε-DABTC-Lys; K_2 = α-PTH-ε-DABTC-Lys; K_3 = α-DABTH-ε-PTC-Lys. Arrow indicates change of chart speed from 5 to 8 mm/min.

Chang[10,11] which allows manual amino-terminal sequencing to be performed at the picomole level. The coloured DABTH amino acids obtained after each cycle can be identified by thin-layer chromatography and, even more sensitively, by HPLC[12]. Both methods allow the separation and identification of all amino acids with the exception of Leu and Ile, which cannot be differentiated. We have therefore performed separation optimization experiments similar to those described for the DABS amino acids. Addition of ion-pairing reagents was particularly effective. Several primary, secondary and tertiary amines, as well as quaternary ammonium bases with different alkyl chain lengths, were tested at different concentrations. Parameters were thus selected that allowed identification of all naturally occuring amino acids, including Leu and Ile, in a single analysis (Fig. 4). This had not been possible previously. The decisive factors were the addition of 2% triethylamine to the eluent and the use of a 3-μm Ultrasphere ODS column. Although the Lys derivatives K_2 and K_3 (see legend to Fig. 4) and DABTH-Pro are not separated. Pro can be identified by the lack of K_1. For the separation of DABTH amino acids, exact control of pH was not as critical as for DABS amino acids and the efficiency of separation was similar between pH 4.5 and 5.5.

The suitability of this assay system for the identification of amino acids during sequencing by the DABITC–PITC double-coupling method has been verified by sequencing peptides of known sequence. The amino-terminal amino acid of the gly-

coprotein has been determined to be serine. Work is currently in progress to identify further sequences of the TBE virus glycoprotein and its proteolytic and chemical cleavage products.

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