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HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF LIVER PLASMA MEMBRANE PROTEINS

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SUMMARY

Plasma membrane proteins from liver were analysed by concanavalin A affinity and immunoaffinity high-performance liquid chromatography. In the method, four peptide bands with apparent molecular weights of 140 000, 120 000, 80 000 and 60 000 could be isolated. In the second method, with two immobilized monoclonal antibodies, two corresponding antigens —the membrane proteins dipeptidyl-peptidase IV and GP 110— could be highly purified from plasma membrane extract with good yield in only one step.

INTRODUCTION

The use of high-performance liquid affinity chromatography (HPLAC) has risen slowly though steadily after publication of the pioneering work by Ohlson *et al.*[1]. The first commercially available supports appeared about 3 years ago[2]; more are being developed or scheduled to be marketed.

This trend has been given a boost by the increasing use of monoclonal and polyclonal antibodies, HPLAC being a promising method in this field. It can be applied to the purification of antibodies by chromatography with immobilized Protein A, and to the isolation of single antigens by means of immobilized purified antibodies (immunoaffinity chromatography)[3].

This paper shows the application of concanavalin A (Con A)- and immunoaffinity-HPLC for membrane protein separation.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies were produced essentially according to the method of Köhler and Milstein[4]. Modifications for the preparation of monoclonal antibodies against rat liver membrane proteins have been described[5,6]. The activity of the antibodies was determined as described previously[6].

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Membrane proteins

Membrane protein samples were obtained from plasma membranes of rat liver. The membranes were isolated and selectively extracted as described[7,8].

Columns

The supports used were as follows: Eupergit 1Z, particle size 1 μm, without pores; Eupergit 30N, particle size 30 μm and pore size about 50 nm. Both are based on a polymer and have an epoxy radical as active group (Röhm Pharma GmbH, Weiterstadt, F.R.G.). Activated silica gel was prepared as described[9]; particle size 10 μm, pore size 50 nm (a gift from Dr. Unger, Mainz, F.R.G.); cyanogen bromide-activated TSK 5PW gel, particle size 10 μm and pore size 100 nm (a gift from Dr. Kato, Toyo Soda, Yamaguchi, Japan).

The ligands, Protein A, monoclonal antibodies and concanavalin A (Con A), were coupled according to the following procedure. Activated resin was first washed twice with "binding buffer", containing 0.1 *M* sodium carbonate (pH 8.0), and 0.5 *M* sodium chloride. Silica gel was an exception, with a binding of buffer of pH 7.0 (see ref. 9). The ligand dissolved in binding buffer was added (0.5 mg of protein/ml) after centrifugation at 1000 *g* for 20 min (centrifuge with refrigeration device; Kontron Analytik, München, F.R.G.). Between 2 and 10 mg of protein were added to 1 g of resin. The suspension was shaken overnight at +4°C and subsequently centrifuged at 1000 *g* for 20 min. The protein in the supernatant was determined according to the method of Lowry *et al.*[10]. The quantity of ligand bound was calculated from the amount of protein before and after coupling. The resin with the immobilized protein was then washed twice with binding buffer. In order to block the remaining active groups of the support, 10 ml of 0.2 *M* ethanolamine hydrochloride (pH 7.2) were added per 1 g of resin. The suspension was then shaken at room temperature for 2 h, centrifuged at 1000 *g* for 20 min and washed three times with the binding buffer as described above. The gel with the immobilized ligand was stored at +4°C in phosphate-buffered saline, pH 7.0, containing 0.1% (w/v) of sodium azide, until required for column packing.

The columns were packed in our laboratory, except for the Eupergit 1Z column which was packed by Bischoff Analysentechnik (Leonberg, F.R.G.).

An alternative to the method described above is to bind the ligands to a column that has already been packed with activated support. For this purpose a Microanalyzer Succinylimide column was used, which is packed with succinylimide activated polymer (see ref. 11); 40 mm × 4.6 mm, particle size 30 μm (Bio-Rad, Richmond, CA, U.S.A.).

The binding of the ligand, here a monoclonal antibody, was carried out according to the following procedure. A 10-ml solution of purified monoclonal antibodies (0.2 mg of protein per ml 0.1 *M* sodium carbonate buffer, pH 8.0) was recirculated within 2 h (flow-rate 0.2 ml/min). The column was washed with 50 ml of 0.1 *M* sodium carbonate buffer, pH 8.0 (flow-rate 1.0 ml/min). The flow-rate was then reduced to 0.4 ml/min and 2 ml of 0.2 *M* ethanolamine hydrochloride, pH 7.2, were injected twice. The column was subsequently washed with 20 ml of carbonate buffer and 20 ml of phosphate-buffered saline, at a flow-rate of 1.0 ml/min. The quantities of antibodies bound were determined by the difference in protein contents before and after coupling[10].

The packed column, Microanalyzer Protein A, 40 mm × 4.6 mm, particle size 30 μm, was obtained from Bio-Rad. The TSK Con A 5PW column, 75 mm × 7.5 mm, particle size 10 μm, was a gift from Dr. Kato (Toyo Soda Co.).

RESULTS AND DISCUSSION

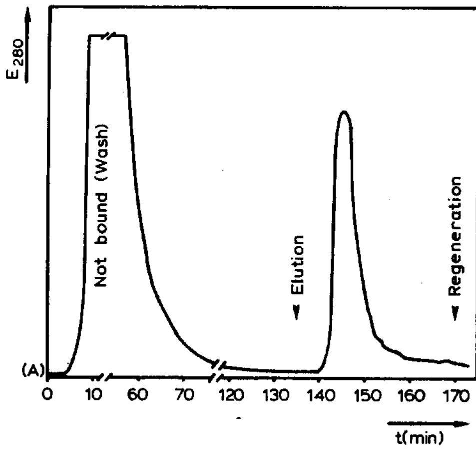
Fig. 1 shows the Con A affinity chromatography of a detergent (Triton X-114) extract from plasma membranes of liver. Four polypeptides with apparent molecular weights of 140000, 120000, 80000 and 60000 could be detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after elution with 0.1 *M* α-methylmannopyranoside. Similar results were obtained with the three columns packed with Con A coupled to different supports. When an Eupergit Con A (1Z) column was used, the same polypeptide bands were detected, but the column capacity was between 4 and 5 times lower (results not shown). Tauber *et al.*[13] have isolated the same polypeptide bands by "classic" affinity chromatography with Con A-Sepharose. In our experiments, however, the protein recovery was much higher. From an extract with 60 mg of protein, 4–5 mg of protein could be obtained by Con A-HPLAC after elution with α-methylmannopyranoside, compared to only 1–2 mg of protein obtained through Con A-Sepharose chromatography. The poorer yield from the "classic" method with Con A-Sepharose results among other factors from the much larger volumes of the eluate. Further treatment of the sample (dialysis, freeze-drying, etc., see ref. 13) leads to inevitable loss of large amounts of these very hydrophobic proteins. This phenomenon can be verified by experiments with ^{125}I-labelled samples[14].

The much longer period of time needed for separation on the soft gel, *e.g.* Sepharose, also contributes to protein loss. Some of the proteins from the Con A eluate are very sensitive to protease[15]. Also the life of a Con A-HPLAC column is considerably longer. A Con A-Sepharose column can be used only four or five times for membrane extract separation before increasingly losing its binding capacity and deteriorating mechanically. However, more than 40 separations have so far been carried out with the Eupergit 30N-Con A column without change in separation performance.

The protein bands isolated by Con A chromatography are partly identified. In the molecular weight range 110000–120000, several proteins are found that can be separated by two-dimensional electrophoresis[16]. We have raised monoclonal antibodies against two of these proteins, dipeptidyl-peptidase IV (DPP IV) and GP 110. The glycoprotein with an apparent molecular weight of 80000 (GP 80) appears in serum as well as in plasma membranes. The serum form however is glycolysed in a different way to that of the plasma membrane form[17]. The possibility of carrying out Con A affinity chromatography quickly and with high yield is likely to help with the isolation and characterization of other proteins in this group. Moreover we are going to isolate antigens in larger quantities directly from the membrane extract by means of monoclonal or polyclonal antibodies against the newly isolated proteins. Experiments with DPP IV and GP 110 are described below.

Immunoaffinity HPLC

In order to make the immunoaffinity column the immunoglobulin G (IgG)



(B)

◄ 94.5
◄ 66
◄ 45
◄ 30
◄ 21
◄ 12.4

Compl. Extr
Wash
Eupergit
TSK 5PW
Silicagel
Calibr. Prot.

first had to be isolated. For this purpose a column with immobilized Protein A can be used, as shown in Fig. 2. One of the monoclonal antibodies used has the identification number 9.2; it belongs to the IgG class 1 and binds to Protein A only in alkaline media. The buffer system MAPS II that was used here for antibody isolation can be used for IgGs of all sub-classes, including IgG 1[18]. In our investigations it gave good results. The isolated antibodies were pure and active. The recovery from 1 ml of ascites was 5–8 mg of IgG.

The purified antibodies were coupled to the activated supports. For antibody immobilization of monoclonal antibody 9.2 against GP 110, we used either activated silica gel (*cf.*, ref. 9) or epoxy-activated Eupergit[19]. For 1 g of support, 2 mg of IgG were used (see Materials and Methods). Binding was almost quantitative. The second monoclonal antibody with identification number 13.4 against DPP IV was used for *in situ* binding to the pre-packed column (see Materials and Methods). In this experiment too, virtually the whole amount of the 2 mg of antibodies used was bound to the column. Immunoaffinity chromatography was performed as shown in Fig. 2. The antigen was applied through pumping of the membrane extract into the column. Subsequently the column was washed with the binding buffer to which 10 m*M* EDTA was added (see Fig. 3).

The bound protein was then eluted in two steps with 0.2 *M* glycine hydrochloride buffer, pH 3.5 and 2.8. The results are shown in Fig. 3. When elution is carried out with the pH 3.5 buffer in the case of the 13.4 (anti DPP IV) column, several bands can be detected by subsequent SDS-PAGE. However, their appearance cannot always be reproduced. It can therefore be assumed that the binding is nonspecific. A part of the DPP IV appearing here (see upper part of Fig. 3) can be further purified by rechromatography. Pure DPP IV can be eluted with 0.2 *M* glycine buffer, pH 2.8.

Similar results were obtained with immobilized monoclonal antibodies against GP 110. The recovery of DPP IV was between 200 and 400 μg of protein per 60 mg of applied membrane extract. In the case of GP 110 the recovery was 200 μg for the same amount of membrane extract. A great advantage of this purification method is that large sample volumes can be applied to the column. The sample concentration takes place on the column. The relatively high flow-rates which are made possible by the supports used shorten the period of time necessary for sample application. The

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Fig. 1. (A) Con A-HPLAC of liver membrane proteins with an α-methylmannopyranoside step gradient. A 50-ml volume of a Triton X-114 extract from liver plasma membranes (about 60 mg of protein) was applied to an Eupergit Con A (30N) column, 80 mm × 8.0 mm. In parallel experiments, a Con A-silica column, 80 mm × 8.0 mm and Con A-TSK 5PW column, 75 mm × 7.5 mm, were used. The column was washed with 60 ml of buffer A. The bound proteins were then eluted with 0.1 *M* α-methylmannopyranoside in buffer A. After elution the column was regenerated by pumping 60 ml of buffer A, injecting 5 mg of Con A and pumping 30 ml of buffer A. Conditions: flow-rate, 0.5 ml/min during sample application and elution, 1.0 ml/min during column washing and regeneration; pressure, 5 and 9 bar respectively for TSK and Eupergit columns, 35 bar for silica column; temperature, +4°C. Buffers: A = 10 m*M* Tris–HCl, pH 7.8, containing 150 m*M* sodium chloride as well as 1 m*M* Mg^{2+}, 1 m*M* Ca^{2+}, 1 m*M* Mn^{2+}, 0.02% (w/v) sodium azide and 0.1% (w/v) detergent Genapol X-100; B (elution buffer) = 0.1 *M* α-methylmannopyranoside in A. (B) Electrophoretic monitoring of the chromatographic experiment. The main components of the α-methylmannopyranoside eluate, polypeptides with apparent molecular weights of 140000, 120000, 80000 and 60000, are marked with arrows.

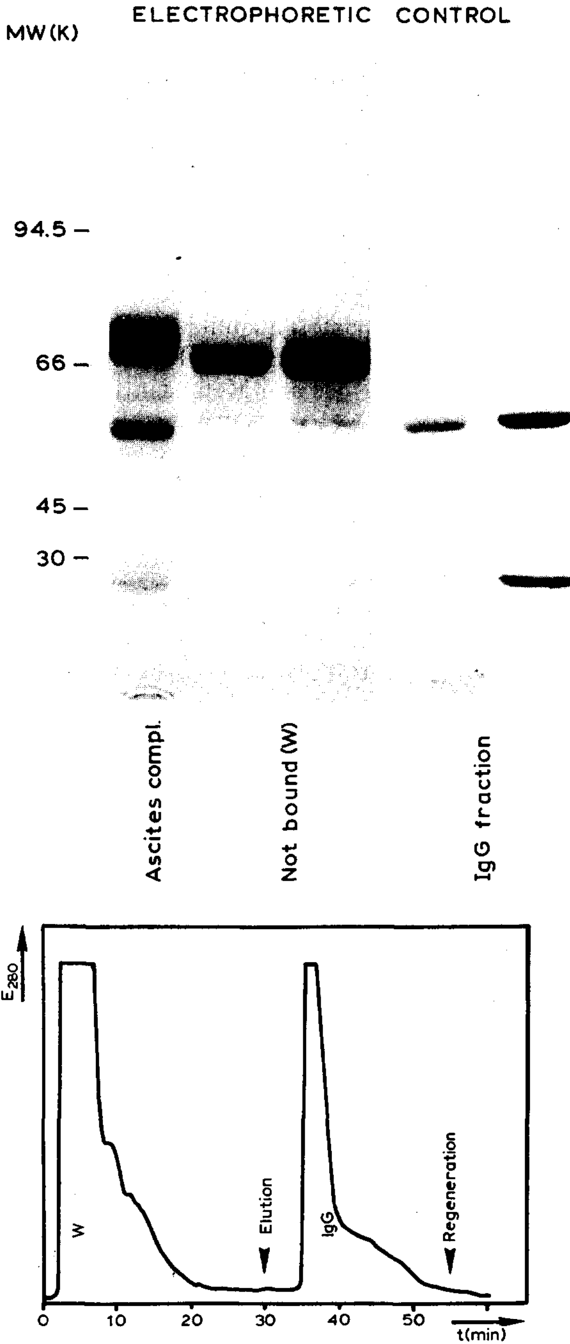

Fig. 2. Purification of monoclonal antibodies by Protein A-HPLAC. A 1-ml volume of ascites containing 7–10 mg of IgG was centrifuged at 100000 *g*. The supernatant was diluted in binding buffer to 5 ml (injection volume). By means of electrophoresis (upper part) the purification of IgG is demonstrated (light and heavy chains are marked with arrows). Chromatographic conditions: column, Eupergit Protein A (30N), 60 mm × 4.6 mm; buffer system, MAPS II (see Materials and Methods) consisting of binding, elution and regeneration buffers; flow-rate, 0.5 ml/min; room temperature; pressure, 5 bar.

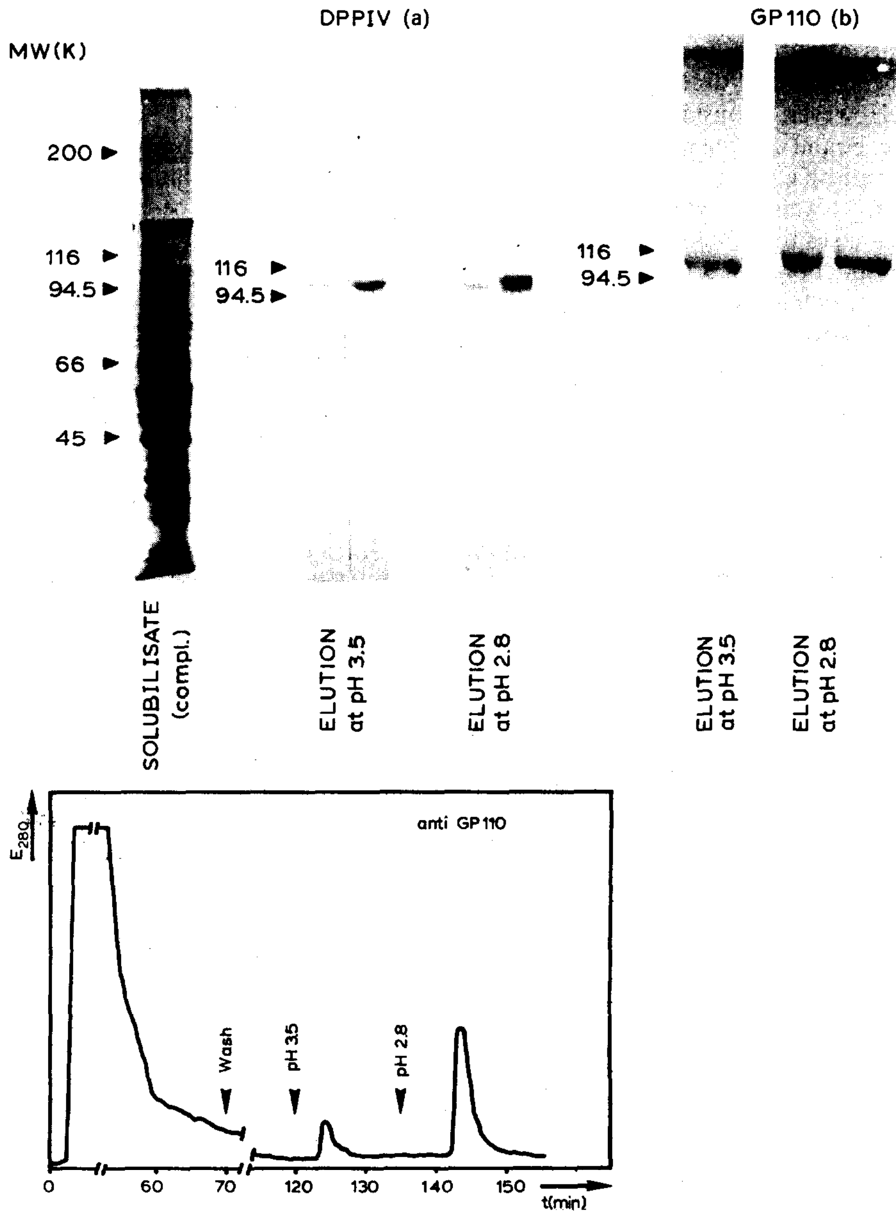

Fig. 3. Purification of membrane proteins by immunoaffinity HPLC. (a) Purification of dipeptidyl-peptidase IV (DPP IV). Purified monoclonal antibodies against DPP IV were bound to a succinylimide activated column (Microanalyzer, Bio-Rad; see Materials and Methods). Then 50 ml of membrane extract (about 60 ml of protein), obtained by detergent solubilization (detergent NP 40; ref. 8), were applied to the column. The column was washed with 50 ml of binding buffer containing 10 m*M* EDTA (see below), and part of the antigen and some other non-specifically bound proteins were first eluted with 0.2 *M* glycine buffer, pH 3.5, then with 0.2 *M* glycine buffer, pH 2.8 (pure DPP IV). About 400 μg of purified protein were obtained. The purification was checked electrophoretically (upper part) of Fig.). (b) Purification of GP 110. Purified monoclonal antibodies against GP 110 were bound to Eupergit 30N (see Materials and Methods). The immobilized antibodies were packed into a 60 mm × 4.6 mm column. The same amount of membrane extract as in (a) was applied. About 200 μg of purified protein were obtained after elution. The electrophoretic monitoring is shown in upper part. The chromatogram is shown for GP 110 isolation only. Conditions: flow-rate, 0.5 ml/min; pressure, 5–7 bar; temperature, 0°C (columns and buffer were kept cold with ice); binding buffer, 20 m*M* Tris–HCl, pH 7.8, with 0.5 *M* sodium chloride and 0.1% detergent Genapol X-100; washing buffer, binding buffer with 10 m*M* EDTA; elution buffer, first 0.2 *M* glycine hydrochloride, pH 3.5, secondly 0.2 *M* glycine hydrochloride, pH 2.8.

amounts of antigens recovered were sufficient for further investigations, above all sequencing, carbohydrate analysis and peptide mapping.

However, a considerable disadvantage of immunoaffinity HPLC lies in the fact that the bound antigen cannot be detached without denaturing the antibody as well. In order to elute the antigen from the column, 0.2 *M* glycine solutions with pH between 3.5 and 2.8, or highly concentrated salt solutions have to be used. This in turn damages the bound antibody after only a few experiments and the column life is correspondingly short.

The column with the bound antibody 13.4 (anti-DPP IV) could be used only for ten separations, before increasingly losing its binding capacity. The antibody with identification number 9.2 is somewhat more stable. We were able to use the column fifteen times before the binding capacity decreased. Despite these shortcomings, we were able to obtain 0.2–0.5 mg of protein per column run.

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