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ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MEMBRANE PROTEINS FROM LIVER AND MORRIS HEPATOMAS

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SUMMARY

The separation of proteins from plasma membranes of liver and two Morris hepatomas with different grades of differentiation (Morris hepatomas 7777 and 9121) is described. Size-exclusion high-performance liquid chromatography under non-denaturing conditions provides only poor separation and, with detergent-soluble fractions, unsatisfactory protein recovery. Much better results are obtained by ion-exchange chromatography.

The amount of a 175 K protein in the membranes decreased in parallel with the increase in tumour malignancy. This protein appears during ion-exchange chromatography in a broad concentration range, between 0.1 and 0.3 *M* sodium chloride. Possible explanations for this phenomenon are discussed.

INTRODUCTION

High-performance liquid chromatography (HPLC) of hydrophobic proteins from plasma membranes of liver and hepatomas presents several difficulties. As the samples investigated are very complex mixtures, each consisting of several hundred proteins, a method was developed that provides a preliminary selection by stepwise extraction[1]. The results of subsequent HPLC are discussed in this paper.

EXPERIMENTAL

Animals and chemicals

Male Wistar rats (Ivanovas, Kisslegg, F.R.G.) or Buffalo rats (Zentrales Tierlaboratorium, Berlin, F.R.G.) weighing about 160–180 g were fed on a commercial diet containing 18–20% (w/w) protein (Altromin R, Altromin, Lage/Lippe, F.R.G.). Chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, F.R.G.) or Serva (Heidelberg, F.R.G.). L-[6-^{3}H]Fucose (4.2 Ci/mol) was purchased from the Radiochemical Centre (Amersham, U.K.).

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Labelling of glycoproteins with L-[^{3}H]fucose

For these studies the rats received an injection of 5 mCi of L-[6-^{3}H]fucose/kg body weight into the tail vein 2 h before removing the 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 assaying of marker enzymes as described by Tauber and Reutter[2]. Protein was determined according to the procedure of Lowry *et al.*[3].

Extraction of plasma membrane proteins

Prior to purification by HPLC, the plasma membrane proteins were extracted in a stepwise manner[1], by freezing–thawing in the first step, alkaline extraction with 1 m*M* sodium hydroxide in the second step and by extraction with the non-ionic detergent Nonidet P-40 in the third step.

HPLC

The HPLC system consisted of two pumps (Model 64, Knauer, Berlin, F.R.G.), a programmer (Model 200, Kontron), a filter photometer with a deuterium lamp (Knauer) and an RH 7125 loop injection valve (Knauer). The separation conditions and columns are shown in the figure legends. All separations by HPLC were carried out at 22°C.

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

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 using the method of Laemmli[4]. An amount of 100–150 μg of protein was applied to each track.

RESULTS AND DISCUSSION

Previous investigations have shown that HPLC of membrane proteins under denaturing conditions provides satisfactory results. This was demonstrated by protein separations by size-exclusion (SE) HPLC (with SDS and mercaptoethanol added to the mobile phase) and by reversed-phase (RP) HPLC[1,5]. The proteins separated under these conditions tend to lose their biological activity, sometimes to a large extent. Our subsequent investigations were aimed at preserving the biological activity of the separated proteins. Therefore, methods that use strongly denaturing conditions had to be discarded.

SE-HPLC, when used without denaturing agents, here shown with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), has proved unsatisfactory. All columns used [Superose 12 (Pharmacia), TSK-4000 SW (Toyo Soda), GF-250 and 450 (DuPont)] gave poor separations.

The recovery of the less hydrophobic proteins from the first fraction (obtained

by freezing–thawing) was satisfactory. When increasingly hydrophobic proteins are applied, the recovery does not exceed 50% and sometimes it is as low as 20%. This agrees with previous results for the SE-HPLC of cells solubilized with Nonidet P-40 and Triton X-400. Whenever hydrophobic proteins were involved, the recovery was low[6]. The results of the SE-HPLC separations of the above two fractions are shown in Fig. 1a and b.

Better results were achieved by ion-exchange HPLC. The separation was much more effective, the protein recovery was above 70% in all instances and between 80 and 90% in most instances. The separations were carried out with the addition of 0.05% CHAPS, a zwitterionic detergent. The results of these separations are shown in Figs. 2–5. Figs. 2–4 show separations of membrane extracts from the liver, from the differentiated, less malignant Morris hepatoma 9121 and the poorly differentiat-

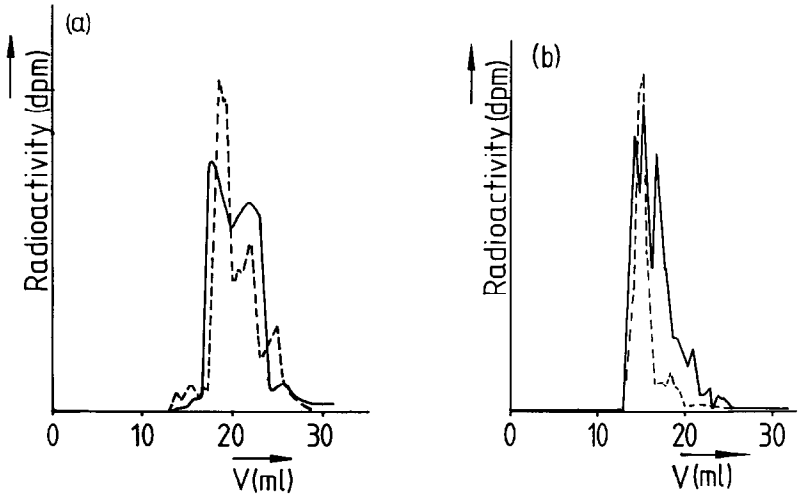

Fig. 1. (a) Separation of less hydrophobic membrane proteins by size-exclusion HPLC. L-[6-^{3}H]Fucose-labelled plasma membranes of liver and Morris hepatoma 7777 were extracted, frozen at −70°C, thawed and centrifugation at 100 000 *g*; 100 000 dpm of L-[6-^{3}H]fucose radioactivity from the supernatant was used for each separation. Column, combined GF 250 and GF 450, each 250 × 9.4 mm I.D. (DuPont–Bischoff Analysentechnik, Leonberg, F.R.G.); mobile phase, 0.155 *M* sodium chloride in 10 m*M* Tris–HCl buffer (pH 7.1) with 0.05% CHAPS added; flow-rate, 0.5 ml/min; pressure, 35 bar. The sample was collected and the radioactivity determined. ——, Extract from plasma membranes of liver; – – –, extract from plasma membranes of Morris hepatoma 7777. The recovery was 80% in both instances. (b) Separation of very hydropohobic intrinsic membrane proteins by size-exclusion HPLC. L-[6-^{3}H]Fucose-labelled plasma membranes of liver and Morris hepatoma 7777 were extracted with 1 m*M* sodium hydroxide and centrifuged. In this way the extrinsic membrane proteins were removed. The pellet was subsequently extracted with a 1% CHAPS solution in 0.155 *M* sodium hydroxide; 100 000 dpm of L-[6-^{3}H]fucose radioactivity was used for each separation. Conditions as in (a). The recovery for liver was 32% and that for Morris hepatoma 7777 40%.

ed, very malignant Morris hepatoma 7777. The extracts were obtained by freezing–thawing of the membranes[1]. The separations by anion-exchange HPLC shown in Fig. 4 can be compared with the previous RP-HPLC separations of the same extracts[1]. The separation conditions are much milder, however. Therefore, the likelihood that the proteins will retain their biological activity is considerably improved.

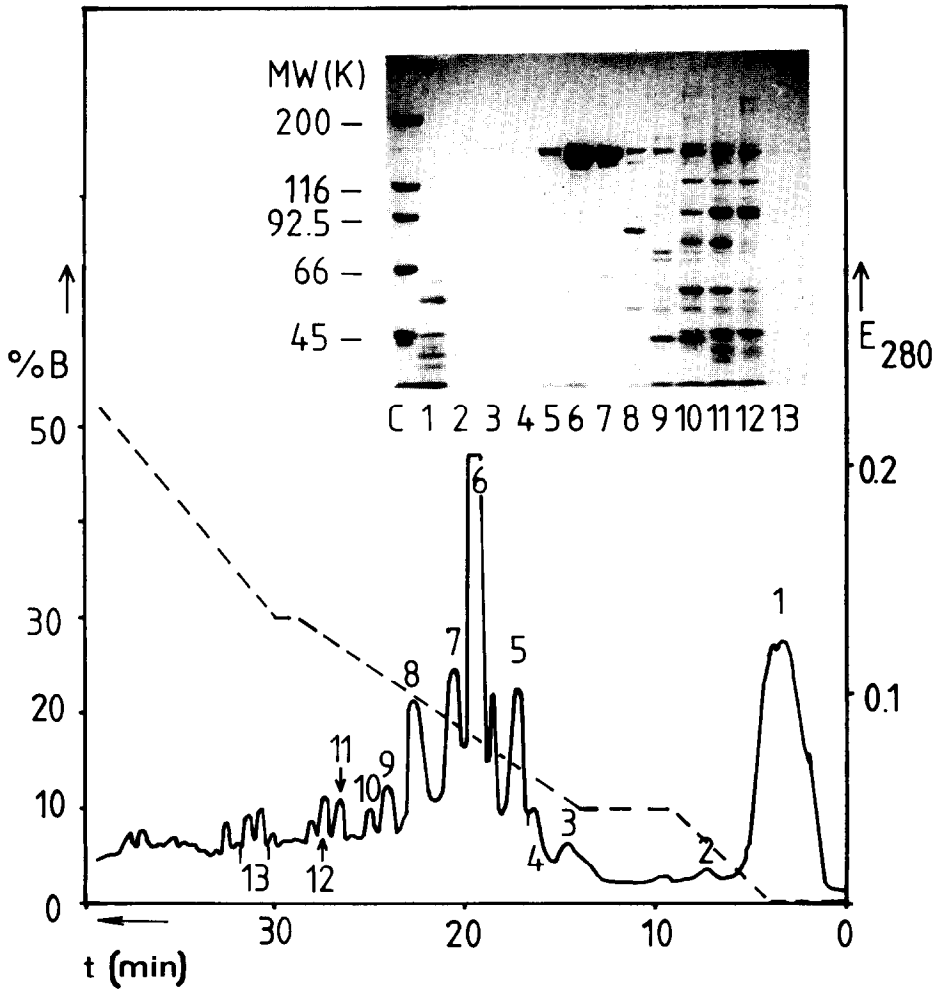

Fig. 2. Anion-exchange HPLC of plasma membrane extract of liver. The membranes were solubilized by freezing–thawing. Column, TSK-DEAE-5PW, 75 × 7.5 mm I.D. (LKB, Munich, F.R.G.); buffer A, 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 6.1) with 0.05% CHAPS; buffer B, 1 *M* sodium chloride in buffer A; flow-rate, 1 ml/min; pressure, 12 bar. The gradient is marked. Samples of 0.5 ml each were collected, and aliquots of 50–100 μl were used for electrophoresis (see upper part). A 5-mg amount of protein was applied; sample loop volume, 1 ml; 90% of the protein was recovered.

As can be seen in Figs. 2–4, the protein patterns clearly change with increasing malignancy of the tissue. The changes are characterized by a decrease in a 175 K protein parallel to an increase in malignancy (see fractions 6 in Figs. 2–4). It has virtually disappeared in the Morris hepatoma 7777. As can be seen in Fig. 2, this protein is eluted in a broad concentration range between about 0.1 and 0.3 *M* sodium chloride. An explanation for this phenomenon has so far not been found. It can be assumed that they are a group of similar glycoproteins, which differ in carbohydrate content and structure. A less welcome explanation would be that the phenomenon

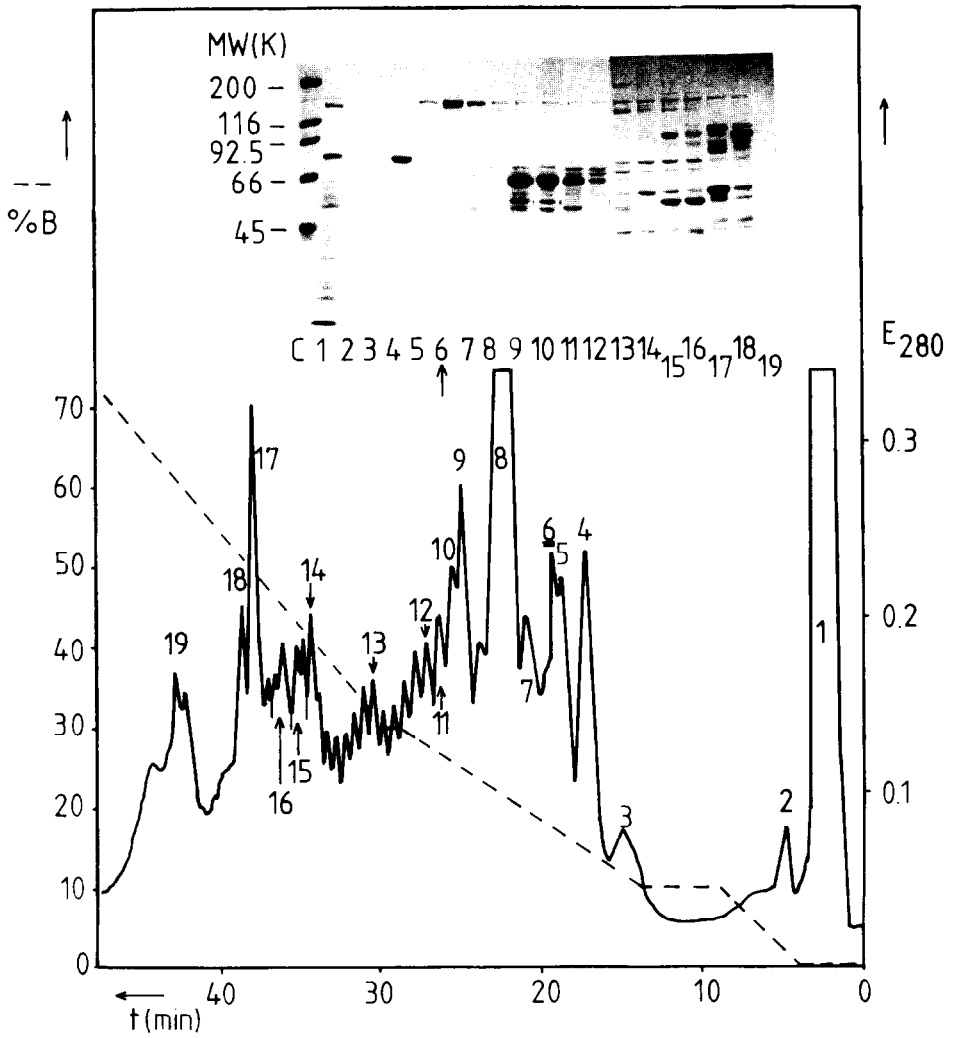

Fig. 3. Anion-exchange HPLC of plasma membrane extract from Morris hepatoma 9121. The membranes were solubilized by freezing–thawing. For conditions, see Fig. 2. A 5-mg amount of protein was applied; recovery, 84% (4.2 mg). Fraction 6, in which the 175 K protein appears, is marked with an arrow.

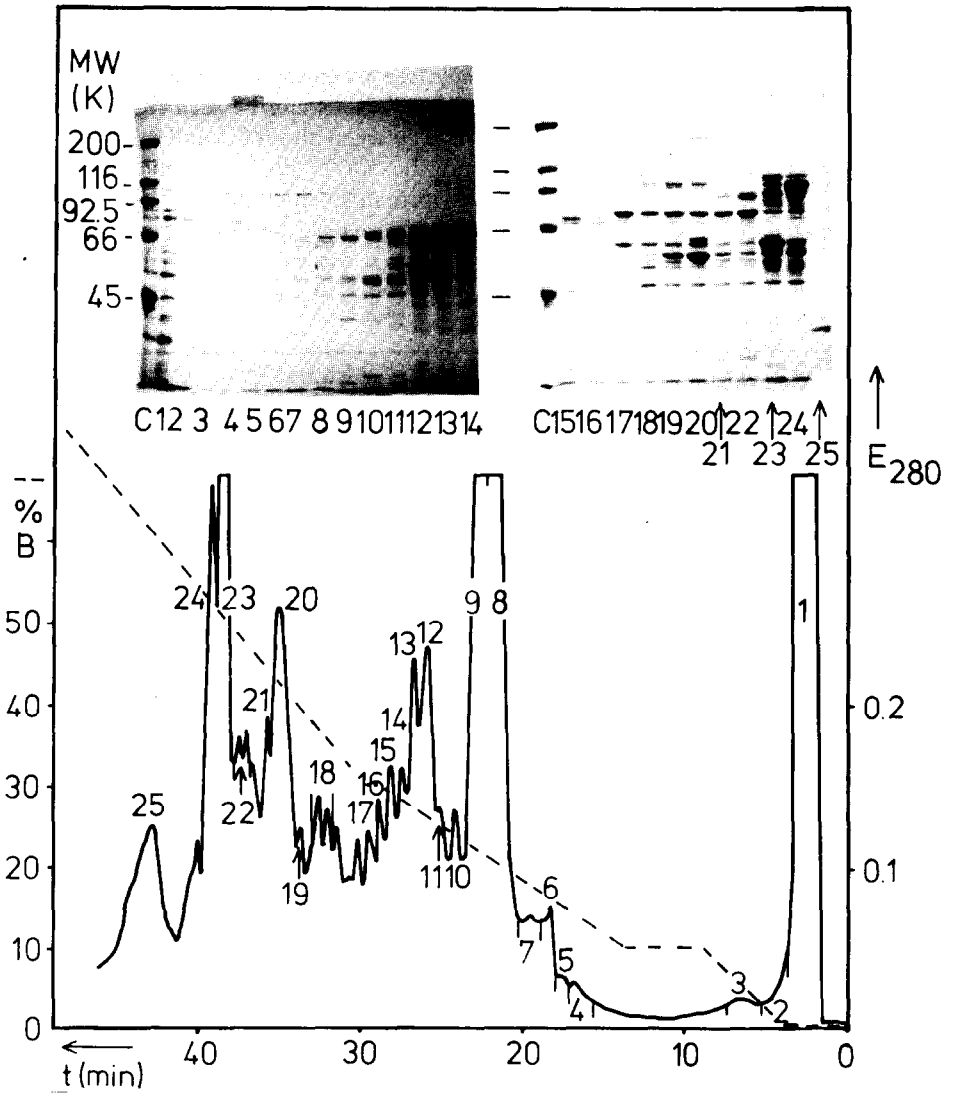

Fig. 4. Anion-exchange HPLC of plasma membrane extract from Morris hepatoma 7777. The membranes were solubilized by freezing–thawing. Conditions same as in Fig. 2. A 5-mg amount of protein was applied; recovery, 82% (4.1 mg).

is prompted by non-specific interaction with the support. Peptide mapping and the investigation of the carbohydrate chain(s) and p*I* values of the proteins from various fractions are planned as a further means of investigation to help provide an explanation of this problem.

Fig. 5 shows an anion-exchange chromatogram of plasma membrane proteins from the liver, obtained by extraction with the non-ionic detergent NP-40. These are hydrophobic intrinsic membrane proteins, which are very difficult to analyse by chro-

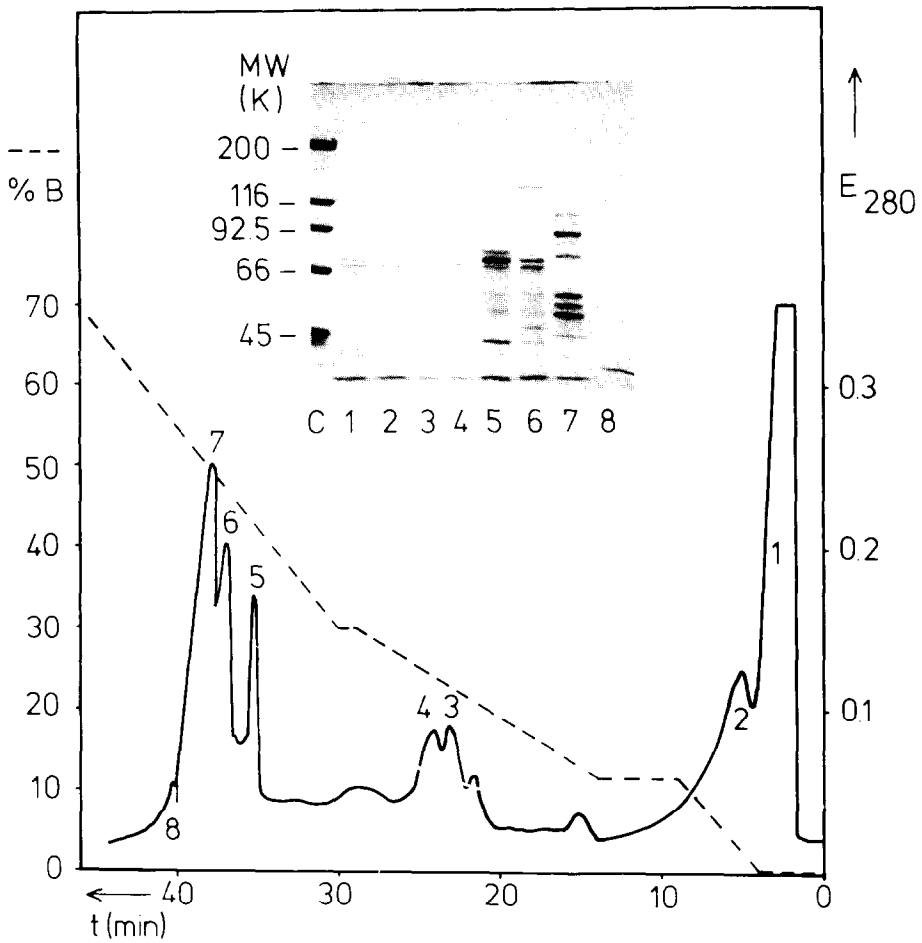

Fig. 5. Anion-exchange HPLC of intrinsic membrane proteins of liver. The plasma membranes were treated with 1 m*M* sodium hydroxide and centrifuged in order to remove the extrinsic membrane proteins (*cf.*, Fig. 1). The pellet was then extracted with a 1% Nonidet P-40 solution in 0.155 *M* hydrochloric acid. Conditions as in Fig. 2. A 3-mg amount of protein was applied in 2 ml of buffer A, the Nonidet P-40 concentration in the sample being about 0.1%; 73% (2.2 mg) of protein was recovered; the additional amount of 0.7 mg of protein was eluted with a 1-ml injection of 40% acetic acid.

matographic methods. As can be seen, a satisfactory separation could be achieved, the protein recovery being 70%. The remaining column-bound proteins could subsequently be washed out by injection of 1 ml of 40% acetic acid[6].

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