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Purification of the integral membrane glycoproteins D of Herpes simplex virus types 1 and 2, produced in the recombinant baculovirus expression system, by ion-exchange high-performance liquid chromatography

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Abstract

Selective elution of Sendai virus integral membrane proteins by ion-exchange high-performance liquid chromatography (HPIEC) using different detergent concentrations was reported before [S. Welling-Wester, M. Feijlbrief, D.G.A.M. Koedijk, M.A. Braaksma, B.R.K. Douma and G.W. Welling, *J. Chromatogr.*, 646 (1993) 37]. In the present study this novel approach was applied to the purification of the integral membrane glycoprotein D of Herpes simplex virus types 1 and 2. The glycoproteins D of types 1 (gD-1) and 2 (gD-2) were cloned into the baculovirus expression system and produced in protein-free cultured insect cells.

Detergent extracts of recombinant baculovirus-infected insect cells containing gD-1 or gD-2 were prepared using pentaethyleneglycol monodecyl ether, for extraction (final concentration 2%, w/v). The same detergent was used as additive in the elution buffers for HPIEC on a Mono Q HR 5/5 column. At low (0.005%) detergent concentration, most of the proteins present in the extract including part of gD were eluted with the sodium chloride gradient whereas a subsequent blank run using the same gradient at higher detergent concentration (0.1%) resulted in selective elution of pure gD.

1. Introduction

One of the structural glycoproteins of Herpes simplex virus, glycoprotein D (gD), which is present in the envelope of the virus particle and as an integral membrane protein in HSV-infected cells, is a confirmed target of the virus-specific immune response [1–5]. Glycoprotein D is a typical transmembrane protein, containing a signal peptide, which is cleaved off and a hydrophobic transmembrane region near the carboxy terminus. Mature gD type 1 (gD-1) contains 369 amino acid residues and mature gD type 2 (gD-2) has 368 residues [6]. Both are glycosylated and have three N-linked glycosylation sites and two to three O-linked sites [6–8]. Multiple polypeptide bands with a molecular mass between 45 000 and 48 000 on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) are found for recombinant gD produced in insect cells. As minor components, bands of lower molecular masses of 33 000, 24 000 and 22 000 may be found [9]. Immunizations with gD proved effective in attenuating the frequency and severity of primary disease in animals and in reducing the likelihood of latency [10]. These properties have led to its being tested as a candidate for a human subunit vaccine [11,12].

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In earlier studies, we used the integral membrane proteins of Sendai virus as a model for the development of methodologies for the purification of membrane proteins using different detergents and different modes of HPLC [13–23]. This resulted in a two step elution protocol with a non-ionic detergent pentaethyleneglycol monodecyl ether ($C_{10}E_5$), at low and high concentration in the eluent for ion-exchange HPLC (HPIEC) [24].

The aim of this study is to investigate whether this method is applicable to the purification of other integral membrane proteins. To this end, the full-length gD polypeptide produced in recombinant baculovirus infected Sf21 insect cells (the cloning strategy will be published elsewhere) in a protein-free insect cell culture medium, will be purified by this procedure.

2. Experimental

2.1. Extraction of recombinant gD from Sf21 cells using non-ionic detergent $C_{10}E_5$ and sample preparation.

Sf21 cells were grown in protein-free insect cell culture medium (Insect X-press; Bio-Whittaker, Walkersville, MD, USA) containing 10 μg/ml gentamicin. Insect cells ($2.5 \cdot 10^8$) were infected at a multiplicity of infection of five plaque-forming units of recombinant baculovirus per cell. After 4 days at 27°C, cells were collected by centrifugation (100 *g*, 10 min, room temperature) and washed three times in ice-cold phosphate buffered saline, pH 7.4 (PBS). For extraction of membrane proteins, the cell pellet was resuspended in 5 ml of ice-cold 20 m*M* Tris–HCl, pH 7.8, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* tosyllysine chloromethyl ketone (TLCK) and subsequently, 5 ml of the same buffer containing 4% (w/v) $C_{10}E_5$, (Kwant-Hoog Vacolie Recycling and Synthesis, Bedum, Netherlands) was added under limited agitation. The cell suspension was incubated on ice for 1 h. Cell debris was removed by low-speed centrifugation (10 min, 2000 *g*). The supernatant (extract) after ultracentrifugation (70 000 *g*, 1 h, 4°C) contains gD and was stored in aliquots at −80°C.

2.2. HPIEC

Chromatography was performed with a system consisting of an LKB Model 2150 pump (Pharmacia–LKB, Woerden, Netherlands), a Rheodyne (Inacom, Veenendaal, Netherlands) Model 7125 injector and a Waters Model 441 detector (Millipore–Waters, Etten-Leur, Netherlands). Anion-exchange HPLC was performed with a Mono Q HR 5/5 column (50 mm × 5 mm I.D.) (Pharmacia–LKB). The flow-rate was 1 ml/min and absorbance was monitored at 280 nm. The samples of the infected cell extracts were 1:1 diluted with 20 m*M* Tris–HCl, pH 7.8, and centrifuged prior to injection at 14 000 *g* at 4°C for 5 min. After isocratic elution for 12 min, retained proteins were eluted with a linear 12-min gradient from 20 m*M* Tris–HCl, pH 7.8, containing 0.005% $C_{10}E_5$ (buffer A) to 0.5 *M* NaCl in the same buffer (buffer B). Following the gradient and subsequent isocratic elution for 15 min using buffer A, the concentration of detergent was increased to 0.1% $C_{10}E_5$ in 20 m*M* Tris–HCl, pH 7.8 (buffer C). During 30 min of isocratic elution with buffer C, the column was equilibrated. This was followed by a "blank" run, *i.e.* an additional gradient elution without sample injection. During the blank run retained proteins were eluted with a 12-min linear salt-gradient from 0 *M* NaCl in buffer C to 0.5 *M* NaCl in the same buffer (buffer D).

Fractions of 1 ml were collected during gradient elution. For enzyme-linked immunosorbent assay (ELISA), 50 μl of each fraction were used and the remainder was dialyzed (4–6 h, 4°C) against water. Samples of 125 μl of the fractions were lyophilized for SDS-PAGE. The remainder of the fractions were stored at − 80°C. Concentrations of gD in the extract and in the fractions were determined by a combination of ELISA and amino acid analysis.

2.3. SDS-PAGE

Dialyzed and lyophilized samples (125 μl) of some of the HPIEC fractions were analyzed by

SDS-PAGE on 12.5% gels under reducing conditions [25]. After electrophoresis, gels were fixed and silver stained as described [26].

2.4. ELISA

Microtiter plates were coated with serial dilutions (in 50 m*M* $NaHCO_3$ buffer, pH 9.6) of collected fractions for 18 h at 4°C. After washing with PBS containing 1 *M* NaCl and 0.3% Tween-20, plates were incubated with 1:6400 diluted monoclonal antibody (mAb) HD1 for 1 h. The mAb HD1 is specific for gD and directed against gD-1 and gD-2. The mAb HD1 is conformation-dependent [27], and reacts only with conformationally intact gD. After washing, plates were incubated for 1 h at 37°C with peroxidase-labelled sheep anti-mouse IgG (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). After colour development with *o*-phenylenediamine dihydrochloride, the absorbance was measured at 492 nm. Glycoprotein D concentrations were calculated at $A_{492} = 1.2$ by using a gD-2 standard in combination with amino acid analysis. The mAb HD1 reacts at an A_{492} of 1.200 with 1.6 μg gD-1 and with 4.0 μg of gD-2.

3. Results and discussion

In a previous study, a two-step purification strategy for a model mixture of integral membrane proteins of Sendai virus was developed. By manipulating the concentration of the non-ionic detergent $C_{10}E_5$ added to eluent buffers during HPIEC, a selective elution of integral membrane proteins could be achieved [24]. This procedure was also successfully applied for the partial purification of a recombinant baculovirus malaria membrane antigen [28]. This prompted us to investigate whether this procedure might be applicable to the purification of other membrane proteins *viz.* the purification of recombinant and full-length gD, produced in baculovirus infected insect cells. Infected Sf21 cells, cultured in protein-free medium, were extracted with 2% $C_{10}E_5$. The extract contained 0.7 mg protein/ml of which 10–15% is gD-1 or gD-2. Samples (110–1000 μl, and containing 77 to 700 μg protein) of a $C_{10}E_5$ detergent extract containing 11.3–104 μg/ml gD-1 were subjected to two consecutive HPIEC-gradient runs with 0.005% $C_{10}E_5$ in the eluent during the first run and 0.1% $C_{10}E_5$ during the second (blank) run. Fractions were collected during the chromatographic steps and analyzed by ELISA to determine the presence of gD-1. In addition, SDS-PAGE was performed on the fractions collected during the salt gradient elution. Table 1 summarizes the purification procedure with the percentage gD-1 present during each purification step. No gD was present in the fractions of the flowthrough, and only trace amounts were found in the first fractions eluted isocratically during equilibration with buffer C.

Glycoprotein D mainly was eluted during the salt gradients, and the SDS-PAGE analyses of these fractions showed that gD that was eluted during the salt gradient in the presence of 0.1% $C_{10}E_5$ (buffer D) was virtually pure (see Fig. 2b). When a 104-μg sample was applied, a relatively large amount of gD-1 was eluted in the first gradient run and the distribution of gD-1 over both gradients resulted in a 85:15 ratio (see Table 2). The elution patterns of the two gra-

Table 1
Purification of glycoprotein D of Herpes simplex virus type 1 from infected Sf21 cells

Purification step	Percentage gD[a] present in purification step
Starting material:	
extract of infected cells	10–15
HPIEC:	
(1) flow-through isocratic elution with 0.005% $C_{10}E_5$ in buffer	0
(2) salt gradient, 0–0.5 *M* NaCl with 0.005% $C_{10}E_5$ in buffer	20–30
(3) isocratic elution with 0.005% $C_{10}E_5$ in buffer	0
(4) isocratic elution with 0.1% $C_{10}E_5$ in buffer	Trace
(5) salt gradient, 0–0.5 *M* NaCl with 0.1% $C_{10}E_5$ in buffer	> 90

[a] The percentage gD is based on the analysis of the fractions by SDS-PAGE and the gD-specific ELISA.

Table 2
Recovery of gD from an extract of insect cells infected with a recombinant baculovirus by HPIEC using a two-step purification procedure

Extract (μg gD)	Percentage of gD eluted during the first salt gradient detergent: 0.005% $C_{10}E_5$	Percentage of gD eluted during the blank run detergent: 0.1% $C_{10}E_5$	Total yield of gD after first and blank run
gD-1 extract			
1.62	22	78	71[a] (1.15 μg)
11.3	21	79	42 (4.7 μg)
26.0	41	59	51 (13.3 μg)
104	85	15	34 (35 μg)
gD-2 extract			
13.4	44	56	42 (5.6 μg)
30.4	60	40	44(13.5 μg)
60.8	64	36	40 (24.4 μg)

[a] Recovery of gD in percentages.

dient runs obtained after HPIEC of a detergent extract containing 26 μg gD-1 are shown in Fig. 1, together with the concentration of gD-1 in each fraction determined by an ELISA using a gD-specific mAb. Fig. 1a shows the elution pattern in the presence of the 0.005% $C_{10}E_5$, and Fig. 1b shows the elution pattern of the blank run in the presence of 0.1% $C_{10}E_5$. The corresponding SDS gels of the fractions are shown in Fig. 2a and b. In this case 41% of gD-1 was found in the first run and 59% in the blank run. Fig. 2b shows that the fractions of the blank run contain mainly two polypeptide bands of M_r 45 000–48 000. ELISA analysis of the fractions with a gD-specific mAb (Fig. 1b, columns below the chromatogram) confirms the presence of virtually pure, conformationally intact gD-1. Probably due to the presence of not fully glycosylated forms, gD-1 is eluted as a broad peak. When a small amount of the gD-1 (11.3 μg) containing extract was subjected to chromatography, gD-1 was mainly found in fractions that were collected during the second (blank) run, resulting in a 21:79 distribution of gD-1 over both gradients.

The total yield of gD-1, varying from 71 to 34% (Table 2), is relatively low. Part of gD is probably degraded during the purification procedure. This proteolytic degradation in the cell extracts is not unusual for HSV glycoproteins and has been described by others [29].

Extracts (containing gD-2, 121.5 μg/ml) of cells infected with recombinant baculovirus expressing gD-2, were prepared and used for HPIEC. Chromatography was performed using identical conditions as used for the purification of gD-1. Samples of 110, 250 and 500 μl of the gD-2 extract were subjected to the two-step elution protocol and analysis of the fractions by SDS-PAGE and ELISA showed results comparable to those obtained after HPIEC of a gD-1 extract (see Table 2).

To investigate whether gD eluted during the first gradient run is different from that eluted during the blank run, a 26-μg sample of gD-1 extract was used. Fractions 8, 9 and 10 (comparable to fractions 8, 9 and 10 of Fig. 1a) containing gD-1 from the first gradient run were pooled (2.7 ml) and briefly dialyzed against buffer A. A sample of 1.5 ml, containing 1.62 μg gD-1, of the dialyzed fractions was subjected to rechromatography, *i.e.* a complete combination of a first gradient run at low detergent concentration and a second (blank) gradient run at high detergent concentration. Glycoprotein D concentrations determined in every fraction showed that gD-1 which was eluted during the first low detergent gradient in the previous

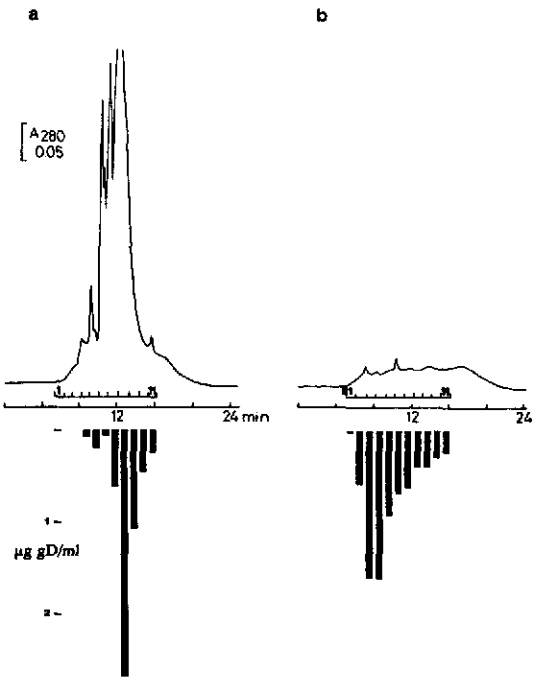

Fig. 1. HPIEC elution profile of a $C_{10}E_5$ extract of insect cells infected with a baculovirus gD recombinant. Chromatography was performed with a Mono Q HR 5/5 column. (a) After isocratic elution retained proteins were eluted with a linear 12-min gradient from 20 m*M* Tris–HCl (pH 7.8) containing 0.005% $C_{10}E_5$ to 0.5 *M* NaCl in the same buffer. This was followed by 15 min isocratic elution with 20 m*M* Tris–HCl (pH 7.8) containing 0.005% $C_{10}E_5$; the column was then equilibrated for 30 min with 20 m*M* Tris–HCl, pH 7.8, containing 0.1% $C_{10}E_5$. (b) The retained proteins were eluted with a linear salt gradient (blank run) in the presence of 0.1% $C_{10}E_5$ in 20 m*M* Tris–HCl buffer, pH 7.8, to 0.5 *M* NaCl in the same buffer. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm. Fractions were collected as indicated and analyzed by ELISA and by SDS-PAGE. The concentration of gD in the fractions was determined by ELISA with the gD-specific mAb HD1 and are indicated by columns below the elution profile.

chromatography, was re-distributed again over both, low (for 22%) and high (for 78%) detergent runs, indicating that there are no differences between gD-1 molecules eluted in the two runs.

It is not unlikely that besides the mainly electrostatic interaction of proteins with ion-exchange resins other contributions, *i.e.* hydrophobic properties of ion-exchange sorbents [30–33] may play a role in the chromatographic process. Non-ionic detergents may interfere with the interaction of the proteins and the ion-exchange ligands. The samples contain 1% of the detergent and consist at least partly out of micelles with the hydrophobic transmembrane region of gD-1 in the hydrophobic bilayer of the micelle. They are subjected to HPIEC with an eluent containing a relatively low concentration of detergent. The detergent monomers in the eluent will try to establish equilibrium with the detergent micelles that are attached to the column through the electrostatic interaction between gD-1 and the ion-exchange ligands. This will only be partially successful, since the detergent concentration in the eluent is too low and a certain concentration of NaCl will be necessary. In the second run the detergent concentration will be sufficiently high to pull all remaining gD-1 molecules into the eluent at the appropriate salt concentration. This may result in relatively pure membrane protein, because all hydrophilic proteins, which did not need a detergent for solubilization, were already eluted during the first run.

4. Conclusions

A highly purified recombinant gD of HSV types 1 and 2 produced by the baculovirus expression system can be obtained by a two-step purification strategy with HPIEC. At low detergent concentration the hydrophilic proteins in the extract are eluted with a salt gradient and a subsequent blank run with the same gradient at higher detergent concentration results in selective elution of the gD polypeptide which is still structurally and immunologically intact.

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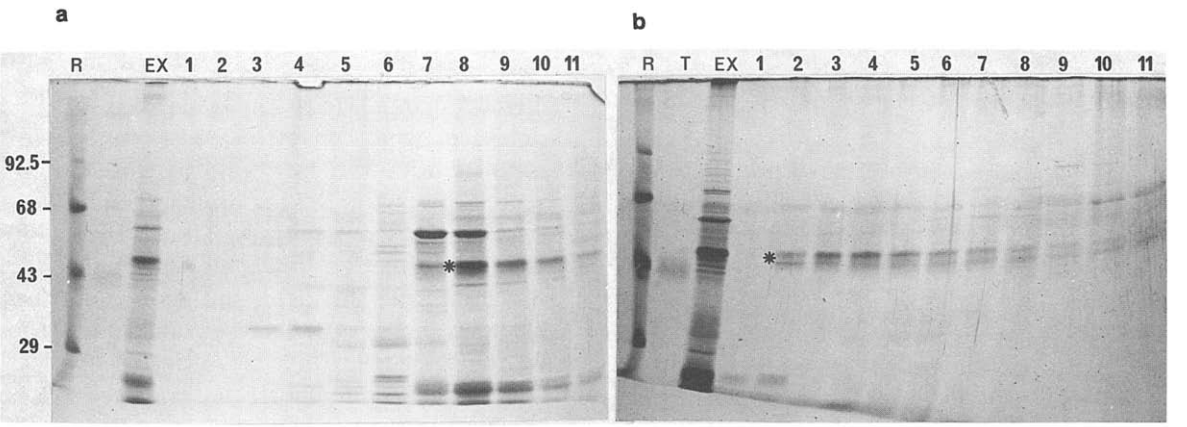

Fig. 2. SDS-PAGE analysis on 12.5% gels under reducing conditions of the fractions collected during chromatography shown in Fig. 1. Lane numbers of the gel correspond to fraction numbers in Fig. 1a and b, respectively. (a) SDS-PAGE analysis of fractions collected during a linear salt gradient in the presence of 0.005% $C_{10}E_5$; (b) SDS-PAGE analysis of the fractions collected during, a blank run, a linear salt gradient in the presence of 0.1% $C_{10}E_5$. Polypeptides were visualized by silver staining. Lanes: T = position of truncated gD-2 (M_r = 37 000–42 000), which was used as control; EX = starting material, extract of the cells infected with the recombinant baculovirus; R = the molecular masses ($\times 10^{-3}$) of reference proteins are given on the left. The asterisk indicates the migration position of glycoprotein D (M_r = 45 000–48 000).

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