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Effect of different amounts of the non-ionic detergents $C_{10}E_5$ and $C_{12}E_5$ present in eluents for ion-exchange high-performance liquid chromatography of integral membrane proteins of Sendai virus

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ABSTRACT

Non-ionic detergents (0.03–0.5%) are used as additives to the eluents when integral membrane proteins are subjected to ion-exchange high-performance liquid chromatography (HPIEC). It is not known whether this concentration should bear some relation to the critical micelle concentration (CMC) of a detergent (the concentration at which micelles begin to form) or that only the amount of detergent is of importance in order to maintain the membrane proteins in solution. This was investigated with a detergent extract of Sendai virus which contains two integral membrane proteins, the fusion protein and the haemagglutinin–neuraminidase protein. Two polyoxyethylene alkyl ethers ($C_{10}E_5$ and $C_{12}E_5$) were used both for extraction (2% final concentration) and as additives in the elution buffers for HPIEC on Mono Q with "classical" HPLC and the micro-HPLC Smart System (Pharmacia-LKB). The CMCs of the two non-ionic detergents $C_{10}E_5$ and $C_{12}E_5$ are 0.026 and 0.002%, respectively. Concentrations below and above the CMC were used in the eluent. The results showed that the concentration of the detergent should be 2–26 times the CMC in order to avoid aggregation. The integral membrane proteins of Sendai virus remain on the column when the detergent concentration is less than 0.026–0.05%, independent of the CMC of the detergent. This may be utilized in HPIEC strategies: at low detergent concentration, hydrophilic proteins are eluted with the salt gradient and a subsequent blank run with the same gradient at higher detergent concentrations results in elution of the integral membrane proteins.

INTRODUCTION

Non-ionic detergents (surfactants) are employed for the extraction of integral membrane proteins from the lipid bilayer in which they are embedded. The extraction results in solubilization of the integral membrane proteins by the formation of complexes with detergent molecules, *i.e.*, micelles [1–4]. At a certain concentration of the detergent, micelles begin to form and this is the critical micelle concentration (CMC). The CMCs of non-ionic detergents may range from 0.002% to 0.32% for the polyoxyethylene (E_y) alkyl (C_x) ethers $C_{12}E_5$ and C_8E_5, respectively [5]. In this study, the importance of the concentration of detergent present during chromatography was investigated, in order to establish whether it is necessary to have the detergent present in the eluent at a concentration above the CMC in order to obtain a satisfactory separation or whether the amount of the detergent is important [6].

In earlier studies, we used the integral membrane proteins of Sendai virus as a model mixture for the development of methodologies for the purification of membrane proteins using different detergents and different modes of

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HPLC [7–19]. The two integral membrane proteins of Sendai virus are the haemagglutinin-neuraminidase protein HN (M_r = 68 000) and the fusion protein F (M_r = 65 000). Both proteins are present in detergent extracts in multimeric forms [20]. 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 one of these studies two polyoxyethylene alkyl ethers were shown to result in relatively high yields of protein after extraction of Sendai virus preparations [5], *i.e.*, $C_{10}E_5$ and $C_{12}E_5$ with CMCs of 0.026% and 0.002%, respectively. In this study, the integral membrane proteins of Sendai virus, HN and F, were extracted with $C_{10}E_5$ and $C_{12}E_5$ from purified virions at a final concentration of 2% (w/w) detergent. The extracts were subjected to ion-exchange HPLC (HPIEC) using concentrations of the detergents in the eluent in a range below and above the CMC.

EXPERIMENTAL

Detergent extraction of Sendai virus and sample preparation for chromatography

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 (10 min, 2000 *g*, 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]. For the isolation of the HN and F proteins, Sendai virions were extracted with $C_{10}E_5$ and $C_{12}E_5$ (Kwant–Hoog Vacolie Recycling and Synthesis, Bedum, Netherlands). 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 contained the extracted HN and F proteins and was stored in aliquots at −80°C.

Ion-exchange and size-exclusion HPLC

Chromatography was performed with a system consisting of an LKB Model 2 150 pump (Pharmacia–LKB, Uppsala, Sweden), a Rheodyne (Inacom, Veenendaal, Netherlands) Model 7125 injector and a Waters Model 441 detector (Millipore–Waters, Etten-Leur, The Netherlands). Anion-exchange HPLC in the "classical system" was performed with a Mono Q HR 5/5 column (50 mm × 5 mm I.D.) (Pharmacia–LKB). In addition, the micro-HPLC Smart System (Pharmacia–LKB) was used with a Mono Q PC 1.6/5 column (50 mm × 1.6 mm I.D.). After isocratic elution for 8 min, retained proteins were eluted with a linear 12-min gradient from 20 m*M* Tris–HCl (pH 7.8) containing different detergent concentrations (buffer A) to 0.5 *M* sodium chloride in the same buffer (buffer B). The detergent $C_{12}E_5$ was added in concentrations ranging from 0.001% to 0.1% and $C_{10}E_5$ in concentrations ranging from 0.013% to 0.1%. The flow-rate was 1 ml/min in the classical HPLC system and 100 μl/min in the Smart System. For HPIEC with the large Mono Q HR 5/5 column (50 mm × 5 mm I.D.), 450 μl of the supernatant (containing 2.0 and 2.5 mg of HN and F proteins for the $C_{10}E_5$ and $C_{12}E_5$ extract, respectively) were diluted (1:1, v/v) with the buffer used in the isocratic elution step and injected. For the Smart System 45 μl of the similarly treated supernatant were used.

Chromatography in the presence of a certain detergent concentration was always followed by a second chromatography (blank run) using the above-mentioned sodium chloride gradient in the presence of 0.1% of the same detergent to elute residual HN and F proteins.

Fractions were collected during gradient elution, 1-ml fractions for the HPIEC with the large Mono Q column and 100-μl fractions for the Smart System.

The amount of protein in the extracts and the yield after HPIEC were determined by high-performance size-exclusion chromatography (HPSEC). From the HPIEC runs, 230 μl of each fraction were pooled, dialysed against demineral-

ized water and lyophilized. To the lyophilized pooled fractions 110 μl of 50 m*M* sodium phosphate (pH 6.5) containing 0.1% sodium dodecyl sulphate (SDS) was added. Subsequently, 4 mg of SDS were added to 100 μl of these dissolved pooled fractions. Prior to chromatography the 100-μl sample was heated for 2 min in boiling water. A Superose 6 HR 10/30 column (300 mm × 10 mm I.D.) was used for HPSEC. The proteins were eluted with 50 m*M* sodium phosphate (pH 6.5) containing 0.1% SDS at a flow-rate of 0.5 ml/min. The absorbance was monitored at 280 nm. The amount of HN and F proteins was then calculated from the peak area. A mixture of similarly treated bovine serum albumin, ovalbumin and trypsin inhibitor (50 μg of each) was used as a standard.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of some 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].

RESULTS AND DISCUSSION

It has been shown that detergents are required as additives in the buffers used for the purification of integral membrane proteins. Concentrations varying from 0.03% to 0.5% have been used in chromatography and mostly detergents are added in concentrations above the CMC [7,24–27]. In previous studies we routinely used polyoxyethylene (E_y) alkyl (C_x) ethers at a concentration of 0.1% in the eluents for HPIEC, which was always above the CMC of the C_{10} and C_{12} series. Both $C_{10}E_5$ and $C_{12}E_5$ are very effective in the extraction of HN and F proteins from Sendai virus particles and therefore preferentially added as detergents in subsequent purification steps. As the CMCs of $C_{10}E_5$ and $C_{12}E_5$ are 0.026% and 0.002%, respectively, the effect of the detergent concentration in the eluent on the separation of HN and F proteins by HPIEC can be studied.

Fig. 1 shows the effect of different concentrations of the $C_{10}E_5$ detergent in the eluent for

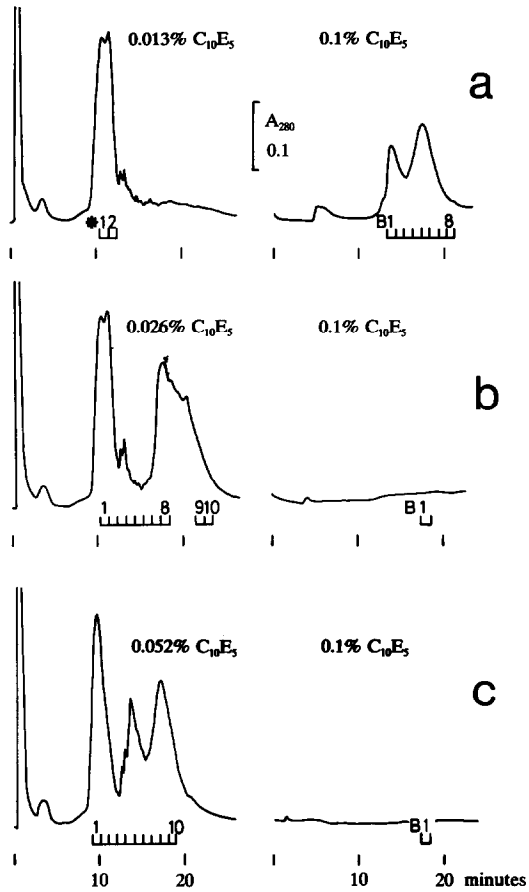


Fig. 1. HPIEC elution profiles of a $C_{10}E_5$ Sendai virus extract, containing HN and F proteins, in the presence of 0.013% [left-hand part of (a)], 0.026% [left-hand part of (b)] and 0.052% [left-hand part of (c)] $C_{10}E_5$. Anion-exchange chromatography was performed with a Mono Q HR 5/5 column. After isocratic elution, retained proteins were eluted with a linear 12-min gradient from 20 m*M* Tris–HCl (pH 7.8) containing different detergent concentrations 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. Chromatography in the presence of a certain detergent concentration was always followed by a second chromatography (blank run) using a sodium chloride gradient in the presence of 0.1% of $C_{10}E_5$ to elute residual HN and F proteins. The elution profiles of these blank runs are shown in the right-hand parts. Fractions were collected during gradient elution as indicated.

HPIEC. A sample of the $C_{10}E_5$ detergent extract, containing 2.0 mg of HN and F proteins, was separated with eluents containing different concentrations (0.013, 0.026 and 0.052%) of $C_{10}E_5$. Each chromatography was followed by a subsequent chromatography (a blank run) in the presence of 0.1% $C_{10}E_5$, in order to elute any

residual HN and F proteins. The elution profiles of the blank runs with the 0.1% detergent are shown in the right-hand parts of Fig. 1. Fractions were collected during elution with a sodium chloride gradient and analysed by SDS-PAGE (see Fig. 2). In the presence of 0.013% $C_{10}E_5$, most of the proteins were retained (see Fig. 1a, and the corresponding SDS-page analysis in Fig. 2a, lanes *1 and 2; numbers correspond to fraction numbers).

HPSEC of the pooled fractions collected during sodium chloride gradient elution revealed that only a small percentage of F protein was eluted. The detergent concentration was probably too low to prevent aggregation of the large membrane proteins. The chromatography was followed by isocratic elution for 41 min with buffer A containing 0.1% $C_{10}E_5$. Subsequently, a blank run was done with a linear gradient of 0–0.5% *M* sodium chloride in the same buffer containing 0.1% $C_{10}E_5$. HN and F proteins were eluted during the latter chromatography with a recovery of 66% (see Fig. 1a, right-hand part of the elution profile, and Fig. 2a, lanes B1–8). In the presence of 0.026% $C_{10}E_5$, which is the CMC, HN and F proteins were eluted (Fig. 1b, left-hand part). However, no separation between HN_4 plus HN_2 proteins and F protein was obtained (see Fig. 2b, lanes 7–10, corresponding to fractions 7–10 in the left-hand part of Fig. 1). Only the truncated forms of HN_4 and HN_2 proteins were eluted earlier during the gradient (Fig. 2b, lanes 3–5). Subsequent elution with the sodium chloride gradient in the presence of 0.1% $C_{10}E_5$ only revealed a small amount of F protein (Fig. 1b and Fig. 2b, lane B1). With a detergent concentration twice the CMC of $C_{10}E_5$ (0.052%) in the buffer, the elution profiles (Fig. 1c) and the corresponding SDS-PAGE analysis (Fig. 2c) show that separation could be obtained between HN_4 plus HN_2 and F proteins. The recoveries of HN and F proteins were 71%, which is in good agreement with earlier studies [5]. The results as shown (Figs. 1 and 2) for different concentrations of $C_{10}E_5$ in the buffers indicate that concentrations above the CMC are essential for separation.

The same experiments as described above were repeated with the Smart System. The

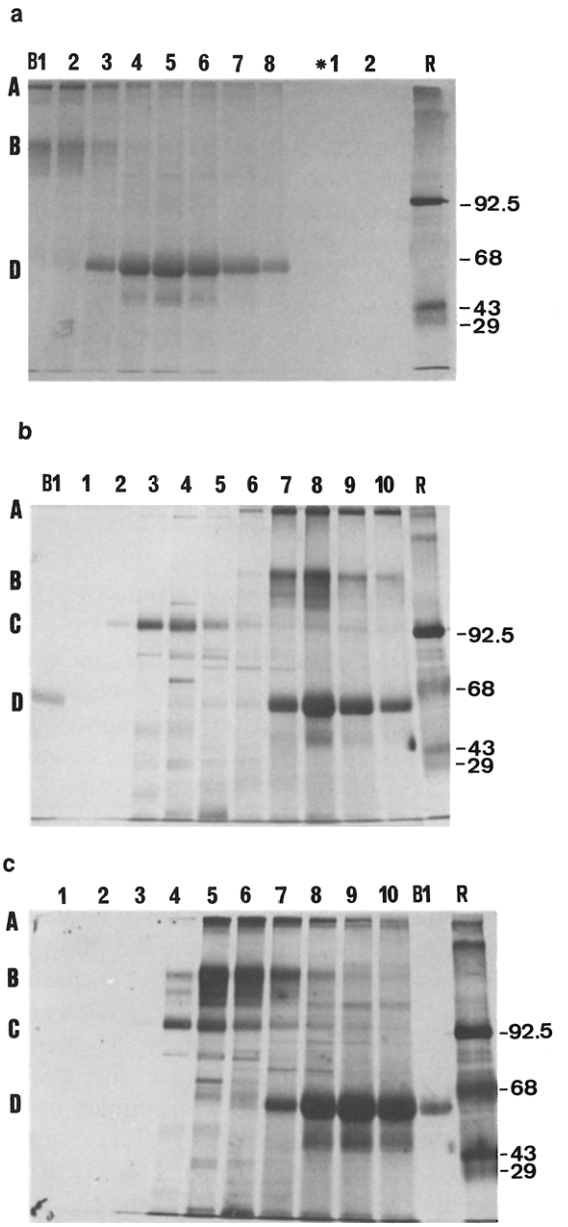
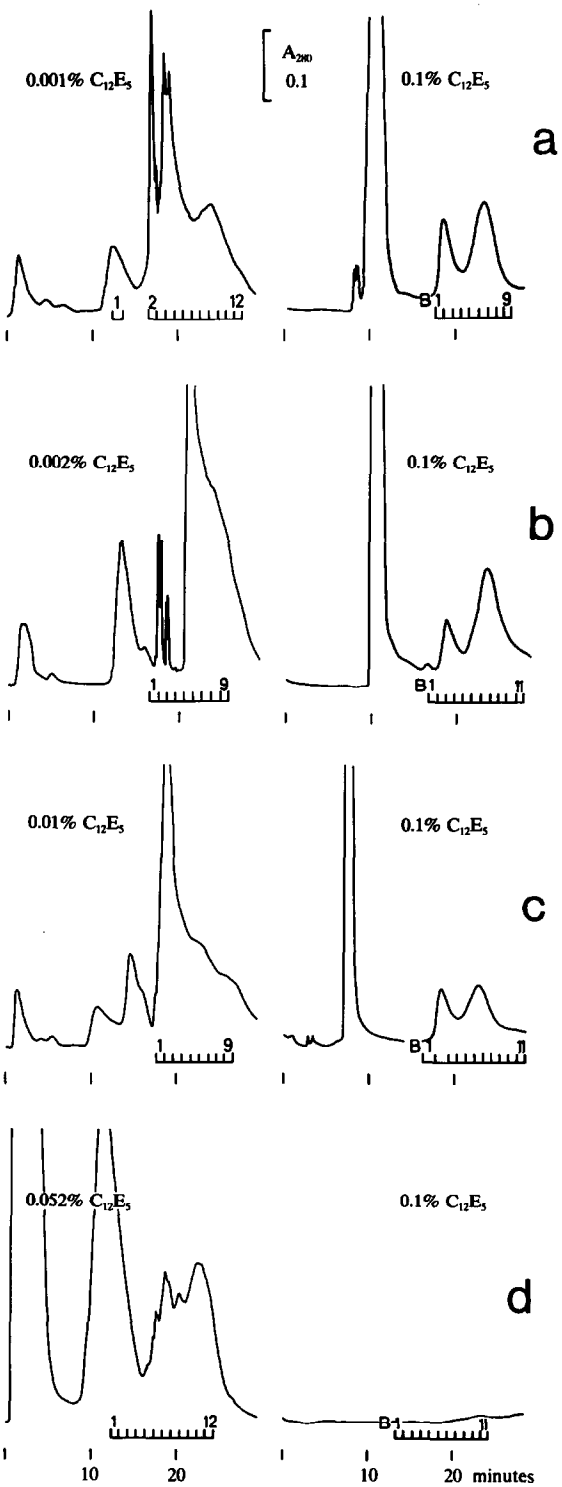


Fig. 2. SDS-PAGE analysis on 12% gels under non-reducing conditions of the fractions collected during chromatography in the presence of different concentrations of $C_{10}E_5$: 0.013% [(a), lanes *1 and 2] followed by a blank run in the presence of 0.1% [(a), lanes B1–8]; 0.026% [(b), lanes 1–10] followed by a blank run with 0.1% [(b), lane B1]; and 0.052% [(c), lanes 1–10)] followed by a blank run with 0.1% [(c), lane B1]. Polypeptides were rendered visible 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 (R) are given on the right.



results were identical with those obtained with the classical system (data not shown). Especially when the protein to be purified is available in relatively small amounts, *e.g.*, viral proteins, the Smart System has the advantage that only one tenth of the amount needed for classical HPLC is required to obtain comparable results. A disadvantage of the Smart System is that a relatively long time is required for equilibration of the column with different detergents.

The detergent $C_{12}E_5$ has a CMC of 0.002%, which is about ten times lower than that for $C_{10}E_5$. Increasing concentrations of $C_{12}E_5$ (0.001, 0.002, 0.004, 0.01, 0.026, 0.052 and 0.1%) present in the elution buffers were investigated. Conditions identical with those for $C_{10}E_5$ were applied, except that a $C_{12}E_5$ extract containing 2.5 mg of HN and F proteins was used. Incorporation of 0.001, 0.002, 0.004 and 0.01% $C_{12}E_5$ in the buffers resulted in partial elution of HN and F proteins, without separation of HN_4 plus HN_2 and F proteins. The more hydrophilic, truncated forms of HN_4 and HN_2 proteins were eluted earlier during the sodium chloride gradient. The elution profiles are shown in the left-hand part of Fig. 3a, b and c and the corresponding SDS-PAGE analyses in Fig. 4a, lanes 1–12, Fig. 4b, lanes 1–9, and Fig. 4c, lanes 1–9, respectively. The data for 0.004% are identical (not shown). Only if the above-mentioned chromatographic runs were followed by equilibration with buffer A containing 0.1%

Fig. 3. Elution profiles of HPIEC of a $C_{12}E_5$ Sendai virus extract, containing HN and F proteins, in the presence of 0.001% [left-hand part of (a)], 0.002% [left-hand part of (b)], 0.026% [left-hand part of (c)] and 0.052% $C_{12}E_5$ [left-hand part of (d)]. Anion-exchange chromatography was performed with a Mono Q HR 5/5 column. After isocratic elution, retained proteins were eluted with a linear 12-min gradient from 20 m*M* Tris–HCl (pH 7.8) containing the different detergent concentrations 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. Chromatography in the presence of a certain detergent concentration was always followed by a second chromatography (blank run) using a sodium chloride gradient in the presence of 0.1% of $C_{12}E_5$ to elute residual HN and F proteins. The elution profiles of these blank runs are shown in the right-hand parts. Fractions were collected during sodium chloride gradient elution as indicated.

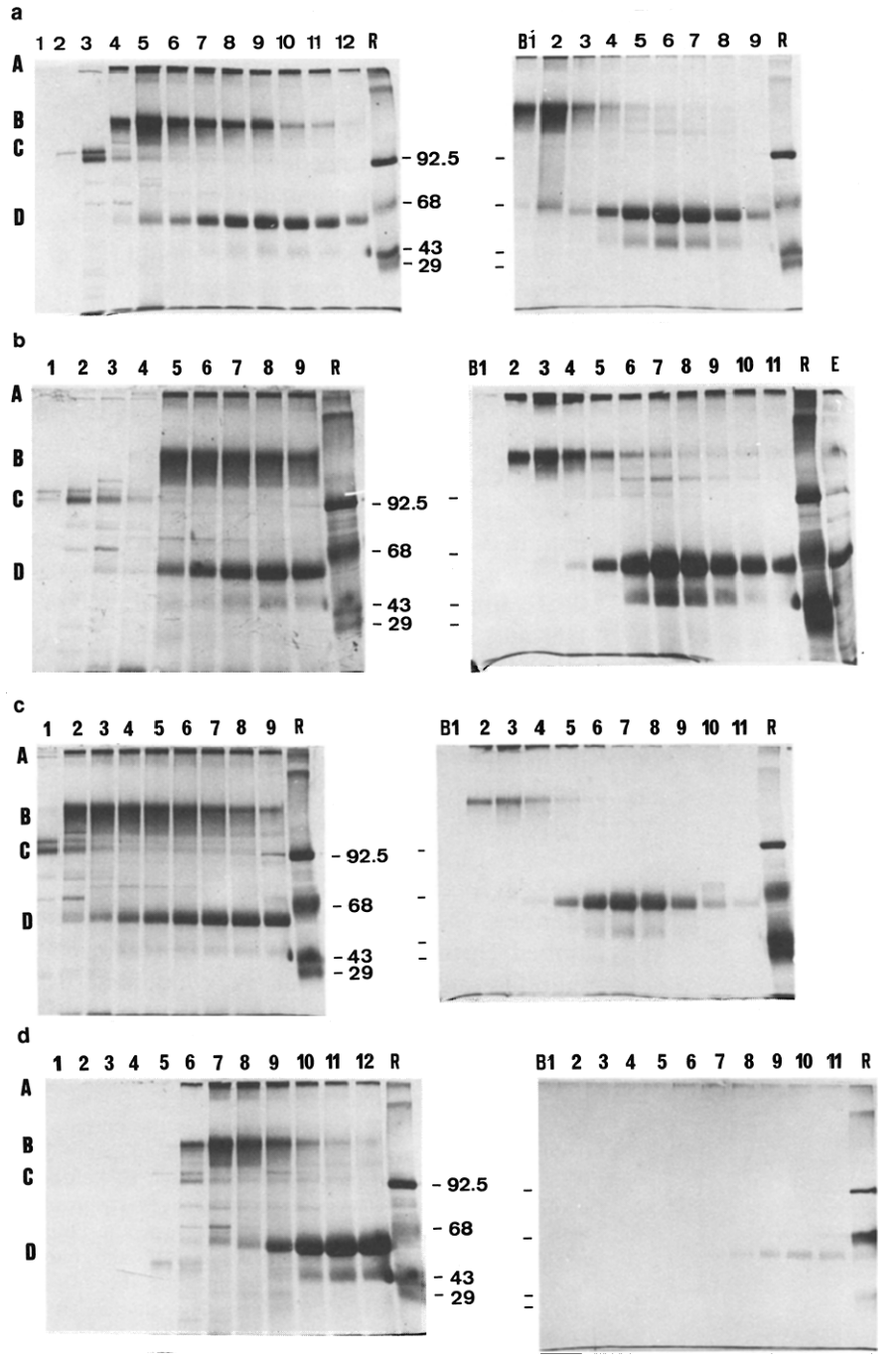


Fig. 4. SDS-PAGE analysis on 12% gels under non-reducing conditions of the fractions collected during chromatography in the presence of different concentrations of $C_{12}E_5$: 0.001% [(a), left gel, lanes 1–12] followed by a blank run in the presence of 0.1% [(a), right gel, lanes B1–9]; 0.002% [(b), left gel, lanes 1–9] followed by a blank run with 0.1% [(b), right gel, lanes B1–11]; 0.026% [(c), left gel, lanes 1–9] followed by a blank run with 0.1% [(c), right gel, lanes B1–11]; and 0.052% [(d), left gel, lanes 1–12] followed by a blank run with 0.1% [(d), right gel, lanes B1–11]. Polypeptides were rendered visible 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 (R) proteins are given on the right. E = $C_{12}E_5$ extract of Sendai virus.

$C_{12}E_5$ and subsequent elution (blank run) with a gradient of 0–0.5 *M* sodium chloride in the same buffer were the remaining HN and F proteins eluted and separated (see right-hand part of Fig. 3a, b and c and the corresponding SDS-PAGE analyses in Fig. 4a, lanes B1–9, Fig. 4b, lanes B1–11, and Fig. 4c, lanes B1–11, respectively). The recoveries for HN and F proteins were 26% after chromatography in the presence of 0.001% $C_{12}E_5$, and after the blank run with 0.1% $C_{12}E_5$ added to the buffers an additional 21% was recovered. In the presence of 0.026% $C_{12}E_5$ the HN and F proteins were almost completely eluted in one run, with a reasonable separation; only a small amount of F protein was found by subsequent elution with 0.1% $C_{12}E_5$ (data not shown). A concentration of 0.052% $C_{12}E_5$ resulted in separation between HN_4 plus HN_2 and F proteins with a recovery of 50% (Fig. 3d and Fig. 4d, lanes 7–12). A small amount of F protein (*ca.* 8% of the injected amount HN and F proteins) was found in the fractions of the blank run (see Fig. 4d, lanes B7–11). Low concentrations of $C_{12}E_5$ in the eluents probably partly prevent HN and F proteins from aggregation, which allows elution of the proteins, but higher concentrations are required for the separation of HN and F proteins. Despite the low CMC value of the detergent $C_{12}E_5$, relatively high detergent concentrations (more than 26-fold above the CMC) are required for a satisfactory separation of HN and F proteins. This concentration is of the same order as for $C_{10}E_5$, which has a higher CMC.

Similar results were recently reported by Casey and Reithmeier [6]. They derived an equation to calculate the minimum concentration of detergent required for dispersion of membrane proteins. This equation shows that when a detergent with a low CMC is used, concentrations well in excess of the CMC are required for dispersion of proteins.

The application of different detergent concentrations may be utilized in HPIEC purification strategies: at low detergent concentrations relatively hydrophilic proteins and truncated forms of membrane proteins are eluted with the sodium chloride gradient; subsequent chromatography with the same gradient at higher detergent concentrations results in the elution of the more hydrophobic integral membrane proteins.

CONCLUSIONS

Detergents have to be added to the mobile phase when separation of membrane proteins is required. In this study the non-ionic detergents $C_{12}E_5$ and $C_{10}E_5$ were added in different concentrations to the eluents. Despite the differences in CMC between the two detergents, both require a minimum concentration of 0.052% in the buffers for a good separation.

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