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PARTIAL PURIFICATION OF A MEMBRANE GLYCOPROTEIN ANTIGEN BY HIGH-PRESSURE SIZE-EXCLUSION CHROMATOGRAPHY WITHOUT LOSS OF ANTIGENICITY

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SUMMARY

A high-pressure size exclusion chromatography system eluted with phosphate saline buffer containing 0.25% sodium deoxycholate has been developed that fractionates both soluble and membrane glycoproteins with good resolution and molecular weight *versus* elution time relationship. Using this system we fractionated membrane glycoproteins from a mutagenized mastocytoma cell that carries a strong transplantation antigen. After dialysis to remove the detergent, the fractions were tested for biological activity by an *in vitro* assay involving T-lymphocyte cell culture. Antigenic activity was found between 43 and 85 kdaltons. This demonstrates the efficiency of the system to resolve complex membrane protein samples without destroying their biological activity.

INTRODUCTION

In vitro treatment of the P815 mouse mastocytoma cell line with the mutagen N-methyl-N′-nitro-N-nitrosoguanidine produces at very high frequency tumour cell variants that are rejected by the syngeneic host via a T-cell dependent immune process. These variants have been called tum$^-$ and most of them carry new antigens that are specific for each variant[1]. For the tum$^-$ variant P35, the presence of the new antigens can be detected in subcellular fractions by their ability to restimulate primed lymphocytes *in vitro*. The cytolytic activity of these lymphocytes can then be measured by a ^{51}Cr release assay on the relevant tum$^-$ variant[2]. For this restimulation test the samples must not be denatured and should be contained in a physiological solvent. Using this assay we first showed that the antigenic activity of tum$^-$ variant P35 is present in the plasma membrane of the cell. After solubilization of the plasma membrane with sodium deoxycholate (DOC), the antigenic activity was retained on

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a lentil lectin affinity column. This suggested the glycoprotein nature of the antigen and demonstrated that the mild ionic detergent sodium deoxycholate allowed the solubilization of the antigen and its recovery after removal of the detergent by dialysis (unpublished results). We therefore used the same solubilization conditions for the next fractionation step of our membrane glycoprotein sample.

We present in this paper the development of a high-pressure size-exclusion chromatography (HP-SEC) system eluted with a phosphate saline buffer containing sodium deoxycholate (PBS–DOC) capable of fractionating such a complex mixture without destroying its biological activity. The non-denaturing conditions to which the proteins are submitted could render the system useful when labile or biologically active proteins must be recovered after HP-SEC fractionation.

EXPERIMENTAL

Chemicals

Inorganic compounds and sodium deoxycholate were purchased from Merck (Darmstadt, F.R.G.). Bovine serum albumin, chicken albumin, α-lactalbumin, soy bean trypsin inhibitor were obtained from Sigma (St. Louis, MO, U.S.A.) and Ferritin, myoglobin, cytochrome c, lactate dehydrogenase and aprotinin from Boehringer (Mannheim, F.R.G.).

HPLC apparatus

We used a Waters liquid chromatograph consisting of a Model 6000A pump, a Model 441 absorbance detector, routinely set at 214 nm, a U6-K injector and a Waters data module.

Procedure for HP-SEC

A Waters guard column filled with I-125 packing was connected in-line to Waters I-250 and I-125 columns. The mobile phase [10 m*M* sodium phosphate, pH 7.4, containing 150 m*M* NaCl and 0.25% DOC (PBS–DOC)] was pumped at flow-rates between 0.1 and 2 ml/min. The maximum column pressure was 1000 p.s.i. at 2 ml/min. The sample volumes varied from 10 to 200 μl and the amount of proteins injected ranged between 10 and 200 μg. Separations were carried out at room temperature. Fractions were collected with a Pharmacia Frac-100 fraction collector.

Sample preparation for HP-SEC

Standard proteins were dissolved in the mobile phase at the appropriate concentration.

The biological sample was prepared as follows: all manipulations were carried out at 4°C; plasma membrane from tum$^-$ variant P35 was prepared according to the method of Esko *et al.*[3], slightly modified by addition of 2 m*M* $MgCl_2$ after the cell disruption to stabilize nuclei. The preparation was dissolved in the PBS–DOC buffer and chromatographed on a lentil lectin affinity column as described elsewhere[4]. The lentil lectin eluate, containing only membrane glycoproteins, was placed in a dialysis bag and concentrated on dry Sephadex G-200 up to a protein concentration of 1 mg/ml. The sample was filtered through a 0.45 μm Millipore filter and stored on ice until fractionated.

Sample preparation for biological assay

Samples solubilized in the PBS–DOC buffer were mixed with a constant amount of P815 plasma membrane solubilized in the same buffer and were extensively dialysed in the cold against PBS to allow the formation of proteoliposomes suitable for lymphocyte restimulation[5]. Liposomes were recovered by a $6 \cdot 10^6$ *g*.min ultracentrifugation. Their protein content was measured by a Lowry assay[6].

Biological assay

DBA/2 mice were immunized intraperitoneally with $2 \cdot 10^5$ living P35 tum$^-$ variant cells. Six weeks later, their spleen cells were used as a source of primed lymphocytes and restimulated *in vitro* with 0.1–30 μg of P35 membrane proteins in proteoliposomes. The cells were cultured for 4 days in 24-well Nunclon Multidishes (NUNC) at $5 \cdot 10^6$ per well in 2 ml of Dulbecco's modified Eagle medium supplemented as described in ref. 7. The cytolytic activity of the cultures was assayed on ^{51}Cr-labelled P35 cells as described in ref. 2.

Electrophoresis

Fractionated samples were freeze-dried and extracted twice with a single-phase Bligh–Dyer solution [methanol–chloroform–water (2:1:0.8)][7] to remove the detergent and salts, and were analysed in a discontinuous 10% polyacrylamide system containing SDS[8]. The proteins were then rendered visible by silver staining[9].

RESULTS

Fractionation of standard proteins

The inclusion of DOC in the phosphate saline buffer resulted in good peak resolution and in a linear relationship between the logarithm of molecular weight and the elution time of the standard proteins (Fig. 1 and 2). This suggested that the combined effects of salt and mild detergent in the mobile phase resulted in a considerable decrease in both ionic and hydrophobic 'non-ideal'[10] interactions responsible for the loss of resolution and of molecular weight deviations commonly observed with proteins on HP-SEC columns. Moreover, the conditions used were as good as those in which much more denaturing eluents are used, including the strong detergent sodium dodecyl sulphate (SDS)[11] or the chaotropic agent guanidinium hydrochloride[12]. As already shown by others, the resolution of the HP-SEC system is inversely proportional to the flow-rate[13]. Indeed, from the elution patterns obtained at flow-rates ranging between 0.2 and 1 ml/min (Fig. 2), we calculated the variation of resolution between the peaks of bovine serum albumin and ovalbumin according to the equation $R = v_e/0.5\ (W_A + W_b)$, where V_e is the difference between the elution volumes of the two peaks and W_A and W_B are their baseline widths. The results in Fig. 3 show that decreasing the flow-rate from 2 to 0.2 ml/min induced an increase in resolution by a factor of 1.5. Further, as the resolution at 0.5 ml/min was only 10% lower than that obtained at 0.2 ml/min, we chose a flow-rate of 0.5 ml/min as a compromise between analysis time and resolution. This may be important when the samples to be fractionated are very labile.

At a flow-rate of 0.5 ml/min, we also found that for a given amount (200 μg) of proteins injected, the resolution was almost the same if the sample volumes were 20 μl ($R = 0.96$) or 200 μl ($R = 0.90$) (Fig. 4).

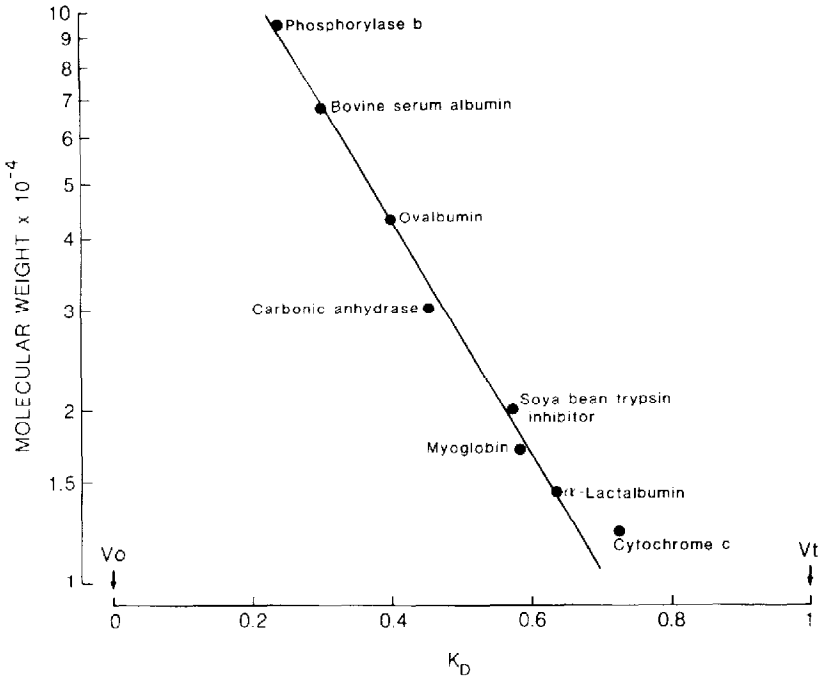

Fig. 1. Relationship between retention time and molecular weight ($\times 10^{-4}$) of standard proteins fractionated on I-250 and I-125 Waters columns eluted with PBS–DOC mobile phase at a flow-rate of 0.5 ml/min.

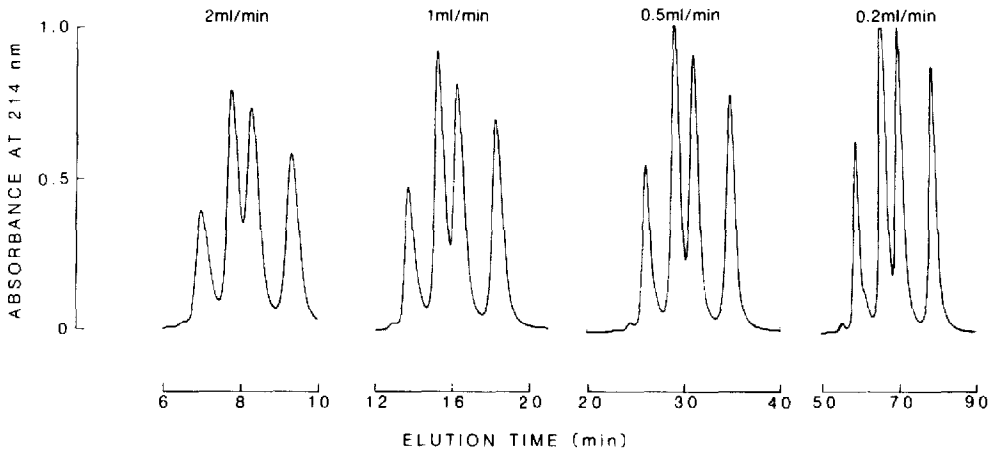

Fig. 2. Relationship between flow-rate and resolution. Horse spleen ferritin, bovine serum albumin, ovalbumin and soya bean trypsin inhibitor (40 μg each) were solubilized in the PBS–DOC mobile phase at a final volume of 80 μl and applied to the two-column system described in the text. Elution was performed at flow-rates varying between 0.2 and 2 ml/min as indicated.

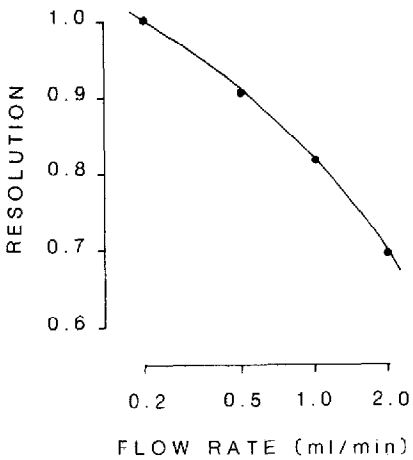

Fig. 3. Relationship between resolution and flow-rate. The resolution of the peaks of bovine serum albumin and ovalbumin was calculated at various flow-rates as described in the text.

P35 antigen fractionation

A 200-μl sample of the membrane glycoprotein fraction of the cell P35 (see Experimental) was applied on the HP-SEC system as described above. Fractions of 0.5 ml were collected, analysed by SDS–polyacrylamide gel electrophoresis and tested for the presence of antigen activity. Fig. 5 shows the gel pattern of the membrane glycoprotein sample after fractionation. The separation was very effective and considerably better than the elution patterns obtained with conventional low-pressure systems.

In addition, it was possible to recover the biological activity after HP-SEC fractionation in three experiments carried out on separate plasma membrane preparations. It should be stressed that the biological assay gives only semi-quantitative information on the presence or absence of the P35 tum$^-$ antigen in a fraction and

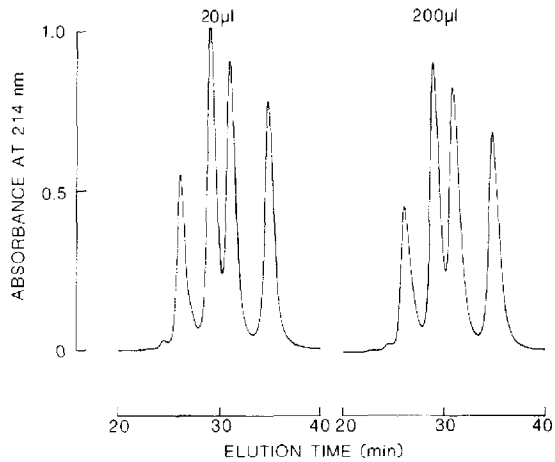

Fig. 4. Influence of sample volume on peak resolution. Samples of 200 μg of the same proteins as in Fig. 2 were dissolved in 20 and 200 μl of PBS DOC and injected at a flow-rate of 0.5 ml/min.

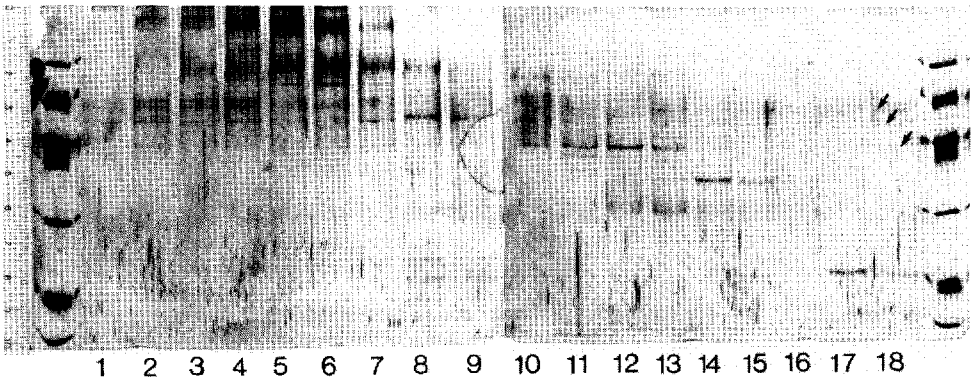

Fig. 5. Gel electrophoresis pattern of the fractionation of 200 μl of membrane glycoproteins containing the P815 tum$^-$ variant antigen. Numbers indicate the fractions collected at 1-min intervals (0.5 ml), starting 1 min before elution of the void volume (track 1). Arrows indicate the non-protein bands whose appearance on silver-stained SDS–polyacrylamide gels is related to the use of 2-mercaptoethanol or DTT in the sample preparation[14]. Molecular weight standards from top to bottom: 94, 68, 43, 30, 20, 14.4 kdaltons.

that, so far, it has not been possible to determine the yield of the HP-SEC fractionation step. Table I shows typical results obtained in one experiment: the antigenic activity of tum$^-$ P35 was eluted as a single peak ranging between 43 and 85 kdaltons.

Gel electrophoresis revealed that the corresponding fractions (Fig. 5, tracks 11, 12 and 13) were devoid of protein aggregates and of high-molecular-weight heterogeneous glycoproteins. They also contain a relatively small number of distinct

TABLE I

CYTOLYSIS BY IMMUNE SPLEEN CELLS STIMULATED *IN VITRO* WITH PLASMA MEMBRANE FRACTIONS FROM TUM$^-$ P35: PERCENT SPECIFIC ^{51}Cr RELEASE FROM P35 TARGET CELLS

Fractions	*Amount (μg)**	*Effector to target ratio*		
		30:1	*10:1*	*3:1*
Medium		8	4	2
Total plasma membrane	0.3	34	19	7
	1	86	57	26
	3	89	79	60
Lentil lectin eluate	0.3	11	9	4
	3	25	12	6
	30	83	59	29
HPLC fractions >85 kdaltons	0.3	10	6	3
	3	12	3	3
	30	21	6	3
HPLC fractions 85 43 kdaltons	0.3	16	6	6
	3	41	21	10
	30	88	70	48
HPLC fractions <43 kdaltons	0.3	14	4	2
	3	17	4	2
	30	17	4	3

* The amount of stimulator is expressed in μg per $5 \cdot 10^6$ responder DBA/2 anti-P35 lymphocytes.

proteins that could probably be resolved by HP-SEC on a system designed for fractionation in this molecular weight range, *e.g.*, an I-300 Waters or a TSK-G3000 column eluted with the PBS–DOC mobile phase.

CONCLUSION

The presence of the mild detergent sodium deoxycholate in the phosphate saline buffer considerably reduces the hydrophobic interactions of proteins and column supports, resulting in good fractionation conditions. Both soluble and membrane proteins are effectively separated on an I-250 and I-125 Waters column system using that eluent. The easy removal of the detergent by dialysis, or column desalting, renders the method very useful for the fractionation of labile proteins or in instances where the preservation of biological activity is of prime importance.

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