Journal of Chromatography, 599 (1992) 157–162
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2535

Comparison of detergents for extraction and ion-exchange high-performance liquid chromatography of Sendai virus membrane proteins

G. W. Welling*, Y. Hiemstra and M. Feijlbrief

Laboratorium voor Medische Microbiologie, Rijksuniversiteit Groningen, Oostersingel 59, 9713 EZ Groningen (Netherlands)

C. Örvell

Department of Virology, Karolinska Institute, School of Medicine and Department of Virology, National Bacteriological Laboratory, S-10521 Stockholm 1 (Sweden)

J. van Ede and S. Welling-Wester

Laboratorium voor Medische Microbiologie, Rijksuniversiteit Groningen, Oostersingel 59, 9713 EZ Groningen (Netherlands)

ABSTRACT

The integral membrane proteins of Sendai virus, haemagglutinin–neuraminidase (HN) and fusion protein (F) were extracted from purified virions with a non-ionic and two zwitterionic detergents, *i.e.*, pentaethylene glycol monolauryl ether ($C_{12}E_5$), lauryldimethylamine oxide (LDAO) and dodecyldimethylammoniopropane-1-sulphonate (SB12), respectively. The extracts were subjected to ion-exchange high-performance liquid chromatography (HPIEC) using 0.1% of the detergent in the eluent on four different columns (MA7Q, Zorbax BioSeries SAX, Mono Q and PL-SAX) with a quaternary amine as interacting ligand and with different pore sizes: non-porous and 30, 80 nm and 400 nm, respectively. The relative recoveries of protein were similar for all the columns. The highest recovery of HN and F protein and the best separation were obtained with $C_{12}E_5$. Analysis of HPIEC fractions with monoclonal antibodies directed against conformational epitopes showed that $C_{12}E_5$ had less effect on the conformation than the other two detergents.

INTRODUCTION

When the conformation of an integral membrane protein has to be retained, purification by ion-exchange high-performance liquid chromatography (HPIEC) in the presence of a detergent (surfactant) is particularly suitable as the elution conditions are generally mild [1–3]. Detergent extraction is often the first step in the purification of an integral membrane protein. Detergents are lipid-like substances and they possess a hydrophilic head and a hydrophobic tail and are able to compete with the lipids in a bilayer. They are also more hydrophilic than the lipids. As a consequence, detergent–protein complexes are soluble in aqueous solutions and the detergent molecules, in mimicking the lipid molecules, help to maintain the native configuration of the membrane proteins during a purification procedure. Extraction can be achieved with a wide variety of detergents [4–8], but in order to preserve biological activity non-ionic or zwitterionic detergents are used. We have used the integral membrane proteins of Sendai virus as a model mixture for the development of methodologies for purification of membrane proteins using different detergents and different modes of high-performance liquid chromatography (HPLC) [9–18]. The two integral membrane proteins of Sendai virus are the haemaggluti-

0021-9673/92/$05.00

nin–neuraminidase protein HN (relative molecular mass, M_r = 68 000) and the fusion protein F (M_r = 65 000). Both proteins are present in detergent extracts in multimeric forms [19]. Dimeric HN (HN_2) and tetrameric HN and F (HN_4, F_4) are observed in addition to truncated forms of HN due to proteolytic degradation.

In an earlier study different polyethylene glycol alkyl ethers were investigated [17]. In this study one non-ionic and two zwitterionic detergents, *i.e.*, pentaethylene glycol monolauryl ether ($C_{12}E_5$) [17], lauryldimethylamine oxide (LDAO) [20] and N-dodecyl-N,N-dimethylammonio-3-propanesulphonate (SB12) [8], respectively, were used for extraction and as additive to the eluents for HPIEC. In addition, the effect on the immunological activity was studied. As the extracts contain relatively large multimeric forms of the Sendai proteins [19], separation and recovery may be adversely affected by the pore size of the column matrix. Therefore, we subjected these proteins to chromatography on four columns (MA7Q, Zorbax BioSeries SAX, Mono Q and PL-SAX) with a quaternay amine as interacting ligand and with different pore sizes: non-porous and 30, 80 and 400 nm, respectively.

EXPERIMENTAL

Detergent extraction of Sendai virus

Sendai virus was grown in 10-day-old embryonated chicken eggs. Allantoic fluid was harvested after incubation at 37°C for 3 days. Cell debris was removed by low-speed centrifugation (2000 *g*, 10 min, 5°C) and virus particles were obtained from the supernatant by centrifugation for 1 h at 70 000 *g* at 5°C. Virus was resuspended in 10 m*M* Tris–HCl (pH 7.2), supplemented with 10% sucrose and stored at −80°C. The amount of protein was determined [21]. Sendai virions were extracted with three detergents with the same hydrophobic tail (C_{12}) (see Fig. 1), *i.e.*, pentaethylene glycol monolauryl ether ($C_{12}E_5$) (Kwant-Hoog Vacolie Recycling and Synthesis, Bedum, Netherlands), lauryldimethylamine oxide (LDAO; N,N-dimethyldodecylamine-N-oxide) (Fluka, Buchs, Switzerland) and N-dodecyl-N,N-dimethyl-3-ammoniopropane-1-sulphonate (SB12) (Serva, Heidelberg, Germany). A Sendai virus suspension containing 40 mg of protein was pelleted and resuspended in 1 ml of 10 m*M* Tris–HCl (pH 7.2). The same volume of buffer containing 4% (w/w) of the detergent was added, resulting in a final detergent concentration of 2%. After incubation for 20 min at room temperature, the suspension was centrifuged for 1 h at 70 000 *g* at 5°C. The supernatant contains the HN and F proteins and was stored at −80°C.

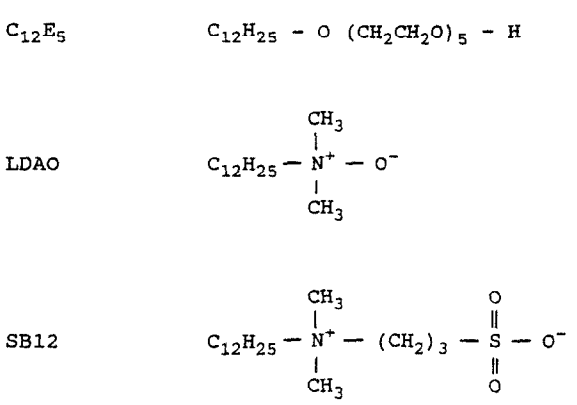

Fig. 1. Structural formula of $C_{12}E_5$, LDAO and SB12.

Ion-exchange and size-exclusion HPLC

Chromatography was performed with a system consisting of an LKB 2150 pump (Pharmacia–LKB, Uppsala, Sweden), a Rheodyne Model 7125 injector (Inacom, Veenendaal, Netherlands) and a Waters Model 441 detector (Millipore–Waters, Etten-Leur, Netherlands). Anion-exchange HPLC was performed with an MA7Q (50 mm × 7.8 mm I.D.) column (Bio-Rad Labs., Richmond, CA, USA) consisting of non-porous polymeric particles with a particle size of 7 μm, a Zorbax BioSeries SAX (80 mm × 6.2 mm I.D.) column (DuPont, Wilmington, DE, USA) consisting of zirconium oxide-stabilized silica with a particle size of 6 μm and a pore size of 30 nm, a Mono Q HR 5/5 (50 mm × 5 mm I.D.) column (Pharmacia–LKB) consisting of polymer beads with a particle size of 10 μm and pores of 80 nm and a PL-SAX 4000 Å (50 mm × 4.6 mm I.D.) column (Polymer Labs. Church Stretton, UK) consisting of polymer beads with a particle size of 8 μm and pores of 400 nm. To determine a gradient time suitable for all four columns, 730–1000 μg of a $C_{12}E_5$ extract of Sendai virus membrane proteins were subjected to HPIEC. After isocratic elution for 10 min, retained proteins were eluted with a linear 6-, 12- and 24-min gradient

from 20 m*M* Tris–HCl (pH 7.8) containing 0.1% $C_{10}E_{5-9}$ (decylPEG-300) [18] to 0.5 *M* sodium chloride in the same buffer. In subsequent experiments, using a gradient time of 12 min, the eluent contained 0.1% of the detergent used for extraction. In that case, 440–510 μg of Sendai virus proteins were subjected to HPIEC.

The amount of protein in the extracts and fractions after HPIEC was determined by size-exclusion HPLC (HPSEC). A Polyol Si500 (100 nm × 4.6 mm I.D.) column (Serva) with 5-μm particles and a pore size of 50 nm was used for HPSEC. To a volume of 100 μl of extract or HPIEC fraction (freeze-dried and after dialysis dissolved in 500 μl of water) sodium dodecyl sulphate (SDS) was added to a final concentration of 4%. After heating for 3 min in a bath of boiling water, 10 μl of the fractions were subjected to HPSEC. The proteins were eluted with 50 m*M* sodium phosphate (pH 6.5) containing 0.1% SDS at a flow-rate of 0.8 ml/min. The absorbance was monitored at 280 nm. The amount of HN and F protein was then calculated from the peak height. Similarly treated bovine serum albumin was used as a standard. Relative recoveries of total protein after HPIEC were calculated from the area of the peaks at 280 nm.

SDS–polyacrylamide gel electrophoresis (PAGE)

Samples of the HPIEC fractions were analysed by SDS-PAGE [22] on 12% gels under non-reducing conditions. After electrophoresis, the gels were fixed and silver-stained as described [23].

Enzyme-linked immunosorbent assay (ELISA)

Selected HPIEC fractions containing predominantly F or HN protein were analysed with the conformation-dependent monoclonal antibodies (mAbs) HN 851 and F 1.216. The properties of these mAbs have been described [16,24]. ELISA trays were coated with 100 μl of twofold dilutions of fractions diluted in coating buffer, consisting of 50 m*M* sodium carbonate (pH 9.6) supplemented with 0.6 *M* NaCl. The starting dilution contained 10 μg protein/ml as determined by HPSEC. After coating overnight at 4°C, the ELISA was performed as described earlier [17]. The absorbance at 492 nm (A_{492}) was measured in a microplate photometer. Absorbances below 0.2 were considered as negative. The amount of protein (ng) giving an absorbance value of 1.2 was recorded. This amount is inversely related to the immunological activity of the protein.

RESULTS AND DISCUSSION

It has been shown that zwitterionic detergents are useful in retaining the biological activity of integral membrane proteins [8]. Two series of such detergents with varying lengths of hydrophobic tails are commercially available, the alkylamine oxides [20] and the alkylammoniopropanesulphonates [8]. We decided to compare the C_{12} variant of these detergents (LDAO and SB12, respectively) with the non-ionic C_{12} oxyethylene ether ($C_{12}E_5$) with which the highest yields of protein were obtained after extraction of purified Sendai virions [17]. Extraction was performed at a final detergent concentration of 2%, which is well above the critical micellar concentration (CMC) (Table I). Extraction of Sendai virions with $C_{12}E_5$, LDAO and SB12 resulted in 204, 189 and 110 μg of protein per 100 μl of extract, respectively. These extracts were compared by HPIEC on four different columns. However, prior to this comparison a suitable gradient time for all four columns was selected by subjecting a Sendai virus $C_{12}E_5$ extract to HPIEC using gradient times of 6, 12 and 24 min after isocratic elution for 10 min. The elution patterns with a gradient time of 12 min are shown in Fig. 2. Non-proteinaceous material and the HN proteins are eluted first, followed by a broad peak containing the F protein (indicated with an asterisk). Despite the differences in column volume, 2.4, 2.4, 1.0 and 0.8 ml of the MA7Q, Zorbax SAX, Mono Q and PL-SAX columns, respectively, the elution patterns with respect to the separation between the first peaks (containing non-proteinaceous material and HN proteins) and the F peak were similar using gradient times of 6, 12 and 24 min, except for a gradient time of 6 min with the Zorbax

TABLE I

CRITICAL MICELLAR CONCENTRATIONS (%)

Detergent	0–0.05 *M* Cl^-	0.2 *M* Cl^-	Ref.
$C_{12}E_5$	0.020		17
LDAO	0.048	0.034	20
SB12	0.144	0.047	8

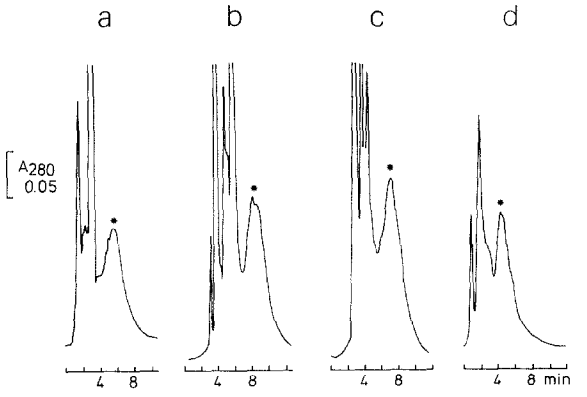

Fig. 2. Anion-exchange HPLC of a $C_{12}E_5$ extract of Sendai virus membrane proteins on four different columns: (a) MA7Q (non-porous), (b) Zorbax BioSeries SAX (30-nm pores), (c) Mono Q (80-nm pores) and (d) PL-SAX (400 nm-pores). Conditions: linear A–B gradient (0 to 0.5 *M* NaCl in 12 min), following 10-min isocratic elution with buffer A, where buffer A is 20 m*M* Tris–HCl (pH 7.8) containing 0.1% $C_{10}E_{5-9}$ and buffer B is buffer A containing 0.5 *M* NaCl; flow-rate, 1 ml/min; absorbance monitored at 280 nm. The amounts of protein applied to columns a, b, c and d were 790, 1000, 980 and 730 μg, respectively. Peak * = fusion protein (F).

SAX column, which resulted in less separation between these peaks. Therefore, a gradient time of 12 min was chosen for further experiments.

Amounts of Sendai virus membrane proteins ranging from 440 to 510 μg were subjected to HPIEC on the four columns. When a detergent is applied as additive to an elution buffer, it is most satisfactorily used at concentration above the CMC [25]. We used a concentration of 0.1% of the detergents in the eluent. Very shortly after starting the gradient, this is above the CMC (see Table I). The elution patterns obtained with the PL-SAX column together with the SDS-PAGE analysis are given as an example (Fig. 3). The most satisfactory separations with all columns were obtained when $C_{12}E_5$ was used for extraction and as an additive to the elution buffer. With SB12 less separation between HN and F protein was obtained and with LDAO hardly any separation was found. The corresponding SDS gels (Fig. 3) also indicate that the highest resolution is obtained with $C_{12}E_5$. This can also be judged from HPSEC analysis of selected HPIEC fractions. In Fig. 3a, b and c, the HN/F ratio was 4.8, 2.0 and 3.3 in fractions 2 plus 3, 2 and 2, respectively. For fractions 6, 6 and 4 in Fig. 3a, b and c the HN/F ratio was 0.35, 0.81 and 0.85, respectively.

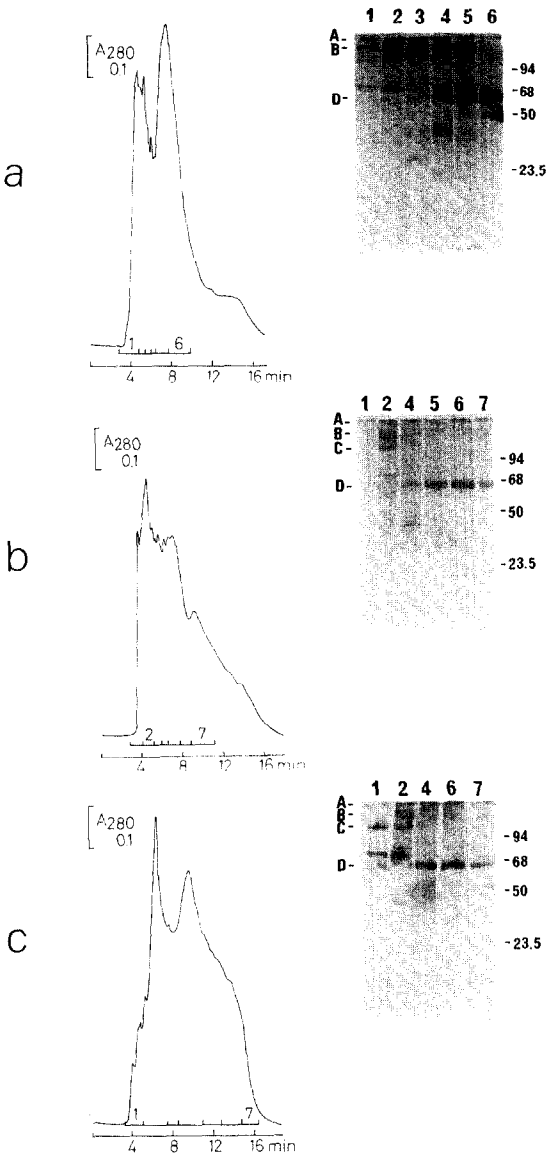

Fig. 3. Anion-exchange HPLC of (a) $C_{12}E_5$, (b) LDAO and (c) SB12 extract of Sendai virus membrane proteins on a PL-SAX column. Elution conditions: linear A–B gradient (0 to 0.5 *M* NaCl in 12 min), following 10-min isocratic elution with buffer A, where buffer A is 20 m*M* Tris–HCl (pH 7.8) containing 0.1% of the detergent used for extraction and buffer B is buffer A containing 0.5 *M* NaCl; flow-rate, 1 ml/min; absorbance monitored at 280 nm. Fractions were collected as indicated and analysed by SDS-PAGE (12% gel) under non-reducing conditions. The sample of reference proteins contained mercaptoethanol, which occasionally affected Sendai proteins in other lanes [*e.g.*] lane 6 in (a)]. Polypeptides were revealed by silver staining. A, B, C and D are the tetrameric, dimeric and truncated forms of HN protein and F protein, respectively. The molecular masses ($\times 10^{-3}$) of reference proteins are given on the right.

TABLE II

RELATIVE RECOVERIES (%) OF PROTEIN FROM THE PEAK AREA AT 280 nm AFTER HPIEC OF SENDAI VIRUS EXTRACTED WITH DIFFERENT DETERGENTS

The highest yield was taken as 100%.

Detergent	Column			
	MA7Q	Zorbax SAX	Mono Q	PL-SAX
C12E5	100	72	98	82
LDAO	88	93	99	100
SB12	90	100	89	93

An interesting difference is that the HN protein is fragmented to greater extent when SB12 and LDAO are used (Fig. 3b and c, fraction 2). Large amounts of the tetrameric and dimeric form of HN are only present when $C_{12}E_5$ is used. Absolute recoveries were higher than 70% and the relative recoveries of total protein are listed in Table II.

None of the columns showed always the highest recovery, regardless of which detergent was used in the eluent. For example, the MA7Q column showed a 100% relative recovery when $C_{12}E_5$ was used and the PL-SAX column showed a relative recovery of 100% when LDAO was used. The average relative recoveries obtained with the three detergents for the four columns were similar and ranged from 88 to 95%. It has been argued that increasing the pore size may improve intraparticle mobile phase mass transfer for large macromolecules [26]. Another solution would be to use a non-porous matrix [27]. Our results do not indicate that the pore size of the matrix is of paramount importance for the separation. One reason may be that relatively small amounts of protein were used consisting of a mixture of differently sized macromolecules. The detergent extract may contain monomeric, dimeric and tetrameric forms of the integral membrane proteins of Sendai virus in addition to aggregated forms. In addition, proteolytically degraded smaller forms may be present. The capacity of the columns will probably be sufficient to accommodate the relatively small amounts of large proteins and aggregated forms on the outside of the column particles resulting in similar performances of all four columns.

Another matter of concern may be the effect of the detergent on the conformation of the membrane proteins during chromatography. This was measured in an ELISA with mAbs directed against conformation-dependent epitopes of HN and F protein. ELISA plates were coated with serial dilutions of the HPLC fractions. The detergent concentration in the fractions which gave an A_{492} of 1.2 was *ca.* 0.0005%. This concentration did not interfere with the coating procedure. Only detergent concentrations higher than 0.015%, 0.020% and 0.025% for $C_{12}E_5$, SB12 and LDAO, respectively, disturbed the coating of HN and F protein. The structural intactness of HN and F protein is expressed as the amount of protein needed to obtain an A_{492} of 1.2. For F protein from the different fractions the amount varies from 3.2 to 7.2 ng (Table III). This shows that the F protein is not affected by HPIEC in the presence of the three detergents, as the amount of F protein necessary for an A_{492} of 1.2 in detergent extracts (the starting material for HPIEC) ranges from 2 to 7 ng [17]. For the HN protein present in the HPIEC fractions, the amount required to obtain an A_{492} of 1.2 varies from 3.5 to 28.6 ng (Table III). This is slightly more than the amount of 4–11 ng of HN protein that is required to obtain an A_{492} of 1.2 for HN protein from detergent extracts [17]. In contrast, more than 1000 ng of HN were required to obtain an A_{492} of 1.2 when HN was denaturated by boiling in 4% SDS [17]. This means that the F protein is not affected by the

TABLE III

IMMUNOLOGICAL REACTIVITY OF HN AND F PROTEIN AFTER HPIEC IN THE PRESENCE OF DIFFERENT DETERGENTS

Immunological activity is expressed as the amount (ng) of HN or F protein needed to give an A_{492} of 1.2.

Column	Detergent					
	$C_{12}E_5$		LDAO		SB12	
	HN	F	HN	F	HN	F
MA7Q	6.6	2.5	13.6	7.2	28.6	3.6
Zorbax SAX	3.9	2.3	27.0	3.4	12.0	3.6
Mono Q	3.5	2.7	12.8	6.9	15.9	3.5
PL-SAX	3.7	2.8	15.5	6.3	15.5	4.4
Average	4.4	2.6	17.2	6.0	18.0	3.8

three detergents used in this study, and that the HN protein is slightly affected when LDAO and SB12 are used as additives to the eluent, independent of the column used for HPIEC. With $C_{12}E_5$ in the eluent neither the HN nor the F protein is affected.

CONCLUSIONS

The results show that the non-ionic detergent $C_{12}E_5$ is more suitable for extraction, chromatography and preservation of conformation of Sendai virus integral membrane proteins than the two zwitterionic detergents SB12 and LDAO. The resolution in HPIEC largely depended on the detergent used for extraction and used as additive in the elution buffer.

ACKNOWLEDGEMENTS

We thank Mr. J. J. van Dijk, Bio-Rad, Veenendaal, Netherlands, for the MA7Q column, Dr. A. Dams, DuPont, 's-Hertogenbosch, Netherlands, for the Zorbax BioSeries SAX column, Dr. L. L. Lloyd, Polymer Labs. Church Stretton, UK for the PL-Sax 400 Å column, Serva, Heidelberg, Germany, for the Polyol Si500 column and Mr. B. Kwant, Bedum, Netherlands, for the non-ionic detergent $C_{12}E_5$.

REFERENCES

1 G. W. Welling, R. van der Zee and S. Welling-Wester, *J. Chromatogr.*, 418 (1987) 223.
2 G. W. Welling and S. Welling-Wester, In C.T. Mant and R. S. Hodges (Editors), *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation*, CRC Press, Boca Raton, FL, 1991, p. 223.
3 M. Rögner, *J. Chromatogr.*, 512 (1990) 219.
4 A. Helenius and K. Simons, *Biochim. Biophys. Acta*, 415 (1975) 29.
5 C. Tanford and J. A. Reynolds, *Biochim. Biophys. Acta*, 457 (1976) 133.
6 A. Helenius, D. R. McCaslin, E. Fries and C. Tanford, *Methods Enzymol.*, 56 (1979) 734.
7 L. M. Hjelmeland and A. Crambach, *Methods Enzymol.*, 104 (1984) 305.
8 A. Gonenne and R. Ernst, *Anal. Biochem.*, 87 (1987) 28.
9 G. W. Welling, G. Groen and S. Welling-Wester, *J. Chromatogr.*, 266 (1983) 629.
10 R. van der Zee, S. Welling-Wester and G. W. Welling, *J. Chromatogr.*, 266 (1983) 577.
11 G. W. Welling, J. R. J. Nijmeijer, R. van der Zee, G. Groen, J. B. Wilterdink and S. Welling-Wester, *J. Chromatogr.*, 297 (1984) 101.
12 G. W. Welling, G. Groen, K. Slopsema and S. Welling-Wester, *J. Chromatogr.*, 326 (1985) 173.
13 G. W. Welling, K. Slopsema and S. Welling-Wester, *J. Chromatogr.*, 359 (1986) 307.
14 G. W. Welling, K. Slopsema and S. Welling-Wester, *J. Chromatogr.*, 397 (1987) 165.
15 G. W. Welling, B. Kazemier and S. Welling-Wester, *Chromatographia*, 24 (1987) 790.
16 S. Welling-Wester, B. Kazemier, C. Örvell and G. W. Welling, *J. Chromatogr.*, 443 (1988) 255.
17 J. van Ede, J. R. J. Nijmeijer, S. Welling-Wester, C. Örvell and G. W. Welling, *J. Chromatogr.*, 476 (1989) 319.
18 S. Welling-Wester, R. M. Haring, H. Laurens, C. Örvell and G. W. Welling, *J. Chromatogr.*, 476 (1989) 477.
19 O. Sechoy, J. R. Phillipot and A. Bienvenue, *J. Biol. Chem.*, 262 (1987) 11519.
20 K. W. Hermann, *J. Phys. Chem.*, 66 (1962) 295.
21 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
22 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
23 W. Wray, T. Boulikas, V. P. Wray and R. Hancock, *Anal. Biochem.*, 118 (1981) 197.
24 C. Örvell and M. Grandien, *J. Immunol.*, 129 (1982) 2779.
25 Y. Kato, T. Kitamura, K. Nakamura, A. Mitsui, Y. Yamasaki and T. Hashimoto, *J. Chromatogr.*, 391 (1987) 395.
26 L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 512 (1990) 365.
27 J. K. Duncan, A. J. C. Chen and C. J. Siebert, *J. Chromatogr.*, 397 (1987) 3.