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High-performance capillary electrophoresis of hydrophobic membrane proteins

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ABSTRACT

Hydrophobic membrane proteins, extrinsic and intrinsic ones, were separated by high-performance capillary zone electrophoresis (HPCZE) and high-performance capillary isotachophoresis (HPCITP). In the case of HPCZE with both coated and uncoated quartz capillaries the addition of 7 *M* urea to the separation buffers was necessary to achieve reproducible results. In the HPCITP experiments PTFE capillaries were used. When spacers were used, *e.g.*, ampholytes, additional splitting of peaks was observed. The splitting was caused by the microheterogeneity of the investigated proteins, which are differently glycosylated and/or phosphorylated.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) has been established in many fields as a separation method for small molecules[1–3]. Good progress has been made also with regard to high-molecular-mass polymers, such as proteins and nucleic acids. For their separation free-flow capillary electrophoresis was mainly used[4,5], but separation in prepacked capillaries has also been attempted[6,7] (for a review, see ref. 8). At a time when other methods were being developed for the same purpose, capillary isotachophoresis (HPCITP) was used for the separation of biopolymers from complex mixtures, such as serum[9] and protein mixtures[10], and also for the separation of small molecules[11].

The separation of hydrophobic proteins in aqueous solutions is very difficult. Experience gained from high-performance liquid chromatographic (HPLC) experiments shows that solubility problems can occur during the separation, owing to a tendency for these proteins to undergo aggregation and self-aggregation. In addition, undesirable, non-specific interactions with the support frequently appear, leading to poor recovery of the sample and results which are no longer adequately repro-

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ducible[12]. These effects can be avoided or at least controlled by the addition of detergents[12] and/or other denaturing agents such as urea or formic acid[13].

In this work we investigated the separation of membrane proteins by different HPCE methods. For the experiments we used two groups of model membrane proteins, intrinsic and extrinsic.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.) or Sigma (Munich, F.R.G.). Liver and Morris hepatoma membrane proteins were isolated as described elsewhere[14,15].

Apparatus

For all separations by high-performance capillary zone electrophoresis (HPCZE) and ITP, a Beckmann (Munich, F.R.G.) system was used. In ITP separations the capillary cassette contained the PTFE capillary. The ITP experiments on the Beckmann system were also carried out with a system developed by Schmitz and co-workers[9,16].

Capillaries

For HPCZE uncoated quartz capillaries (20 cm × 25 μm I.D.) (Beckmann and Bio-Rad Labs. Munich, F.R.G.) and coated capillaries (20 cm × 50 μm I.D.) (Bio-Rad Labs.) were used. According to information provided by the manufacturer, the coated capillaries contain a hydrophilic polymer which is covalently bound on the inner surface. PTFE capillaries (20 cm × 250–400 μm I.D.) (Labochron, Sinsheim, F. R. G.) were used for HPCITP.

Buffers

Sodium borate buffer (0.2 *M*, pH 9.2) was used for HPCZE, with or without the addition of 7 *M* urea. As the leading electrolyte in ITP, 5 m*M* H_3PO_4 was used. The electrolyte contained 0.25% (w/v) hydroxypropylmethylcellulose (HPMC) in order to increase the viscosity and to suppress electroendosmotic movement in the capillary. Ammediol (2-amino-2-methyl-1,3-propanediol) was added as a counter ion to achieve pH 9.2. The terminating electrolyte contained 100 m*M* valine and was adjusted with ammediol to pH 9.4.

Separation conditions

HPCZE was carried out at a constant voltage of 30 kV and a current between 80 and 180 μA.

HPCITP experiments with the system from Beckmann were carried out at a voltage of 5–6 kV, the current being 240 μA (beginning) to 50 μA (end of separation). With the system developed by Schmitz and co-workers, the separation was started with a constant current of 150 μA and during the 10-min run the current was reduced to 100 μA (the corresponding voltage was 7 kV), and subsequently to 50 μA (6 kV) before detection.

The proteins were monitored spectrophotometrically at 280 nm. The tem-

perature during the separation was kept constant at 20°C (Beckmann apparatus) or 10°C (Schmitz and co-workers' system).

Gel electrophoretic methods

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the Laemmli method[17], using a mini-system (Bio-Rad Labs). Two-dimensional electrophoresis was performed according to the method described by O'Farrel[18].

RESULTS AND DISCUSSION

High-performance capillary zone electrophoresis

HPCZE separations were carried out with calcium-binding proteins from liver plasma membranes and Morris hepatoma 7777 plasma membranes, and also with a mixture consisting of three different intrinsic membrane glycoproteins, dipeptidyl peptidase IV (DPPIV), cell-CAM and a glycoprotein with an apparent molecular weight in SDS-PAGE of 95 000 dalton. The CBP solutions contained 0.1% (w/v) of CHAPS detergent; with the intrinsic membrane proteins the detergent Triton X-100 (0.1%, w/v) was used.

When the membrane proteins were separated in an uncoated fused-silica capillary using a buffer without denaturing reagents, no sample recovery was achieved (not shown). This result can be compared with those obtained by HPLC. When, in the course of separating hydrophobic proteins, non-specific interactions with the matrix could not be prevented, the proteins were only partly or were not eluted from the column[12]. In HPLC these difficulties can be avoided by coating the silica gel matrix or by introducing hydrophilic groups into the polymer supports. With coated silica capillaries some substances showed a modest improvement in terms of their peak surface. However, the time for the appearance of single peaks was not reproducible (not shown). This suggests that the coated capillaries that are commercially available at present do not allow a satisfactory separation of membrane proteins under the described conditions. Only after addition of 7 mol/l urea, a denaturing reagent, are reproducible results obtained in terms of peak height and running time for single substances.

HPCZE of calcium-binding proteins with the addition of a denaturing reagent is shown in Fig. 1. The low-molecular-mass proteins CBP 33 and CBP 35, which in Morris hepatoma 7777 plasma membranes appear at much higher concentrations than in liver plasma membranes (*cf.*, SDS-PAGE in Fig. 1), are seen as additional, separate peaks (*cf.* Fig. 1b).

The separation of intrinsic membrane proteins, which were solubilized and kept in solution by means of the non-ionic detergent Triton X-100 , requires more complicated conditions. If the separation is carried out without urea, a single peak is detected at 280 nm. A run with injection of a 1% solution Triton X-100 without protein showed that this peak could not be attributed to the protein, but to the detergent (not shown). Under these conditions, the detergent is separated from the proteins, which consequently aggregate and are precipitated. Fig. 2 shows a separation of intrinsic membrane proteins after 7 *M* urea has been added. The detergent, or at least part of it, is again separated, but the proteins are kept in solution all the same by the urea and can

be recovered. The first peak in the electropherogram in Fig. 2 has to be attributed to the Triton X-100. The additional peaks are due to the membrane proteins.

The results shown in Figs. 1 and 2 illustrate the guidelines that have to be followed in the separation of hydrophobic proteins by HPCZE. It is to be expected that the separation and recovery can be improved through adequate derivatization of the surface of the capillaries. However, the experiments with coated capillaries have shown that precipitation of the proteins occurs, because during the separation process the detergent is itself separated (data not shown). It can be avoided only by adding denaturing reagents to the separation buffer. The strongly denaturing 7 *M* urea solution that has been used so far can probably be replaced with more adequate reagents.

High-performance capillary isotachophoresis

The experiments on membrane protein separation by HPCITP were based on

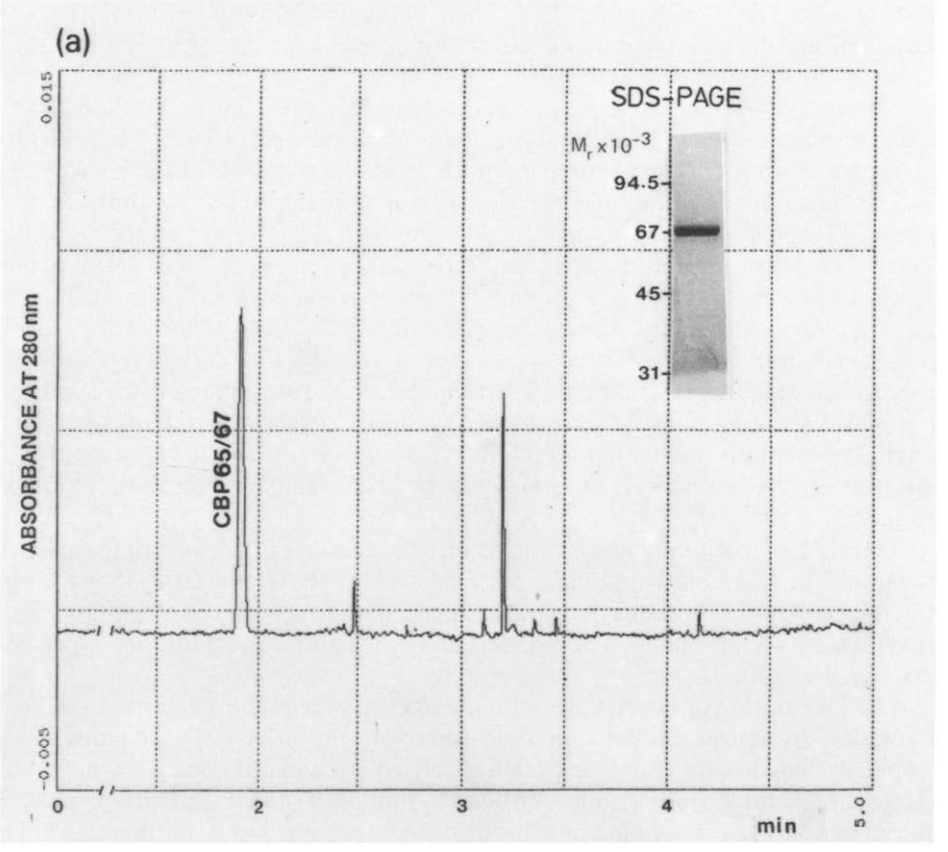

Fig. 1.

experience gained with the separation of serum lipoproteins[9,16]. The buffer systems that were optimized for the separation of lipoproteins can also be used for the separation of hydrophobic membrane proteins. Fig. 3 shows the separation of calcium-binding proteins by HPCITP. In preliminary experiments that were carried out chiefly in order to check reproducibility, the influence of spacers on the separation process was not investigated. The CBP from liver and also from Morris hepatoma 7777 plasma membranes could be separated and recovered by HPCITP (see Fig. 3). An additional splitting of single peaks occurred through the use of spacers, as shown in Fig. 4. In the case of CBP, ampholytes in the pH range 5–7 were chosen.

The intrinsic membrane proteins DPP IV, cell-CAM and the 95 000-dalton protein could be separated very well by HPITP, as shown in Fig. 5. Through the addition of spacers, in this instance ampholytes in the pH range 4–6, an additional splitting of single peaks also occurred (see Fig. 6). Cell-CAM (the first peak in Fig. 5) consists of two isoforms, which can be separated in two-dimensional electrophoresis.

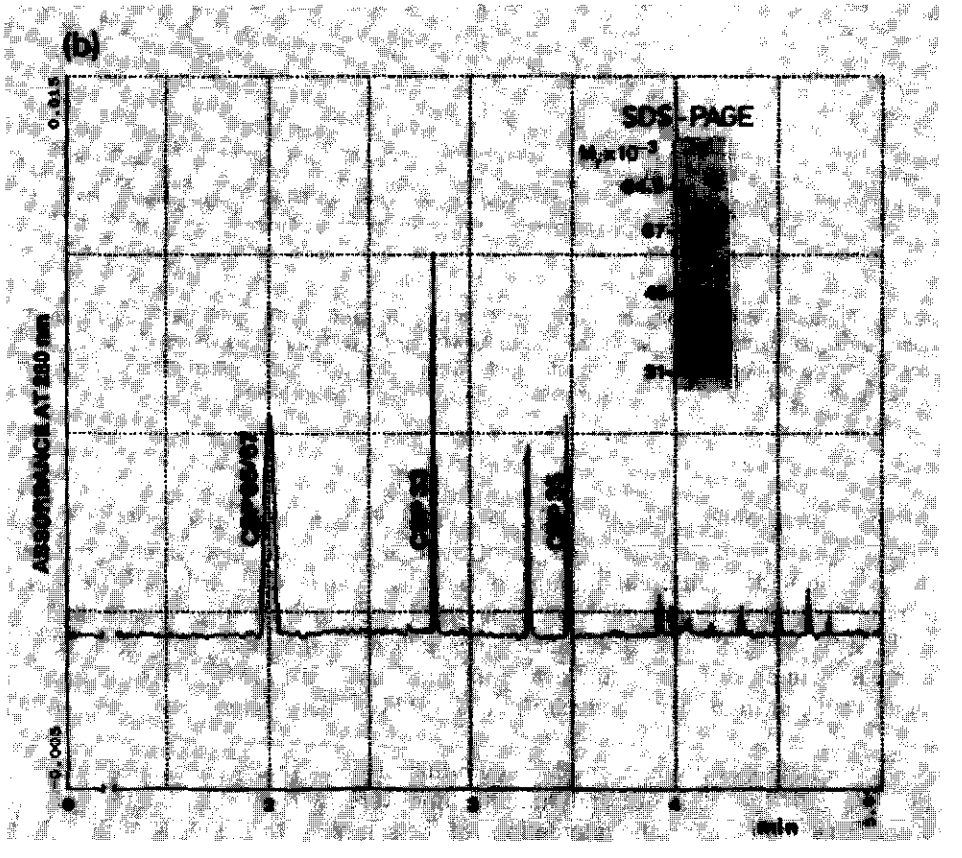

Fig. 1. Separation of calcium-binding proteins from (a) liver and (b) Morris hepatoma 7777 plasma membranes by HPCZE with addition of 7 *M* urea. The isolated membrane proteins (SDS-PAGE is shown in the inset) were injected into the capillary by pressure (0.5 bar). Injection time, 3 s. Other conditions are given under Experimental. M_r = Molecular mass.

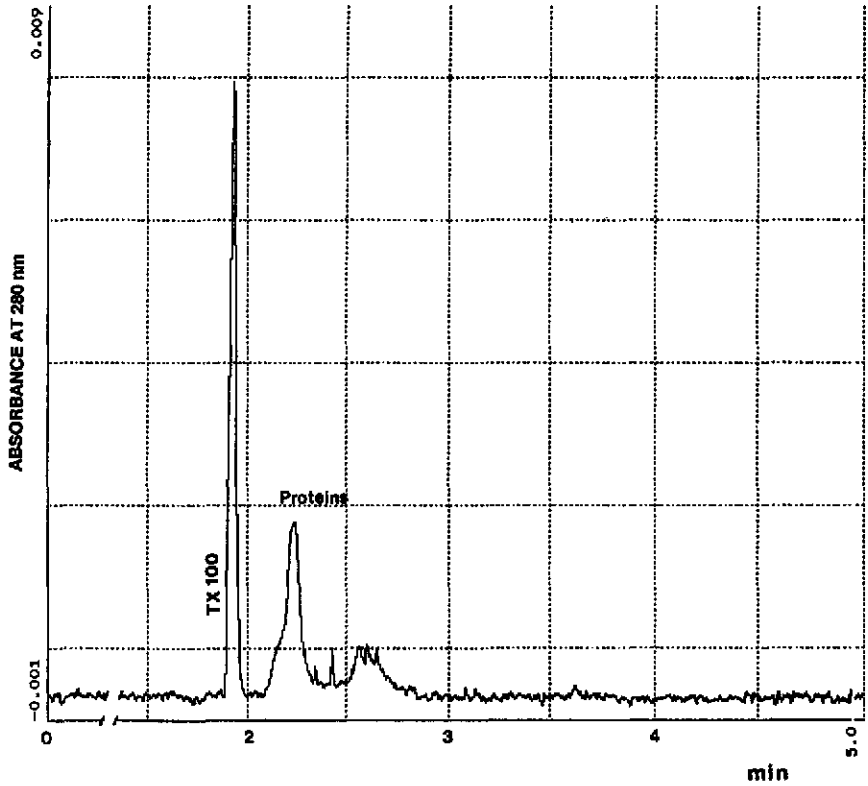

Fig. 2. Separation of intrinsic membrane proteins from liver by HPCZE with addition of 7 *M* urea. The proteins with an apparent molecular mass between 95 000 and 120 000 dalton in SDS-PAGE were injected under the same conditions as in Fig. 1 and separated. The first peak in the electropherogram was attributed to the detergent Triton X-100. The other peaks result from separated proteins.

Moreover, the protein is highly glycosylated and contains more than 50% carbohydrate. The carbohydrate part consists of at least twelve chains, which terminally contain sialic acid[19]. Different amounts of sialic acid can lead to further differences in the moblity of the isoforms of this protein. DPP IV contains about 24% carbohydrate in at least six chains[20]. Therefore, microheterogeneity in this glycoprotein also can be assumed, owing a different sialic acid content. The third protein (95 000 dalton) is probably an enzymatically inactive form of DPP IV, which is glycosylated in another way[21].

In all the separations of model membrane proteins that were carried out with HPCITP, both the peak size and running time were reproducible, because of two factors. First the non-specific interactions of the sample with the walls of the PTFE capillary are minimal, and second the HPMC that is added to the leading electrolyte to increase the viscosity also acts as a detergent an helps to keep the proteins in solution during separation.

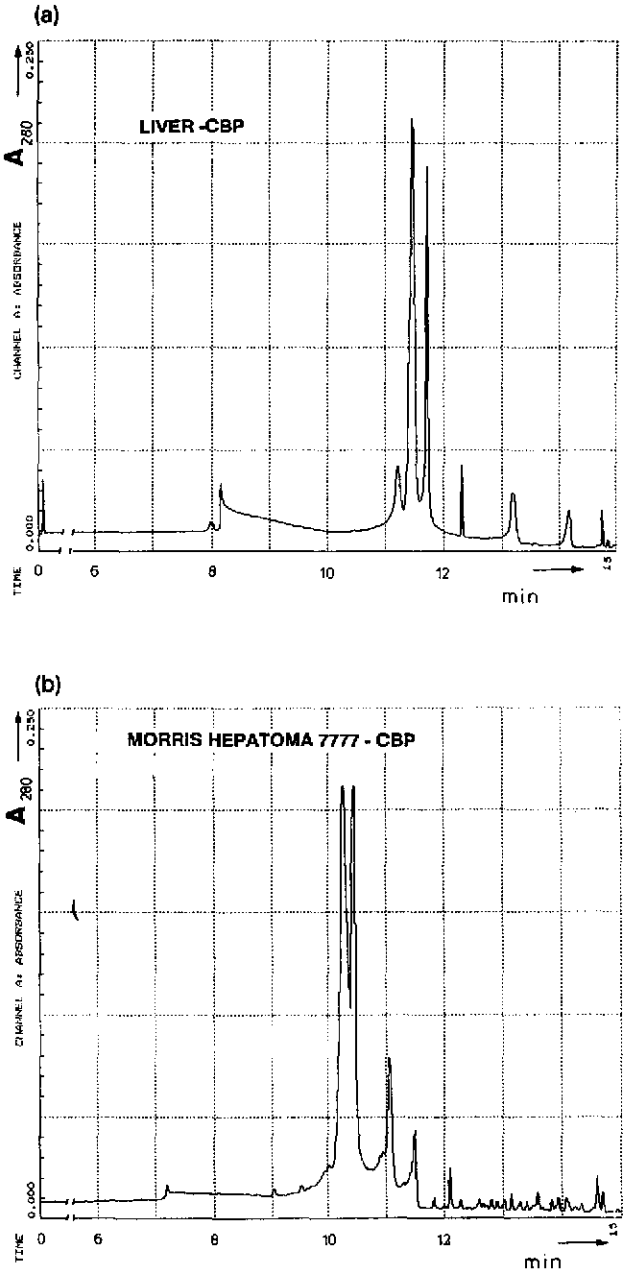

Fig. 3. Separation of calcium-binding membrane proteins from (a) liver and (b) Morris hepatoma 7777 by HPCITP without addition of spacers. The separation was carried out on a Beckmann HPCE system. Injection conditions as in Figs. 1 and 2. For SDS-PAGE, see Fig. 1b. Other conditions are given under Experimental.

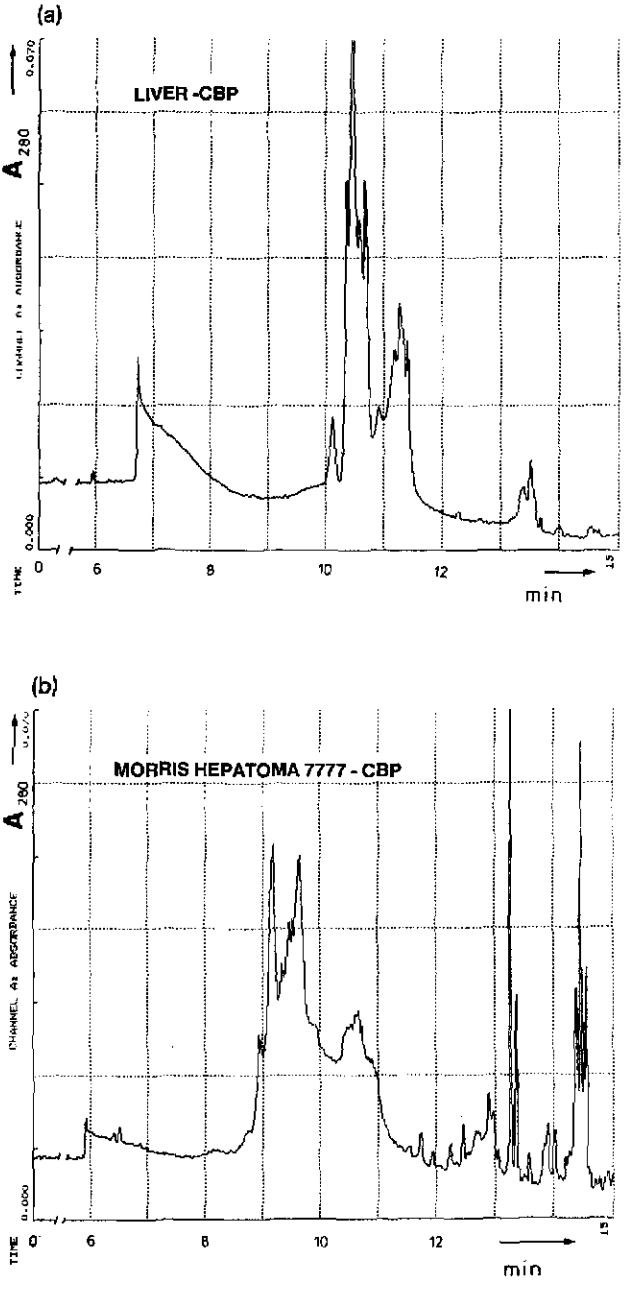

Fig. 4. Separation of calcium-binding membrane proteins from (a) liver and (b) Morris hepatoma 7777 by HPCZE with addition of ampholytes as spacers in the pH range 5–7. Other conditions as in Fig. 3.

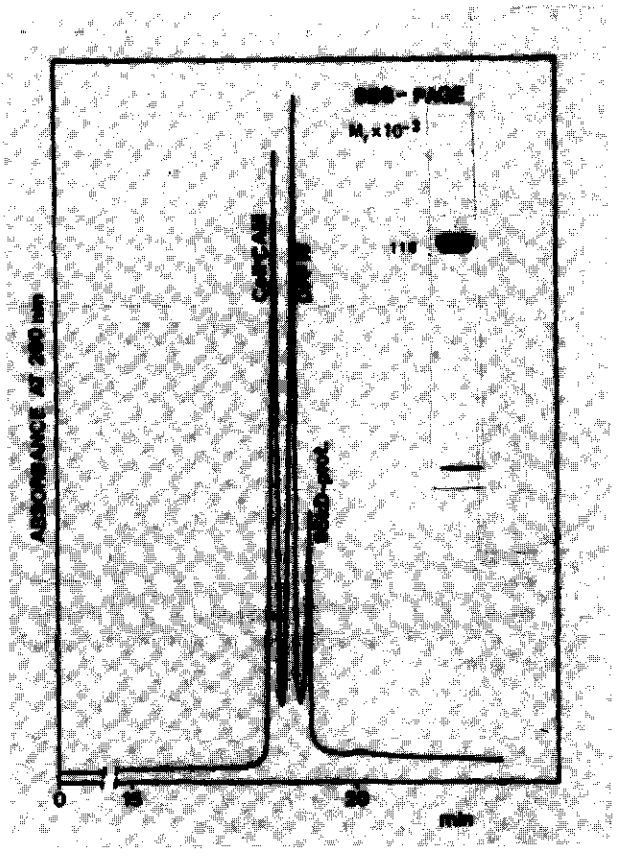

Fig. 5. Separation of intrinsic membrane proteins from liver by HPCITP without spacers. The three peaks belong to the three main components in the mixture, DPP IV, cell-CAM and 95 000-dalton protein (95kD-prot.)[12]. One dimensional SDS-PAGE is shown in the inset. Other conditions as in Fig. 3.

CONCLUSIONS

The HPCZE and HPCITP methods described can be applied to the separation of membrane proteins. Precipitation of the proteins, which can occur during separation either on reaching the isoelectric point or through the separation of detergents, must be avoided. This can be achieved by applying detergents and/or denaturing reagents in HPCZE and HPCITP. The non-specific interactions with the surface of the capillary have to be suppressed. In this respect the nature of the capillary walls (PTFE or coated capillaries) plays an important role.

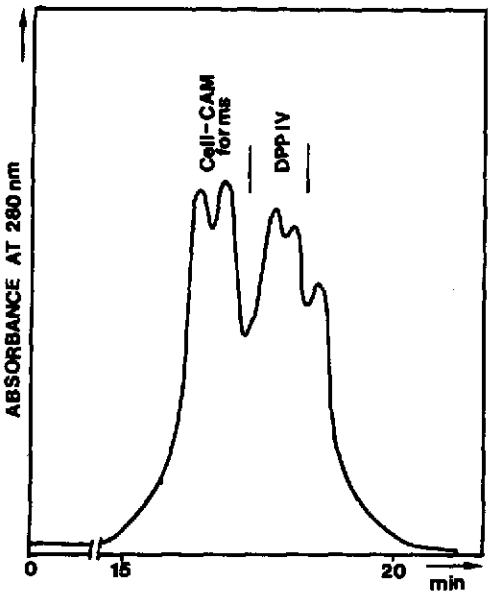

Fig. 6. Separation of intrinsic membrane proteins from liver by HPCITP with addition of ampholytes as spacers in the pH range 4–6. The same mixture as in Figs. 2 and 5 was separated. The splitting of single peaks is due to the microheterogeneity of these glycoproteins.

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