Journal of Chromatography, 444 (1988) 29–39
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1296

HIGH-PERFORMANCE CONCANAVALIN A AFFINITY CHROMATOGRAPHY OF LIVER AND HEPATOMA MEMBRANE PROTEINS

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SUMMARY

Although the separation of water-soluble glycoproteins by high-performance (HP) concanavalin A (ConA) affinity chromatography (AC) is feasible, irregularities may be encountered with hydrophobic glycoproteins. The separation of plasma membrane glycoproteins from liver and Morris hepatoma 7777, used as a model, showed that not only the interaction between the lectin and the oligosaccharide portion of the glycoproteins plays a role in the chromatographic process, but also the hydrophobic interactions between sample and lectin and between sample and support. In this, the characteristics of the support, such as surface hydrophobicity and pore size, play an important part.

It was found that a portion of the ConA is not covalently bound to the column, especially when elution is carried out with buffers containing detergents. Moreover, some extremely hydrophobic proteins could only be eluted from the column when high concentrations of detergents [1% (w/v) or higher] were applied. Despite these difficulties, four membrane glycoproteins from the liver with apparent molecular weights of 60, 80, 100 and 110–120 kilodaltons could be highly enriched by ConA HPAC. These proteins were further fractionated according to their strength of binding to the ConA and their different hydrophobic characteristics, using various detergents as eluents.

INTRODUCTION

Concanavalin A (ConA) affinity chromatography is a popular method for the isolation and analysis of glycoproteins and glycopeptides[1]. Ohlson *et al.*[2] have shown that ConA can be bound to activated silica gel and used for high-performance affinity chromatography (HPAC). Renauer *et al.*[3] have immobilized lectins other than ConA and showed that lectin HPAC has a wide range of possible applications. We have shown in a previous paper[4] that ConA HPAC can be used successfully to enrich ConA-binding liver plasma membrane proteins. However, a more thorough study of our results showed that the behaviour of single ConA-binding membrane proteins depends to a great extent on the character of the surface of the support, on its pore size and on the spacer that is covalently bound to the ConA. This paper

0021-9673/88/$03.50

describes changes in the behaviour of several proteins caused by variations in the charactveristics of the column support material. These differences in behaviour can be exploited for fractionating the membrane proteins according to their binding capacity to the ConA and their hydrophobic characteristics.

EXPERIMENTAL

Animals and chemicals

Male Wistar rats (Ivanovas, Kisslegg, F.R.G.) and buffalo rats (Zentrales Tierlaboratorium, Berlin, F.R.G.), weighing about 160–180 g, were fed a commercial diet containing 18–20% (w/w) protein (Altromin R; Altromin, Lage/Lippe, F.R.G.). The non-ionic detergent Genapol X-100 was purchased from Hoechst (Frankfurt a.M., F.R.G.). All the other chemicals, of analytical-reagent grade, were purchased from Merck (Darmstadt, F.R.G.), Serva (Heidelberg, F.R.G.) or Sigma (Munich, F.R.G.). L-[6-^{3}H]Fucose (4.2 Ci/mol) and L-[^{35}S]methionine (1020 Ci/mmol, 1 mCi/ml of 0.9% sodium chloride) were obtained from the Radiochemical Centre (Amersham, U.K.).

Labelling of proteins with L-[6-^{3}H]fucose and L-[^{35}S]methionine

For protein labelling the rats received an injection of 5 mCi of either L-[6-^{3}H]fucose or L-[^{35}S]methionine per kilogram body weight into the tail vein 2 h before removal of their liver and hepatoma.

Isolation of plasma membranes

Plasma membranes were isolated by zonal centrifugation using a Kontron centrifuge (Kontron, Munich, F.R.G.). Membrane purity was routinely checked by electron microscopy and by assays for marker enzymes, as described[5]. Protein was determined according to the procedure of Lowry *et al.*[6]

Extraction of membrane proteins

Prior to purification by high-performance liquid chromatography (HPLC), the membrane proteins were extracted in a stepwise manner[7], by alternate freezing and thawing, then alkaline extraction with 1 m*M* sodium hydroxide and, finally, extraction with the non-ionic detergents Nonident P-40 or Triton X-114. In order to obtain extremely hydrophobic proteins that are not Triton-soluble, the 1 m*M* sodium hydroxide extraction was performed after the Triton X-114 extraction[8].

HPLC

The HPLC system consisted of two pumps (Bischoff Analysentechnik, Leonberg, F.R.G.), a programmer (Shimadzu C-R 3A; Bischoff Analysentechnik), a spectrophotometer with a deuterium lamp (Bischoff), an RH 7125 loop injection valve (Bischoff Analysentechnik) and a Frac-100 fraction collector (Pharmacia, Freiburg, F.R.G.). The chromatographic conditions are given in the figure legends.

Protein recovery was determined by counting the radioactivity of the L-[6-^{3}H]fucose- or L-[^{35}S]methionine-labelled fractions (Kontron β-counter) and by determination of protein in the unlabelled fractions.

Columns

The following columns were used: Eupergit C1Z ConA, particle size 1 μm, non-porous; Eupergit C30N ConA, particle size 30 μm, pore size 50 nm (Eupergit with 20-nm pores was not a commercially available sample), all from Röhm Pharma (Weiterstadt, F.R.G.); TSK 5PW, particle size 2 μm, non-porous, and particle size 10 μm, pore size 100 nm (Toyo Soda, Yamaguchi, Japan); a column containing ConA, coupled to "bound-phase" *p*-nitrophenyl-activated silica, particle size 40 μm and size pore 27.5 nm (Baker Chemicals, Gross-Gerau, F.R.G.). ConA–Sepharose was purchased from Pharmacia. The ligand ConA was bound to the support by a previously described method[4]. The amount of bound ConA was 10–12 mg per gram of support in all instances, except for the ConA–Sepharose column. The Eupergit columns were packed by Bischoff Analysentechnik and the TSK and Baker columns were packed in our laboratory. The dimensions of the columns were 60 × 8.0 mm I.D., unless stated otherwise in the figure legends.

Buffers

The buffers used for ConA HPAC were as follows. Buffer A was 10 m*M* Tris–HCl containing 150 m*M* sodium chloride and 1 m*M* each of Ca^{2+} and Mg^{2+}. An amount of 0.1% (w/v) of a detergent was added to buffer A according to sample solubility, either Genapol X-100 (Gen), octylglucose (OG) or CHAPS (CHA). The elution buffer was buffer A, with or without detergent, to which 0.2 *M* methyl α-D-mannopyranoside (Man) was added.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Dialysed and freeze-dried samples were dissolved in 62.5 m*M* Tris–HCl buffer (pH 6.8), containing 3% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. SDS-PAGE was performed by the Laemmli method[9]. An amount of protein between 100 and 150 μg was applied to each track.

RESULTS AND DISCUSSION

All the investigated columns gave similar results in routine tests carried out with a mixture of human transferrin and bovine serum albumin, as described previously[10]. However, when the extract from liver plasma membranes was chromatographed, the column performance was different. As has been reported earlier[4], all columns bind the glycoproteins with apparent molecular weights of 60, 80 and 110–120 kilodaltons (Fig. 1), but the relative amount of protein bound varies considerably from protein to protein. The TSK 5PW ConA column (porous matrix) and, similarly, the ConA–agarose column bind predominantly glycoproteins in the range 110–120 kilodaltons, whereas the portion of bound glycoproteins with apparent molecular weights of 80 and 60 kilodaltons is much larger with the two Eupergit columns with 50- and 20-nm pores. Close scrutiny of the bands in the 110–120 kilodalton range of apparent molecular weights reveals additional bands with apparent molecular weights of about 100 kilodaltons using the TSK column and the Eupergit column with 50-nm pores. For the other two columns this band is much less obvious. It can also be seen that all columns lose a certain amount of ConA when 0.2 *M* methyl α-D-mannopyranoside and 0.1% Genapol is used for elution (*cf.*, bands around 26–30

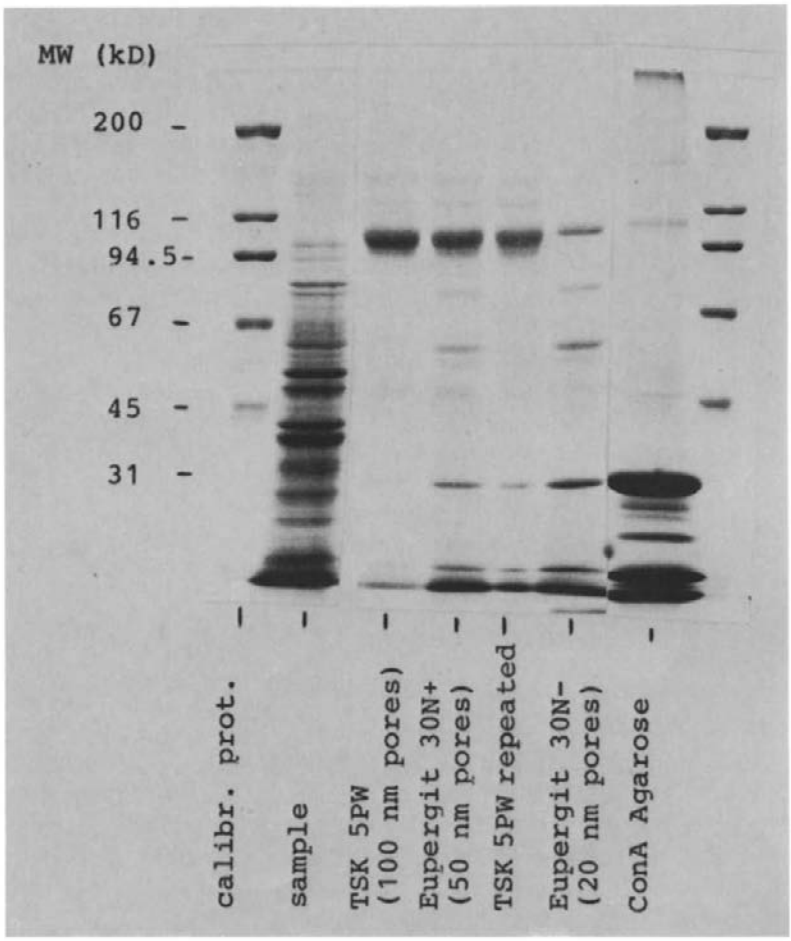

Fig. 1. Behaviour of different ConA HPAC columns in the separation of glycoproteins from liver plasma membranes. A 10-ml volume of Triton X-114 extract from liver plasma membranes, containing 30 ml of protein[4,8], was applied to each column. The HPAC columns were then washed with buffer A, containing 0.1% (w/v) Genapol X-100, for 20 min at a flow-rate of 1 ml/min. The ConA-binding proteins were subsequently eluted with the elution buffer, containing 0.1% Genapol X-100. A 150-μl aliquot (40–80 μg of protein) was taken from each eluate for subsequent SDS-PAGE. HPAC conditions: flow-rate, 0.5 ml/min during sample application, 1 ml/min during washing and elution; pressure, 1–5 bar. Chromatography was carried out at 4°C. The result of a subsequent experiment with the same sample on the ConA TSK 5PW column is shown as "TSK 5PW repeated". Chromatography on the ConA-agarose column: the same amount of Triton X-114 extract from liver plasma membranes was applied to the ConA-agarose (ConA-Sepharose) column (100 mm gel bed, 100 mm I.D.). The extract was recycled through the column four times. The column was then washed with 60 ml of buffer A, containing 0.1% Genapol X-100, and eluted with 30 ml of elution buffer containing 0.1% Genapol X-100. The flow-rate was *ca.* 4 ml/h throughout; temperature, 4°C. A 3-ml aliquot from the eluate was dialysed against doubly-distilled water, freeze-dried and subjected to SDS-PAGE. Only the results of SDS-PAGE are shown. kD = kilodaltons.

kilodaltons). The amount of ConA eluted is very high with the agarose column (see Fig. 1).

It is well known that ConA molecules form dimers, tetramers and polymers

in solution. In this process, hydrophobic interaction occurs between monomers[1]. When such a solution is used for immobilizing the protein on the support, ConA may be bound in this form, *i.e.*, as monomer, dimer or polymer. As a result, it is possible that only one monomer is covalently bound, whereas the other molecules are associated only by hydrophobic interaction. It is also possible that two or more monomers are covalently bound simultaneously, interacting with one another. The loss of ConA during elution, especially under hydrophobic conditions, suggests the first alternative.

To verify this assumption, an experiment was carried out, which is shown in Fig. 2. First 2 mg and then 5 mg of ConA in solution were injected into the columns packed with Eupergit C1Z ConA, Eupergit C30N ConA (both 20- and 50-nm pore size), TSK 5PW ConA (porous) and Baker *p*-nitrophenyl ConA. After washing the column with buffer A, it was washed with buffer A containing 0.1% Genapol X-100, then with buffer A containing 1.0% Genapol X-100. The behaviour of the different columns differed in this procedure. The non-porous Eupergit C1Z ConA column bound only a small amount of ConA, whereas the two porous Eupergit C30N ConA columns bound larger amounts. The TSK 5PW and Baker columns were capable of binding more than 2 mg of free ConA. The amount of ConA that could be washed out with Genapol increased in the same order as the amounts of ConA bound by each column.

The results indicate that the separation is influenced not only by interaction between the lectin and the glycoprotein, but also by interaction between the support and the glycoprotein. The factors that can play a part in this interaction are the hydrophobicity of the support surface, the pore size and the presence and length of the spacer.

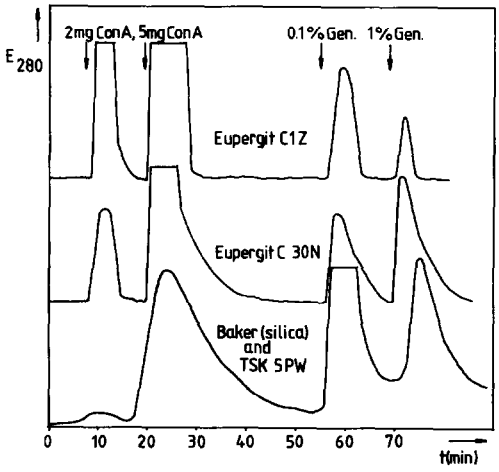

Fig. 2. Binding of free ConA to different ConA HPAC columns. Each column was first saturated by injection of 10 ml of 50 m*M* calcium chloride solution. First 2 mg and then 5 mg of ConA in 10 ml of buffer A were then injected. The column was washed with buffer A for 20 min and subsequently with 0.1% Genapol X-100 in buffer A. Conditions: Flow-rate, 1 ml/min; room temperature; pressure, 4–8 bar for columns with porous materials and 90 bar for the Eupergit C1Z column. For other conditions see Fig. 1 and Experimental.

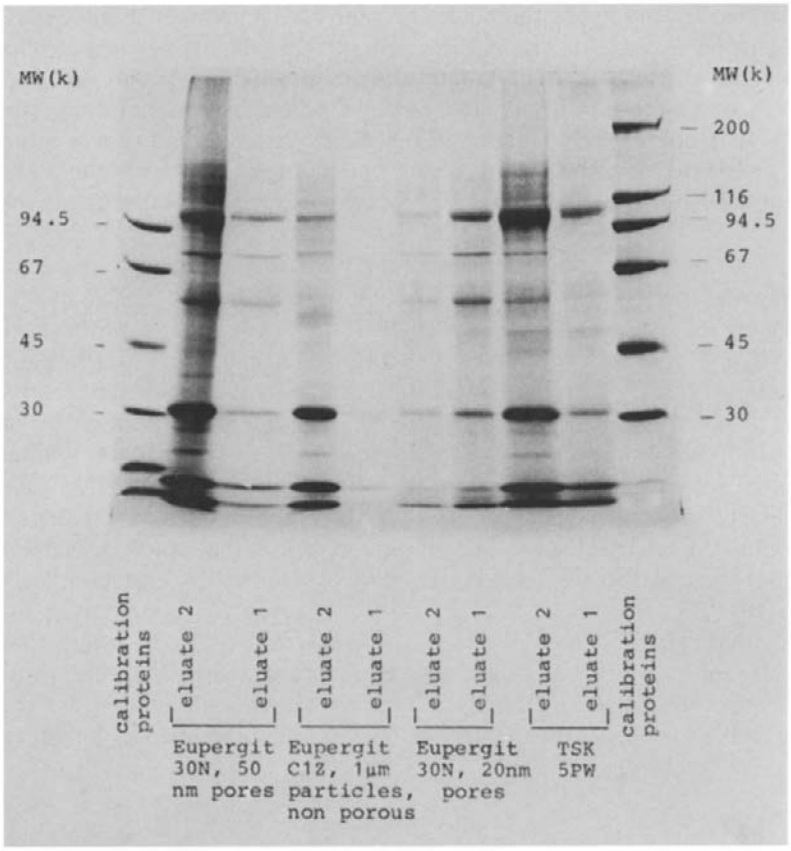

Fig. 3. Fractionation of glycoproteins from liver plasma membranes on different ConA HPAC columns according to their water solubility. Triton X-114 extracts from liver plasma membranes, each containing 30 mg of protein, were applied to single columns. The columns were then washed with 20 ml of buffer A, containing 0.1% Genapol X-100 (flow-rate, 1 ml/min), and subsequently washed with 60 ml of buffer A without Genapol X-100 in order to remove the detergent. Elution was carried out in two steps: (1) with elution buffer without detergent (eluate 1); (2) with elution buffer containing 0.1% Genapol X-100 (eluate 2). For other conditions see Fig. 1. Only the SDS-PAGE results are shown; k = kilodaltons.

Fig. 3 gives an idea of how a certain separation between less and more hydrophobic proteins can be achieved in relation to ConA affinity and to hydrophobic interaction of the proteins with the lectin and the column matrix. After applying the membrane extract, the columns were washed with buffer A without detergent and then eluted with 0.2 *M* methyl α-D-mannopyranoside (eluate 1 in Fig. 3). The hydrophobic proteins were subsequently eluted with 0.2 *M* methyl α-D-mannopyranoside solution containing 0.1% Genapol X-100. The different behaviour of particular columns can be seen clearly. The two extremes are the TSK 5PW ConA column (porous) and the Eupergit C1Z ConA column (non-porous). From the first column only the glycoprotein with an apparent molecular weight of 110–120 kilodaltons could be eluted with mannopyranoside without detergent, whereas from the second column

the bound proteins could only be eluted when detergent was added to the mannopyranoside. Obviously, this material is hydrophobic to such an extent that water-soluble glycoproteins can bind only weakly to the ConA, being repelled by the matrix. The control experiment shows that the hydrophilic glycoproteins which are weakly bound to this support can be washed out by buffer A alone, without added methyl α-D-mannopyranoside (not shown here).

Fig. 4 shows the fractionation of liver plasma membrane proteins by a combination of different detergents. The weakly bound proteins were washed out by buffer A, the water-soluble proteins were eluted by 0.2 *M* methyl α-D-mannopyranoside, and the hydrophobic proteins were eluted by a combination of methyl α-D-mannopyranoside and various detergents with differently hydrophobic residues (octylglucose, CHAPS and Genapol X-100). The glycoproteins (GP) with apparent molecular weights of 60, 80 and *ca.* 110–120 kilodaltons (*cf.*, Figs. 1 and 3) are clearly discernible here also. The GP 80 is mainly eluted with methyl α-D-mannopyranoside alone, the GP 60 with methyl α-D-mannopyranoside and 0.1% Genapol X-100 (eluate 2 in Fig. 3), while the glycoproteins with apparent molecular weights between 110 and 120 kilodaltons appear in all the eluates. This is hardly surprising, as they are several glycoproteins having roughly the same apparent molecular weight[4]. Therefore, the differences between these proteins cannot be discerned by SDS-PAGE, but become evident only after 2D electrophoresis[8]. The glycoproteins with an apparent molecular weight of 100 kilodaltons (*cf.*, Fig. 1) are eluted with methyl α-D-mannopyranoside without detergent, but they also appear in the fractions obtained with 0.1% CHAPS and 0.1% Genapol X-100.

In order to show electrophoretically the differences demonstrated in Fig. 4, a much larger amount of sample was applied compared with the experiments shown in Figs. 1 and 3 (30 mg of protein in Fig. 3 and 100 mg of protein in Fig. 4). The results shown in Fig. 4 demonstrate that a large amount of proteins other than the 110–120, 80 and 60 kilodalton proteins are retarded, especially those with low apparent molecular weights. An indication of this can also be found in Fig. 3 (see the line

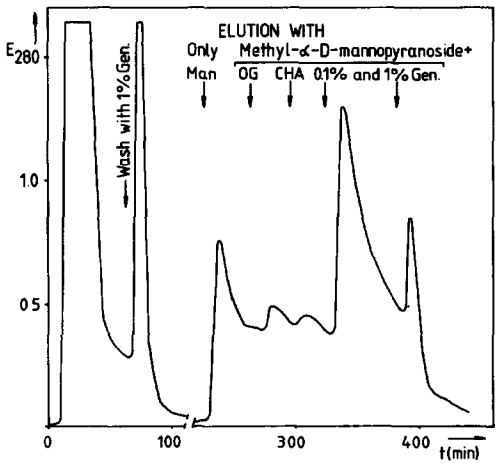

Fig. 4.

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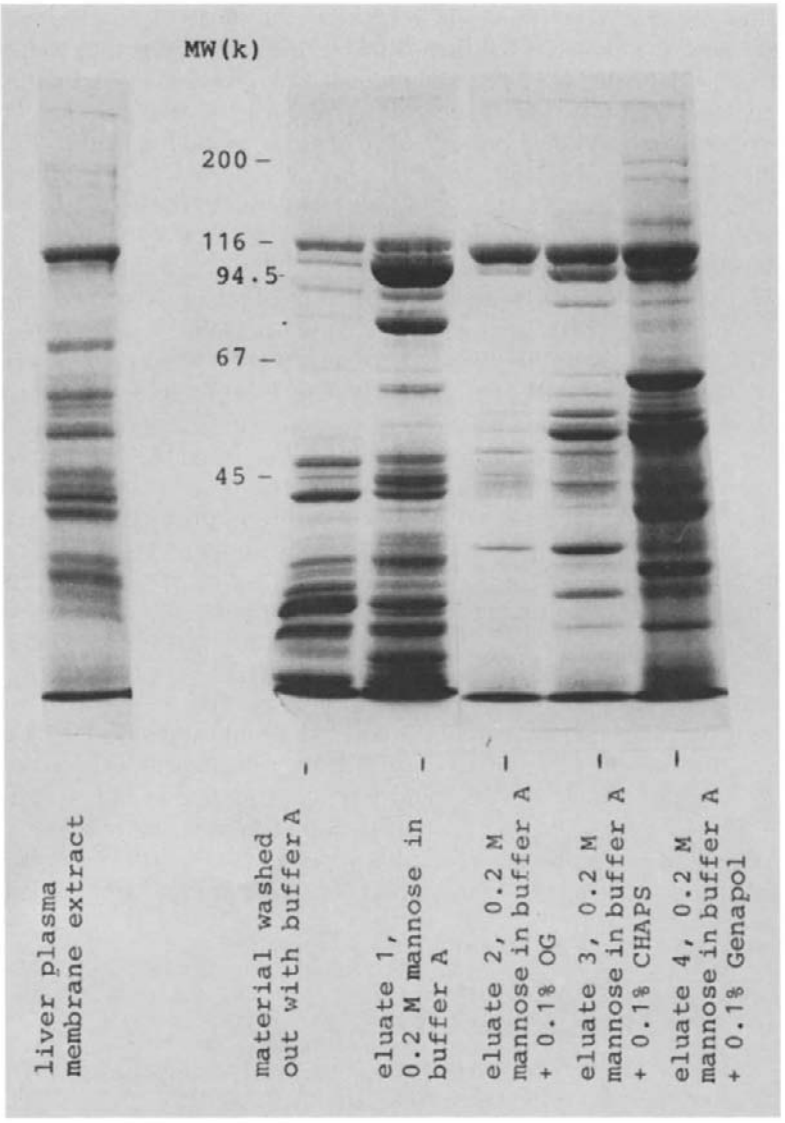

Fig. 4. Fractionation of glycoproteins from liver plasma membranes according to their binding to ConA and their hydrophobicity. A 25-ml volume of Triton X-114 extract from liver plasma membranes, containing 100 mg of protein, was applied to a Eupergit C30N ConA column with 50-nm pores. The column was first washed with a 10-ml injection of 1% Genapol X-100 in buffer A, then with about 50 ml of buffer A without detergent. The bound glycoproteins were eluted stepwise in the following order: (1) with elution buffer without detergent, eluate 1; (2) with elution buffer and 0.1% octylglucose, eluate 2; (3) with elution buffer and 0.1% CHAPS, eluate 3; (4) with elution buffer and 0.1% Genapol X-100, eluate 4; (5) with elution buffer and 1% Genapol X-100, eluate 5. The amount of eluate corresponding to 400 μg of protein was dialysed against water, freeze-dried and analysed by SDS-PAGE. The remaining material, washed out of the column with buffer A, was also collected, dialysed and lyophylized. About 250 μg of protein were taken for SDS-PAGE (second line on the left). Both the chromatogram (p. 35) and the SDS-PAGE results (this page) are shown; k = kilodaltons.

"Eupergit 30N, 50-nm pores, eluate 2"). The proteins shown in this line have been fractionated into three lines in Fig. 4, as three different detergents were used.

Fig. 5 is intended to show why the two main difficulties with ConA chromatography, *viz.*, the loss of ConA during elution and the incomplete recovery of highly hydrophobic proteins, have been given very little attention. As Fig. 5a shows, water-soluble proteins can easily be eluted with 0.2 *M* methyl α-D-mannopyranoside from a ConA column, in this instance TSK 5PW (porous) or Eupergit C30N (50-nm pores) (see also ref. 8). However, if extremely hydrophobic proteins are applied to the column, as in Fig. 5b, they can only be fully eluted, and as a very broad peak, if 1.0% Genapol X-100 is added to the 0.2 *M* methyl α-D-mannopyranoside. The loss of ConA from the column is negligible with water-soluble proteins, but extremely high when elution is carried out in the presence of 1.0% Genapol X-100. Experiments with these extremely hydrophobic proteins have so far not been undertaken very often.

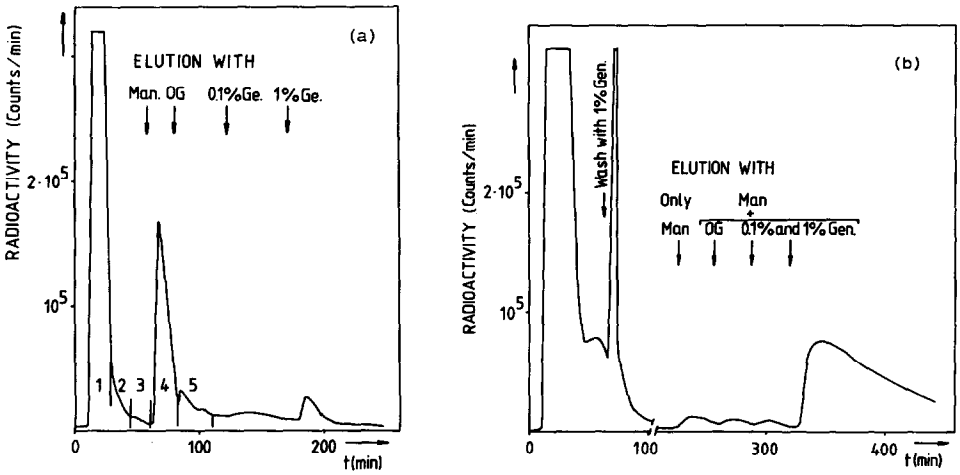

Fig. 5. (a) Separation of water-soluble membrane proteins by ConA HPAC. A 10-ml volume of freeze–thawing extract from [^{3}H]fucose-labelled hepatoma 7777 plasma membranes was applied to the Eupergit C30N ConA column (50-nm pores) and eluted in the same order as in Fig. 4, except for the step with 0.1% CHAPS; $3.3 \cdot 10^6$ counts/min were applied and recovered in (1) $2.11 \cdot 10^6$ counts/min (64%) (not bound); (2) $0.21 \cdot 10^6$ counts/min, washed out with buffer A (6.4%) (not bound); (3) $0.13 \cdot 10^6$ counts/min (4%); (4) $0.4 \cdot 10^6$ counts/min (12.1%), eluted with elution buffer without detergent; (5) $0.15 \cdot 10^6$ counts/min (4.5%) eluted with elution buffer, containing 0.1% octylglucose. Finally, the remaining $0.2 \cdot 10^6$ counts could be eluted with 1% Genapol X-100 in elution buffer. Total recovery, $3.2 \cdot 10^6$ counts/min or 97%. (b) Separation of extremely hydrophobic membrane proteins by ConA HPAC. A 10-ml volume of extract from [^{3}H]fucose-labelled hepatoma 7777 plasma membranes were applied to the Eupergit C30N ConA column (50-nm pores). The proteins were obtained by extraction with 1 m*M* sodium hyudroxide solution and 0.5% Triton X-114, after membrane extraction with 1% Triton X-114[8]; $6.75 \cdot 10^6$ counts/min were applied and recovered in (1) $3.98 \cdot 10^6$ counts/min (59%) (not bound); (2) $0.48 \cdot 10^6$ (7.1%) washed out with buffer A and 0.1% Genapol X-100; (3) $0.46 \cdot 10^6$ counts/min (6.8%) washed out with buffer A and 1% Genapol X-100; (4, 5, 6) $0.13 \cdot 10^6$ counts/min (*ca.* 2%), subsequently eluted with elution buffer without detergent, with elution buffer and 0.1% octylglucose, and with elution buffer and 0.1% Genapol X-100; (7) $1.16 \cdot 10^6$ counts/min (17.2%) were obtained with elution buffer and 1% Genapol X-100. Total recovery, $6.2 \cdot 10^6$ counts/min or 92%. Chromatographic conditions as in Fig. 4. A 1-ml volume of each fraction was collected; a 25-μl aliquot was taken and its radioactivity was measured.

After treatment under these fairly vigorous conditions, *i.e.*, with 1% Genapol X-100 in the eluent, the binding capacity of the column was reduced. In the experiment repeated with water-soluble proteins, the column bound only about 15% of the amount shown in Fig. 5a. The proteins that were bound to the column could be eluted to a great extent only with the elution buffer to which 0.1% Genapol X-100 was added. Therefore, the binding mechanism appears not to be the desired interaction between the lectin and the glycoprotein, but hydrophobic interaction with the matrix. This in turn indicates that the residual covalently bound ConA in the column forms only monomers. These have hardly any binding affinity to the glycoproteins[1]. After regeneration with 10 ml of 20 m*M* calcium chloride solution and injection of 5 mg of ConA, dissolved in buffer A, the original binding capacity could be restored. The Eupergit C30N column with 20-nm pores and the TSK 5PW column with 100-nm pores showed the same characteristics as the Eupergit C30N column with 50-nm pores.

In our laboratory, ConA HPAC has become a routine method for the fractionation of membrane glycoproteins from liver and Morris hepatomas. The application of Eupergit C30N ConA columns has proved a rapid and economical method of enriching large amounts of ConA-binding proteins from different membrane extracts. In the eluates from the ConA HPAC columns the same proteins appear that can also be enriched by chromatography on ConA-Sepharose[11]. However, the amounts of single proteins differed widely and varied with the different columns. When the TSK 5PW column with 10-nm pores is used, the amount of glycoproteins is highest in the range 110–120 kilodaltons, similarly to the case of ConA–Sepharose. With the Eupergit columns (C1Z and C30N), the amount of protein with an apparent molecular weight of 80 kilodaltons was much higher than that of the above-mentioned glycoproteins of 110–120 kilodaltons. This indicates that in ConA HPAC the characteristics of the support material play a significant part in column selectivity.

ConA can often be found in solution as the dimer, tetramer and polymer. Every molecule binds calcium and magnesium, which are necessary for sugar binding[1,12]. The ConA molecule also has hydrophobic areas on the surface, and hydrophobic interaction is an important factor in binding to the lectin, especially in the case of membrane glycoproteins[13]. We were able to verify these observations in our experiments on HPAC columns with immobilized ConA. It could be demonstrated that hydrophobic interactions are particularly important for membrane glycoprotein separation, not only as it occurs between ConA and the glycoprotein, but also between ConA and the support or the support and the glycoprotein.

By specific application of detergents during elution, this interaction can be exploited for further fractionation of glycoproteins according to their hydrophobic characteristics. In this paper only basic results are shown. The different selectivity of the columns, as shown here, indicates that separation can be influenced by changes in the support material, such as surface characteristics and pore size.

The loss of ConA during elution, especially with methyl α-D-mannopyranoside, presents a serious problem in both classical ConA chromatography and in ConA HPAC. It was shown in the experiments with extremely hydrophobic membrane proteins that the column performance can be seriously impaired by the loss of ConA. The option of regenerating the column by adding calcium and soluble ConA and thereby restoring its former capacity, as is also recommended by some column pro-

ducers, cannot be regarded as an ideal answer to the problem. A possible improvement would be to cross-link the associated ConA, as described by Lloyd[14].

The results of these investigations show that the commercially available supports for affinity chromatography have yet to be optimized with regard to surface chemistry and applicability to single ligands. Despite the difficulties, ConA has provided the enrichment of single membrane proteins, which could subsequently be isolated, *e.g.*, by preparative gel electrophoresis or other HPLC methods. It was the application of HPAC that revealed the above-mentioned problems, giving us the opportunity to find more complete answers in the future.

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