Purification of liver and hepatoma membrane proteins by high-performance liquid chromatography

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This paper documents the recovery of selected proteins from hepatic plasma membranes. Initial purification, achieved by a series of stepwise extractions, facilitates the subsequent purification by HPLC. Examples are provided to illustrate the recovery of specific proteins from two Morris hepatoma lines and the liver.

Liver *Hepatoma* *Membrane protein* *High-performance liquid chromatography*

1. INTRODUCTION

Recent significant advances have been made in the development of techniques designed to improve the yield and purity of proteins isolated by HPLC. However, the application of these techniques to the resolution and recovery of detergent-solubilized plasma membranes has been less than satisfactory. We have found that much of the difficulty encountered may be overcome by a simple stepwise-solubilization procedure based on the hydrophobicity and solubility properties of individual membrane components. This report outlines a pre-purification scheme which, combined with subsequent chromatography by HPLC, has permitted the isolation of several intrinsic and extrinsic membrane proteins. It is envisaged that continuing adaptation of these techniques to the unique properties of individual membrane constituents will provide a rational basis for the recovery of as yet unidentified, specific membrane proteins.

Abbreviations: HPLC, high-performance liquid chromatography; SE, size exclusion; RP, reversed phase; PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonyl fluoride; CHAPS, 3-(3-chloramidopropyl) dimethylammonio-1-propane-sulfonate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Animals and chemicals*

Male Wistar rats (Ivanovas, Kisslegg, FRG) or Buffalo rats (Zentrales Tierlaboratorium, Berlin) weighing about 160–180 g each were fed on a commercial diet containing 18–20% (w/w) protein (Altromin R, Altromin, Lage, Lippe, FRG). Chemicals of analytical grade were purchased from Merck (Darmstadt) or Serva (Heidelberg) FRG.

2.2. *Isolation of plasma membranes*

Plasma membranes were isolated by zonal centrifugation, using a Kontron centrifuge (Kontron, München). Membrane purity was routinely checked by electron microscopy and by assaying of marker enzymes as reported [1]. Protein was determined according to the procedure of Lowry et al. [2].

2.3. *Extraction of plasma membrane proteins*

Prior to purification by HPLC the plasma membranes were extracted in the following stepwise manner:

(1) Freezing and thawing

Purified plasma membranes were suspended in 1 mM sodium carbonate buffer, pH 7.0, containing 0.5 mM $CaCl_2$. The protein concentration

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was 10–12 mg/ml. This suspension was frozen twice at −70°C and thawed. The plasma membranes were centrifuged for 45 min at $10^5 \times g$. The supernatant was decanted, the pellet was solubilized as follows (see also fig.1).

(2) Salt extracting

The pellet from the first step was treated with PBS, the final concentration was 5–10 mg protein/ml. The suspension was homogenized 12 times by 2–3 strokes at ½ h. intervals in a loose fitting Dounce type homogenizer (Braun, Melsungen, FRG). In both of the above steps the temperature was maintained at 4°C. The suspension was centrifuged for 45 min at $10^5 \times g$ at 4°C.

(3) Alkaline extraction

To the pellet from step 2, 5 mM NaOH was added at a final concentration of 5 mg protein/ml. The suspension was homogenized in a loose-fitting Dounce homogenizer for 1 h at room temperature. To inhibit proteolysis PMSF at a final concentration of 1 μM was added. The suspension was centrifuged for 45 min at $10^5 \times g$ at 20°C.

(4) Non-ionic detergent

A 1% solution of Genapol X-100 (Hoechst, Frankfurt) or Nonidet P-40 (Sigma, München) in PBS was added to the pellet in the third step. The suspension was homogenized and centrifuged in the same manner as in step 2.

(5) Calcium chelation

10 mM EDTA in PBS containing 1% (w/v) zwitter ionic detergent CHAPS was added to the pellet from step 4. The suspension was homogenized and centrifuged in the same manner as described in step 2.

2.4. *HPLC*

The HPLC system consists of two pumps (model 64, Knauer, Berlin), a programmer (model 200, Kontron Analytik, München), FRG a filter-photometer (Knauer) and/or spectrophotometer both with deuterium lamp (BT 3030, Biotronik, Frankfurt), FRG, an RH 7125 loop injection valve and HPLC column oven (Knauer). For SE-HPLC a 600 × 7.5 mm TSK-4000 column with 10 μm particle size is used ('Blue column', LKB, München). The mobile phase used for SE-HPLC was PBS. The final concentration of appropriate detergent was 0.1% (w/v), the temperature was 22°C.

For RP-HPLC a SyncroPac RP-4 column (250 × 4.1. mm) with 10 μM particle size and 300 Å pore size was chosen (SynChrom, Linden, IN). The solutions used are: (A) 10% (v/v) formic acid and 0.1% (w/v) trifluoroacetic acid and (B) isopropanol or acetonitrile. The temperature was 62°C.

2.5. *SDS-polyacrylamide gel-electrophoresis*

Dialysed and freeze-dried samples were dissolved in 62.5 mM Tris-HCl buffer, pH 6.8, containing 3% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromphenol blue. SDS-PAGE was performed using the method of Laemmli [3]. 100–150 μg protein in each line was applied.

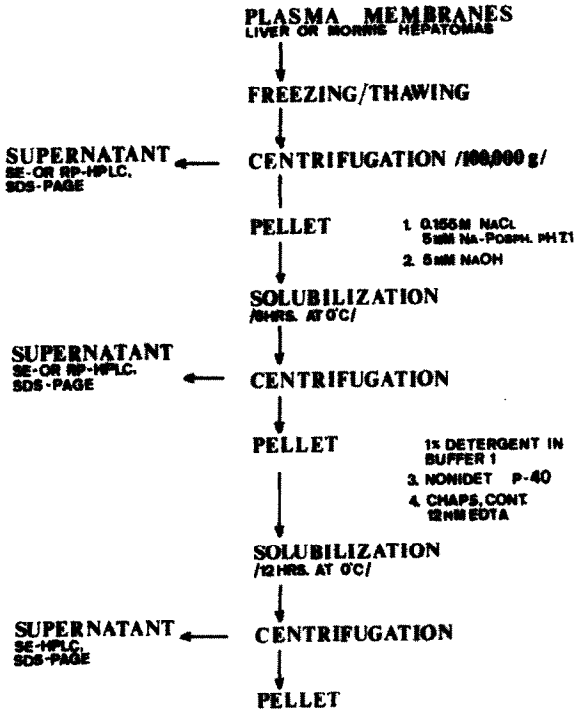

Fig. 1. Stepwise extraction scheme for plasma membranes.

Gels were fixed and stained in a solution of Coomassie blue G250 (0.1% in water:methanol: acetic acid, 5:4:1, by vol.) and finally destained in water:methanol:acetic acid (175:10:15, by vol.)

3. RESULTS AND DISCUSSION

In the first 3 steps (see fig.1), especially in the second (PBS) and third (alkaline extraction) extrinsic membrane proteins constitute the major extracted fraction. Alkaline extraction removes about 30–40% of the total protein content of the membranes.

By application of different HPLC methods, several proteins from these extracts could either be isolated or enriched. Two samples are shown in figs 2 and 3.

Fig. 2 shows RP-HPLC of an extract obtained after freezing/thawing of the plasma membranes of Morris hepatoma 7777. A 70 kDa protein was isolated (see peak 1), which also occurs in somewhat smaller quantities in the plasma membranes of Morris hepatoma 9121, but not in liver plasma membranes. Based upon selective precipitation with anti-rat serum albumin and cochromatography by HPLC, this protein has been identified as rat serum albumin (not shown). Since it has been established that neither of those hepatoma lines have the ability to secrete serum proteins [4], it would appear that the albumin remains with the cell presumably bound to the plasma membrane.

Fig. 3 Shows the SE-HPLC of an extract obtained from the liver plasma membranes by freezing/thawing. Employing SE-HPLC, several proteins from this extract could be enriched. Shown is the enrichment of a protein with an apparent molecular mass of 105 kDa in SDS-PAGE. This protein (see fig.3, line 5), or a group of related proteins, is characteristic of the liver and does not appear in hepatoma cells. It has been isolated recently by Hixson et al. [5]. They could show that this protein is a glycoprotein and plays a role in the interaction between hepatocytes and extracellular

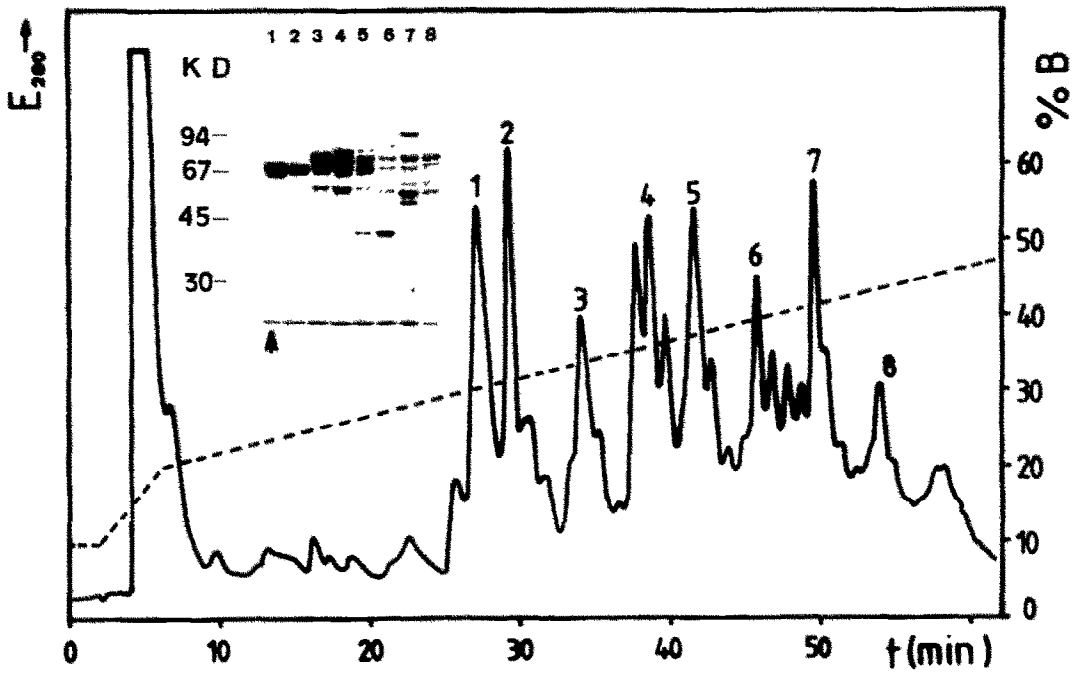

Fig. 2. RP-HPLC of membrane proteins from Morris hepatoma 7777, extracted in the first step, by freezing/thawing. A protein with apparent molecular mass of about 70 kDa (arrow) could be isolated. Chromatographic conditions: column, SyncroPac RP4 (250 × 4.1. mm, 10 μM particle size and 300 Å pore size); mobile phase, buffer (A) 0.1% (w/v) trifluoro acetic acid, buffer (B) isopropanol; temperature, 62°C; flow rate; 1 ml/min; pressure, 40–60 bar. 0.8 ml Protein dissolved in 500 μl buffer A was applied to the column.

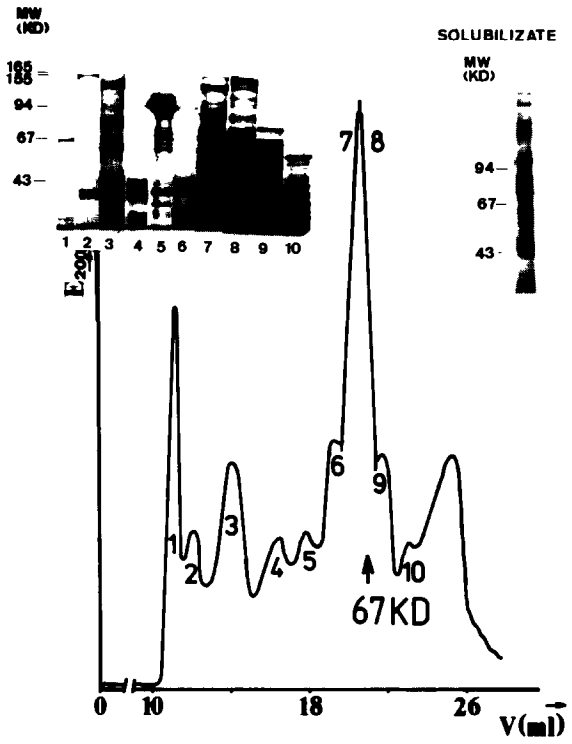

Fig. 3. Size-exclusion-HPLC of membrane proteins from liver, extracted in the first step by freezing/thawing. A polypeptide with an apparent molecular mass 105 of kDa could be highly enriched (line 5, peak 5). Chromatographic conditions: column, TSK 3000 (600 × 7.5 mm); mobile phase, phosphate-buffered saline, pH 7.2; flow rate, 0.1 ml/min; pressure, 5 bar; room temperature. 0.5 mg protein was dissolved in running buffer and applied to the column.

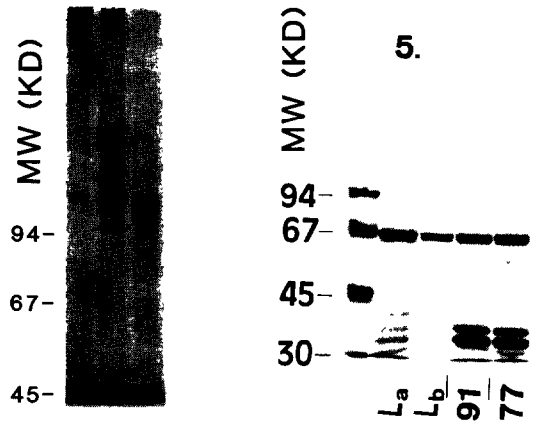

Fig. 4. Proteins with apparent molecular masses of 120 kDa (two lines left) and 105 kDa (third line) isolated by SE-HPLC from NP-40 extract of liver plasma membranes. For more details see fig. 3 and section 2.

Fig. 5. Fifth extraction step : chelating calcium by EDTA. Two polypeptides with apparent molecular masses of 65 and 67 kDa could be extracted from both liver and Morris hepatoma plasma membranes (lines 'L' for liver, '91' for Morris hepatoma 9121 and '77' for Morris hepatoma 7777). Two additional polypeptides 33 and 35 kDa appear in the extracts from both hepatoma plasma membranes, but not from the liver. To keep these proteins in solution, 1% (w/v) CHAPS was added to the solubilization buffer.

matrix. This protein can be released only partly by freezing/thawing. The remaining moiety can then be solubilized by extraction with the non-ionic detergent, Nonidet P-40 (NP-40), as shown below. Consequently this component posesses, at least to some extent, the properties of an intrinsic membrane protein.

From the material obtained by extraction with NP-40, several proteins were isolated or enriched by HPLC. The partial isolation and characterization of an adhesion molecule from liver and Morris hepatoma has been described elsewhere [6].

In fig. 4 the isolation of 120 and 105 kDa proteins from the above detergent extract of liver plasma membranes is shown. The 120 kDa protein is a glycoprotein and has been isolated previously [7]. The present method offers the advantage that it is quicker and leads to higher yields. The aforementioned 105 kDa protein also appears in this step and can be separated from the 120 kDa protein by SE-HPLC (see fig.4).

Fig. 5 shows the last step of solubilization. In this step the proteins are dissociated from the membranes by removing Ca^{2+}. The presence of the zwitterionic detergent CHAPS is not essential for extraction. However, as these proteins are very hydrophobic, the detergent increases their solubility and maintains them in solution.

As can be seen in fig. 5 the appearance of two polypeptides with apparent molecular masses of 65 and 67 kDa is typical for both the liver and the two hepatoma lines. With the two hepatoma lines two major polypeptides appear with the apparent molecular masses of 33 and 35 kDa. These proteins are barely detectable in the liver. The 65–67 kDa and the 33–35 kDa polypeptides from the

hepatoma extracts can be separated by SE-HPLC with 0.1% SDS added (not shown). The 65 and 67 kDa polypeptides which seem to be subunits of a protein complex described in [8].

Similar polypeptides were isolated from the plasma membranes of lymphocytes by Ca^{2+} chelation [9]. Their function, however, is not yet clear.

The significant difference between liver and hepatoma extracts in this calcium-dependent step lies in the appearance of the two additional polypeptide chains at 33 and 35 kDa. Current investigation indicate that these are not products of proteolytic cleavage. (Josić, Schuett, Reutter, in preparation). Whether or not these polypeptides are correlated with malignant transformation remains to be seen.

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REFERENCES

[1] Tauber, R. and Reutter, W. (1978) Eur. J. Biochem. 83, 37–45.
[2] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Rendall, R.J. (1951) J. Biol. Chem. 195, 265–275.
[3] Laemmli, U.K. (1970) Nature 227, 680–685.
[4] Schreiber, G., Boutwell, R.K., Potter, V.R. and Morris, H.P. (1966) Cancer Res. 26 (Part I), 2357–2361.
[5] Hixson, D.C., De Lourdes Pouce, M., Allison, J.P. and Walborg, E.F. Jr. (1984) Exp. Cell. Res. 152, 402–414.
[6] Neumeier, R., Josić, Dj. and Reutter, W. (1984) Exp. Cell Res. 151, 507–512.
[7] Tauber, R., Park, C.-S. and Reutter, W. (1983) Proc. Natl. Acad. Sci. USA 80, 4026–4029.
[8] Josić, Dj., Baumann, H. and Reutter, W. (1984) Anal. Biochem. 142, 473–479.
[9] Owens, R.J. and Crumpton, M.J. (1984) Biochem. J. 219, 309–316.