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PREPARATIVE ISOLATION OF GLYCOPROTEINS FROM PLASMA MEMBRANES OF DIFFERENT RAT ORGANS

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SUMMARY

By a combination of high-performance affinity chromatographic (HPAC) methods, several membrane proteins from liver, Morris hepatoma and kidney were isolated. The use of a tandem system, consisting of a concanavalin A (ConA) and a wheat germ agglutinin (WGA) high-performance liquid chromatographic (HPLC) column, as a first purification step allowed the isolation of proteins directly from organ homogenates. In a subsequent step, the membrane proteins can be isolated by simply using a combination of immunoaffinity HPLC and preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). However, with these methods most proteins lose their biological activity. If native proteins are required, a combination of different HPAC methods has to be applied. Several membrane proteins were isolated in milligram amounts under non-denaturing conditions using either HPAC columns or Mem Sep membranes with immobilized lectins, collagen, amino acids, crown ethers or heparin.

INTRODUCTION

The isolation of plasma membranes from animal organs and the separation of membrane glycoproteins by different chromatographic methods has become a matter of routine in many laboratories[1–3]. The introduction of high-performance liquid chromatography (HPLC) and high-performance affinity chromatography (HPAC) has made isolation methods quicker, simpler and more reproducible. The yield of isolated proteins has been increased and the biological activity could be retained more often as a consequence of shorter isolation times[4–6]. However, the problem of low yields persists in the preparative isolation of membrane glycoproteins. The greatest loss of material does not occur in the chromatographic processes, but during plasma membrane isolation. In liver plasma membrane isolation, for example, the yield does not exceed 10%, regardless of whether zonal centrifugation is used according to the method of Neville[7] or isolation with a sucrose gradient[1]. In order to avoid these losses, we have tried to isolate the membrane proteins directly from the organ homogenates through a combination of preparative HPAC, HPLC and gel electrophoretic methods.

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EXPERIMENTAL

Animals and chemicals

Male Wistar or Buffalo rats, weighing about 180–200 g, were fed on a commercial diet (Altromin R, Altromin, Lage/Lippe, F.R.G.). The non-ionic detergents Triton X-100 and Triton X-114 were purchased from Aldrich (Milwaukee, WI, U.S.A.). All 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.).

Production of plasma membranes and organ homogenates

Plasma membranes were isolated by zonal centrifugation using a Kontron (Munich, F.R.G.) centrifuge. Membrane purity was routinely checked by electron microscopy and by assays for marker enzymes, as described[1]. Plasma membranes were extracted selectively with detergents.[8] For the production of homogenates, the organs (liver, Morris hepatoma 7777 or kidney) were cut into small pieces after removal of connecting tissue. Each organ was then suspended in 100 ml of 5 m*M* Tris–HCl (pH 7.2) and 1 m*M* calcium chloride per 10 g of weight and homogenized (Ultra-Turrax homogenizer; Janke & Kunkel, Staufen, Breisgau, F.R.G.). The nuclei were removed by centrifugation at 1000 *g* for 10 min. The enriched membrane fraction was obtained through centrifugation at 40 000 *g* for 35 min (Kontron centrifuge). The supernatant after centrifugation was discarded. The pellet was suspended in 10 m*M* Tris–HCl (pH 7.4) containing 155 m*M* sodium chloride, 1 m*M* magnesium chloride, 1 m*M* calcium chloride and 1% (w/v) Triton X-100 and then homogenized in a Dounce homogenizer. It was subsequently solubilized for at least 2 h[2,8]. The protein content in the membrane fractions and in the homogenates was determined according to the method of Lowry *et al.*[9].

HPLC

The HPLC system consisted of two pumps, a programmer, a spectrophotometer with a deuterium lamp, a loop injection valve (all from Knauer, Berlin F.R.G.) and a Frac-100 fraction collector (Pharmacia–LKB, Freiburg, F.R.G.). The chromatographic conditions are given in the figure legends. Recovery was determined either by measuring the amount of protein or the activity of the membrane-bound enzyme dipeptidyl peptidase IV (DPP IV)[10].

Columns

The following column materials were used: Eupergit C 30N concanavalin A (ConA), particle size 30 μm, pore size 50 nm (Röhm Pharma, Weiterstadt, F.R.G.), Fractogel (Tosohaas, Yamaguchi, Japan) with wheat germ agglutinin (WGA), immobilized in our laboratory[11], Eupergit C 30N protein A (Röhm Pharma) and protein G Sepharose "Fast Flow" (Pharmacia–LKB). Ligands such as arginine, collagen and crown ethers were immobilized either on Eupergit C 30N or on Fractogel. Mem Sep 1.000, epoxy activated, "membrane" columns were obtained from Knauer. The ligands immobilized on this column were collagen, arginine, heparin and protein G. The procedures for ligand immobilization have been described elsewhere[4,11,12]. The dimensions of the HPLC columns were 250 × 20 mm I.D. unless stated otherwise in the figure legends.

Buffers

The buffers used for ConA and WGA HPAC were as follows. Buffer A was 10 m*M* Tris–HCl (pH 7.8), containing 155 m*M* sodium chloride and 1 m*M* each of Ca^{2+} and Mg^{2+}. An amount of 0.1% (w/v) of Triton X-100, reduced, was added to buffer A. For column rinsing the amount of Triton X-100, reduced, was 1%. The elution buffer for the ConA column was buffer A with 0.1% or 1% Triton X-100, reduced, to which 0.2 *M* methyl-α-D-mannopyranoside was added. The elution buffer for the WGA column was also buffer A with 0.1% or 1% Triton X-100, reduced with the addition of 0.2 *M* N-acetylglucosamine.

Buffer A for collagen and arginine HPAC was 5 m*M* Tris–HCl (pH 8.0) with 0.1% Triton X-100, reduced. Buffer B was buffer A containing 500 m*M* sodium chloride.

Buffer A for crown ether HPAC was 5 m*M* Tris–HCl (pH 7.5) containing 0.1% Triton X-100, reduced, and 10 m*M* potassium chloride. Buffer B was buffer A to which 500 m*M* sodium chloride was added.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of 10–25 μl were mixed with 3–7 μl of 300 m*M* Tris–HCl buffer (pH 6.8) containing 15% (w/v) SDS, 25% (v/v) mercaptoethanol, 30% (v/v) glycerol and 0.005% (w/v) bromophenol blue. SDS-PAGE was performed according to the method of Laemmli[13], using the Bio-Rad mini system (Bio-Rad Labs., Munich, F.R.G.). An amount of protein between 5 and 20 μg was applied to each track.

Preparative SDS-PAGE was carried out in a disk gel, 60 mm long and 12 mm diameter, with a system from Bethesda Research Labs. (Bethesda, MD, U.S.A.).

RESULTS AND DISCUSSION

ConA and WGA HPAC

Although a purification step consisting of centrifugation at 40 000 *g* (see Experimental) is carried out in the processing of the material in order to remove the soluble components from the cytosol, the homogenates are still cloudy and contain large amounts of serum albumin. This happens especially in liver processing. In the first step, lectin HPAC, the glycoproteins and eventually the glycolipids are bound. No other components bind to the column. If ConA HPAC is used as a first purification step, only between 60 and 70% of the membrane-bound enzyme DPP IV binds to the column. The remaining 30–40% runs through the column, even if applied several times. In these experiments, DPP IV is used as a marker enzyme in order to control the complete binding of membrane proteins. If the sample, which does not bind to the ConA column, is subsequently applied to a WGA column, between 20 and 30% of the DPP IV does bind, and the yield after the runs on two lectin columns will come to a total of 90%. We have also successfully operated the ConA and WGA columns in tandem. About 80–90% of the DPP IV activity was bound and could be eluted. Fig. 1 shows the SDS-PAGE of glycoproteins from liver and kidney homogenates bound to the ConA and WGA columns. They were eluted with α-methyl mannopyranoside and N-acetylglucosamine, respectively.

When organ homogenates are applied according to the method described here, some points require special attention. The extracted proteins have a tendency towards

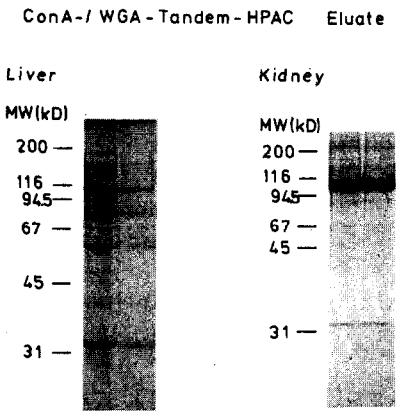

Fig. 1. SDS-PAGE of glycoproteins which were eluted from a tandem column system consisting of one ConA HPAC and one WGA HPAC column. The bound glycoproteins were eluted from the ConA column with 0.2 *M* α-D-methyl mannopyranoside and from the WGA column with 0.2 *M* N-acetylglucosamine. They were subsequently pooled. Recovery was controlled by measuring DPP IV activity; homogenate of the liver and/or kidney, containing 420 000 mU, were applied to a lectin affinity column. Of the kidney DPP IV, 400 000 mU (95%) bound to the column, of which 380 000 mU (90%) could be eluted. Of the liver DPP IV, the same amount was bound and 364 000 mU (87%) could be eluted. MW = Molecular weight; kD = kilodalton.

aggregation and precipitation. Therefore, the sample has to be newly centrifuged before being applied to the column, at at least 50 000 *g* for 30 min. The columns used must allow a quick and simple exchange of the frits. Apart from these risks, aggregation and precipitation lead to a loss of material. It is therefore recommended that the samples are used promptly after production.

Isolation of membrane glycoproteins under denaturing conditions with indirect immunoaffinity HPLC

The eluate from lectin HPAC can be used for the further isolation of membrane proteins. If the proteins are expected to retain their biological activity, as with the enzymatic activity of DPP IV, a number of successive purification steps have to be applied, as described below. If, however, the proteins are required for analytical purposes, such as sequencing or sugar analysis, the use of immunoaffinity HPLC is best.

Several monoclonal and polyclonal antibodies were raised against membrane glycoproteins in our laboratory and routinely used for different investigations[14,15]. We purified these antibodies and bound them to activated HPLC supports. In this way several membrane glycoproteins could be isolated by immunoaffinity (IA) HPLC[4,12]. A problem that arises when immobilized antibodies are used in IA-HPLC is the short life of the columns. Some monoclonal antibodies, including the monoclonal antibody No. 13.4 against DPP IV, lose almost 90% of their binding activity upon immobilization. This loss of activity could not be avoided, although different supports were tested. Therefore, we tried to bind the antibodies non-covalently to protein A or protein G supports, and to elute the antigen–antibody complex from the

column, after the binding of the antigen and extensive rinsing. The method has been described by Phillips *et al.*[16] We modified it and were able to use it successfully as an analytical method instead of immunoprecipitation[12,17]. "Indirect" IA-HPLC with protein G Sepharose "Fast Flow" as support has been sucessful also as a preparative method. A 3-mg amount of monoclonal antibody No. 13.4 against DPP IV was applied to a protein G column. The amount of bound antigen was about 5 mg. The antibody did not have to be purified previously. In the first step, 1 ml of ascites fluid was applied, from which only the antibody bound to the column. In contrast to the method suggested by Phillips *et al.*[15], the antibody is not chemically cross-linked. Instead, the antigen is applied in a subsequent step.

Fig. 2a shows the SDS-PAGE of the eluted antigen–antibody complex. Almost 90% of the antigen has bound. This was verified by measuring the enzymatic activity of DPP IV in the plasma membrane extract before and after the run on the immunoaffinity column. As the antigen against the light and the heavy chain of the immunoglobulin has an apparent molecular weight in SDS-PAGE of 110 000, it is much larger and can therefore be separated by preparative SDS-PAGE in another step. This is shown in Fig. 2b. When DPP IV is isolated with this combination of "indirect" IA-HPLC and preparative SDS-PAGE, a loss of its enzymatic activity has to be expected. For each chromatographic run, new antibody has to be used. It cannot be applied a second time, as it is inactivated in preparative SDS-PAGE and thereby lost. The advantage of this method is that pre-purification of ascites is unnecessary and that the protein G column has a long life. The columns in our laboratory have been in continuous use over a period of 6 months without showing any weakining in their binding activity for antibodies. Non-specific binding of other proteins was minimal with the use of the protein G Sepharose "Fast Flow". This applied to proteins from ascites fluid, from antiserum and from plasma membrane extract.

With the method shown here, several membrane proteins could be isolated in

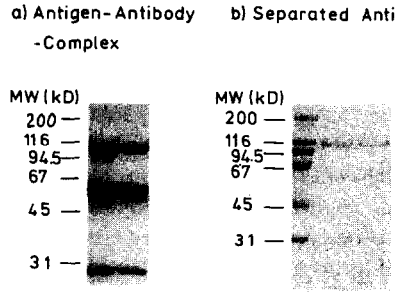

Fig. 2. Isolation of DPP IV of liver by a combination of "indirect" immunoaffinity HPLC and preparative SDS-PAGE. First 1 ml of ascites fluid with antibody No. 13.4 against DPP IV was applied to a protein G Sepharose "Fast Flow" column (30 × 8.0 mm I.D.). The column was then rinsed with 20 ml of Tris-buffered saline (pH 7.2) containing 1% Triton X-100. Then the ConA/WGA eluate of the liver homogenate with 300 000 mU DPP IV was applied, of which about 265 000 mU (about 5 mg of protein) bound to the column. The antigen–antibody complex was eluted with 0.1 *M* citrate buffer (pH 2.4). In a second step, the DPP IV with an apparent molecular weight of 110 000 was separated by preparative SDS-PAGE from the light and heavy immunoglobulin chains. (a) SDS-PAGE of the antigen–antibody complex, eluted from the protein G column; (b) SDS-PAGE of DPP IV after separation of the light and the heavy immunoglobulin chain by preparative SDS-PAGE.

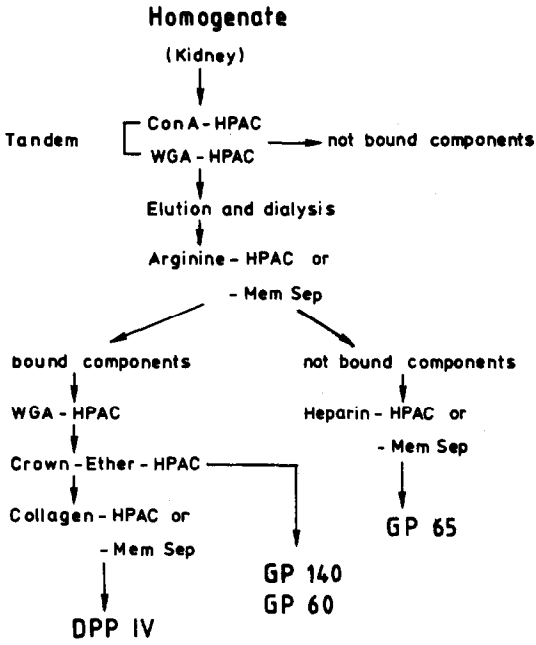

Fig. 3. Scheme for isolation of membrane proteins from kidney homogenate by a combination of different HPAC methods.

addition to DPP IV, such as cell–cell adhesion receptor Cell-CAM (apparent molecular weight in SDS-PAGE of 110 000) and the membrane proteins GP 120 and GP 190 (not shown here). The requirement for a successful separation of the proteins from immunoblogulin chains by preparative SDS-PAGE is a considerable difference in the apparent molecular weight. In our experiments we were able to separate from the immunoglobulin chains the proteins that had an apparent molecular weight of at least 80 000 in SDS-PAGE.

Isolation of membrane proteins under non-denaturing conditions

Many HPAC methods, especially IA-HPLC, have the disadvantage that the elution of the bound ligand from the column has to be carried out under conditions that inevitably lead to their denaturation and loss of biological activity. Lectin HPAC and HPAC with immobilized collagen with heparin and with immobilized low-molecular-weight ligands all have the advantage that elution is carried out under very mild conditions. In most instances a sugar or sodium chloride gradient is used. Fig. 3 shows a scheme for the isolation of different membrane proteins through a combination of HPAC methods. For these purification methods HPAC columns are used in addition to Mem Sep membranes with a bound ligand. The use of Mem Sep membranes was particularly successful when only a step gradient, not a linear gradient, was used for elution. In Fig. 3 these are the purification steps with arginine, heparin and collagen affinity chromatography.

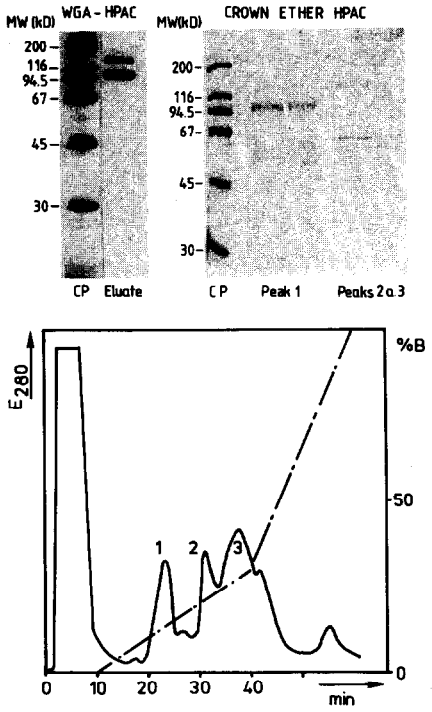

Fig. 4. Isolation of kidney membrane proteins by crown ether HPAC. A 20-ml volume of solution containing 20 mg of proteins that had bound to the arginine and WGA columns (see Fig. 3) was dialysed against a buffer with 5 m*M* Tris-HCl (pH 7.5) and applied to a Eupergit C 30N crown ether column. Elution was carried out with a sodium chloride gradient. In peak 1 the DPP IV was enriched. The glycoproteins with apparent molecular weights of 140 000 (GP 140) and 60 000 (GP 60) in SDS-PAGE were eluted later (peak 2). SDS-PAGE of the isolated proteins is shown in the upper part. Chromatographic conditions: buffer A, 5 m*M* Tris–HCl (pH 7.5) with 20 m*M* potassium chloride and 0.1% Triton X-100, reduced; buffer B, buffer A containing 1 *M* sodium chloride; column, 120 × 8.0 mm I.D.; flow-rate, 1 ml/min; pressure, 5 bar; room temperature. The gradient is shown.

In Figs. 4–6 some of the isolation steps are shown separately. With the combination of affinity chromatographic steps we isolated DPP IV and the glycoproteins with apparent molecular weights in SDS-PAGE of 140 000, 65 000 and 60 000 (GP 140, GP 65 and GP 60, respectively). Their activity was retained, as the elution of the bound proteins was carried out under mild conditions. This was controlled by measuring the DPP IV activity. The amounts of single proteins isolated through the combined affinity chromatographic methods were between 2 mg (GP 60 from kidney) and 50 mg (DPP IV from kidney).

The process as a whole can be scaled up. The answers that we have presented to the varions problems can serve as a model for the isolation of glycoproteins from complex mixtures, such as serum, cell supernatants and cell lysates.

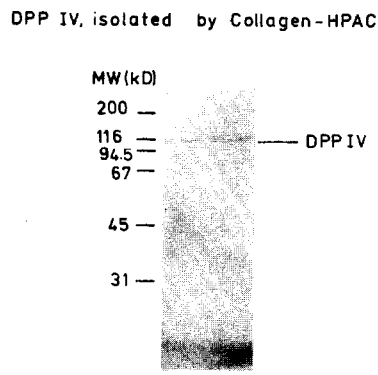

Fig. 5. SDS-PAGE of DPP IV after the last purification step, collagen HPAC. A 13-mg amount of protein in 5 ml after crown ether HPAC was dialysed against 5 m*M* Tris–HCl (pH 8.0) and applied to a Eupergit C 30 N collagen column. The column was rinsed with 10 ml of 5 m*M* Tris–HCl (pH 8.0) and 0.1% octylglucose, then elution was carried out with a step gradient with 0.2 *M* sodium chloride and 0.1% octylglucose. A similar result was obtained with the Mem Sep membrane, with immobilized collagen. A 1-mg amount of protein in 2 ml was applied to the Mem Sep collagen membrane. Rinsing and elution were carried out as above. Chromatographic conditions: HPAC column, 120 × 8.0 mm I.D.; flow-rate, 0.5 ml/min; pressure, 2 bar; temperature, 0°C; membrane, Mem Sep 1000 (Knauer); pressure, 1 bar; other conditions as above.

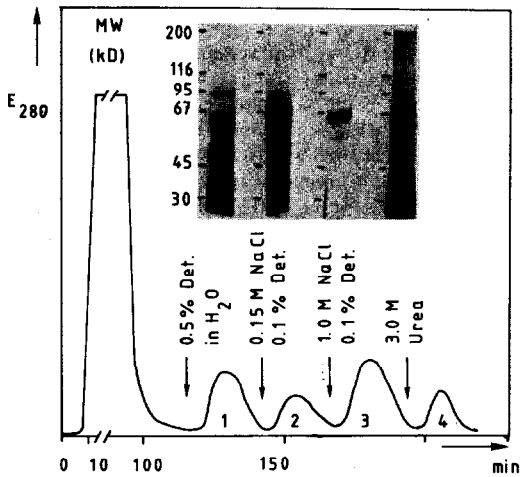

Fig. 6. Heparin HPAC of proteins that were not bound to the arginine column. A 30-mg amount of protein in 30 ml of 5 m*M* Tris–HCl (pH 8.0) was applied to a Eupergit C 30N heparin column and eluted in several steps as shown. SDS-PAGE of single eluted fractions is shown. The same result was obtained with the Mem Sep heparin membrane, but only one fifth of the material was applied and eluted. Chromatographic conditions; column, 120 × 8.0 mm I.D. (or Mem Sep heparin membrane); flow-rate, 0.5 ml/min; pressure, 2 bar; temperature, 0°C. Det = detergent Triton X-100, reduced.

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