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High-performance membrane chromatography of serum and plasma membrane proteins

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ABSTRACT

Porous discs made of poly(glycidyl methacrylate) were used for high-performance membrane chromatography (HPMC) of proteins. In model experiments, separations of standard proteins by anion-exchange HPMC using a DEAE disc were carried out. The influences of sample distribution and disc diameter and thickness on separation performance were studied. The separation disc allowed a scaling-up from analytical (diameter 10 mm) to semi-preparative (diameter 50 mm) dimensions. In an application study, separations with anion-exchange and affinity HPMC were carried out using different complex samples such as rat serum and plasma membrane proteins. In all experiments the results on poly(glycidyl methacrylate) discs were comparable to those achieved on adequate high-performance liquid chromatographic (HPLC) columns. However, the separations on HPMC discs could be carried out faster than corresponding separations on HPLC columns. The pressure drop on the discs was low even at high flow-rates. The experiments show that the poly(glycidyl methacrylate) discs used are especially suitable for the isolation of proteins and other biopolymers which occur in a diluted state in complex mixtures.

INTRODUCTION

High-performance membrane chromatography (HPMC) was introduced several years ago, chiefly for biopolymer separation. Initial investigations revealed that HPMC can be used in affinity chromatography with results similar to those achieved with high-performance liquid chromatographic (HPLC) columns [1–4]. Several techniques of membrane construction exist. One strategy consists in bundling several thin membranes made of synthetic hollow fibres or cellulose, another involves compact, porous, disc-shaped membranes, made of silica gel or polymer supports, and a third option is a combination of these two strategies. The beads, which are non-porous in most instances, are polymerized between the fibres. Remarkable results in biopolymer separations have been achieved especially with separation devices that consist of several bundled membranes and with compact discs [1–4]. The membranes with beads polymerized into hollow-fibre matrices have a low capacity for larger molecules. However, they can be used for sample preparation, *e.g.*, desalting of sugar mixtures before HPLC analysis [5].

Apart from analytical and preparative separations of biopolymers, membranes can be used for the immobilization of enzymes. With such a reactor, containing the immobilized enzyme carbonic anhydrase, synthetic substrates could be converted successfully in a very rapid manner [4].

Membranes have several important advantages over HPLC columns. The chromatographic separation is carried out on a wide, thin disc, so that there is only a pressure drop even at high flow-rates. The fact that separations on membranes can be carried out very quickly is due to the fast reaction kinetics in these systems [6]. Improved reaction kinetics in a disc also explain a phenomenon that occurs in membrane reactors with immobilized carbonic an-

hydrase, which may appear puzzling at first, namely increased enzymatic activity at higher flow-rates [4]. Technical problems have hampered further increases in the use of HPMC. The bundling of thin membranes often leads to leakages in the system, which in turn causes the mobile phase (and the sample) to flow beyond the edges and therefore past the membranes. Another equally important problem is the distribution of the sample or the mobile phase, coming out of a narrow capillary, on the wide disc surface. If the dead volume is large enough, distribution is ensured before and after the disc, but the separation performance is impaired owing to peak broadening.

This paper deals with the HPMC of standard proteins and serum and membrane proteins with diethylaminoethyl (DEAE) groups, heparin and collagen as ligands. Compact discs were used, made of poly(glycidyl methacrylate). Through the introduction of distribution discs, the problems of sample distribution have been solved to a great extent. The identical chemical and mechanical properties of the separation disc allowed a scaling-up from analytical to (for the time being) semi-preparative dimensions.

EXPERIMENTAL

Animals and chemicals

Male or female Wistar or Buffalo rats (Institut für Molekularbiologie und Biochemie, Berlin, Germany), weighing about 160–180 g each were fed on a commercial diet containing 18–20% (w/w) of protein (Altromin R, Altromin, Lage/Lippe, Germany). Chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Sigma (Munich, Germany). All detergents were purchased from Sigma.

The following standard proteins were used: ferritin, immunoglobulin G, transferrin, bovine serum albumin (BSA), ovalbumin, conalbumin and soybean trypsin inhibitor (STI). Other samples containing protein were rat serum and rat kidney, liver and Morris hepatoma plasma membranes. Plasma membranes were isolated by zonal centrifugation using a Kontron centrifuge (Kontron Analytik, Munich, Germany) as described elsewhere [3,7]. Membrane purity was routinely checked by electron microscopy and by assaying of marker enzymes as described by Tauber and Reutter [7]. Protein in membrane and serum samples was determined according to the procedure of Lowry *et al.* [8] or in the presence of detergent according to Smith *et al.* [9], using a protein determination kit (Pearce, Rodgau, Germany).

Enzyme assays

Enzyme units are given as micromoles per minute. Nucleotide pyrophosphatase (NPPase) was routinely assayed according to a modified method of Elovson [10]. An amount of 100 μl of substrate solution, containing thymidine 5′-monophosphate *p*-nitrophenyl ester was added to 290 μl of 100 m*M* Tris–HCl (pH 8.9) and 10 μl of sample. The mixture was incubated at 37°C for 30 min. Enzymatic reaction was stopped by adding 600 μl of 0.1 *M* NaOH, and the absorbance at 405 nm was measured using a Eppendorf (Hamburg, Germany) spectrophotometer. Dipeptidyl peptidase IV (DPP IV) activity was determined according to the method of Nagatsu *et al.* [11], using tosylglycineproline *p*-nitroanilide (Bachem, Bubendorf, Switzerland) as substrate. Between 10 and 100 μl of sample were added to 90–180 μl of 0.1 *M* Tris–HCl buffer (pH 8.0) and 10 μl of substrate, and incubated at 37°C for 30 min. The reaction was stopped by adding 800 μl of 1 *M* sodium acetate (pH 4.5) and the absorbance was measured at 405 nm using an Eppendorf spectrophotometer.

HPLC

The HPLC system consisted of two pumps, a programmer, a spectrophotometer with a deuterium lamp and a Knauer loop injection valve (all from Knauer, Berlin, Germany) and a fraction collector (Bio-Rad, Munich, Germany). In ion-exchange chromatography the salt gradient was controlled by measuring the osmotic pressure (Halbmikro-Osmometer, Type Dig. L; Knauer).

Columns and membranes (discs)

The membranes and the poly(glycidyl methacrylate) discs that were used for separation were produced using the method of Tennikova *et al.* [1]. The thickness of the membrane layers was 1, 2, 3 and 7 mm. They were cut out in a disc shape of 10, 20, 25, 40 and 50 mm diameter using moulds of high-grade

steel and then installed in the corresponding cartridges. When the separation discs were used for ion-exchange or hydrophobic-interaction HPMC, the chosen ligands were previously bound synthetically [1,12,13]. In affinity chromatography the ligand was positioned on the membrane in epoxy form *in situ*, according to the following procedure (this applies to the 25 mm diameter disc): after installing the disc in the appropriate cartridge, any remaining non-polymerized components were washed out with 20 ml of methanol. The disc was then rinsed with 40 ml of doubly distilled water and 40 ml of 0.1 *M* sodium phosphate buffer (pH 8.0) containing 0.5 *M* NaCl. The ligand, *e.g.*, heparin, was added in amounts of 50 mg per gram of support. For a 25 mm diameter disc of thickness 2 mm (about 0.5 g of support), 25 mg of heparin in 25 ml of 0.1 *M* sodium phosphate buffer (pH 8.0) (binding buffer) were mixed with 0.5 *M* NaCl and pumped at a flow-rate of 1 ml/min. This solution was left to circulate on the disc for at least 2 h. Subsequently the disc was rinsed with 50 ml of binding buffer. Any remaining free epoxy groups were blocked with 0.2 *M* Tris–HCl buffer (pH 8.0). The disc was then rinsed with phosphate-buffered saline (PBS) (pH 7.4) and stored at 4°C after further use. The immobilization of collagen has been described elsewhere [3].

Other anion-exchange membranes were Mem Sep DEAE (Millipore, Eschborn, Germany), 20 mm in diameter and *ca.* 10 mm thick, Zeta Prep DEAE (Atlanta, Heidelberg), 50 mm in diameter and *ca.* 10 mm thick and Acti Disc DEAE (FMC, Rockland, ME, USA), 40 mm in diameter and 2 mm thick. Membranes from the above producers with different active groups were also used for immobilization of heparin or collagen.

For HPMC experiments with porous discs, a disc cartridge developed in our laboratory was used (see below). For some experiments an Amicon (Danvers, MA, USA) ultrafiltration cell of 40 mm diameter and 50 ml volume was used [4].

For comparison, HPLC columns packed with silica gel or polymer beads were used. These were TSK DEAE 5 PW, 10 μm particle size, 1000 Å pore size, column dimensions 75 × 7.5 mm I.D. (Tosohaas, Yamaguchi, Japan) and Euramid WAEX, 7 μm particle size, 300 Å pore size, column dimensions 60 × 8.0 mm I.D. (Säulentechnik Knauer, Berlin, Germany). Eupergit C30N polymethacrylamide beads, particle size 30 μm, pore size *ca.* 500 Å, with active epoxy groups for ligand immobilization (Röhm Pharma, Weiterstadt, Germany) were used as the support for affinity chromatography. The immobilization of the ligands on Eupergit C30N beads has been described elsewhere [12].

Buffers

The buffers used for anion-exchange HPMC and HPLC were (A) 10 m*M* Tris–HCl (pH 7.6) and (B) was buffer A containing 1 *M* sodium chloride. In some experiments 0.1–0.25% (v/v) of Triton X-100 (reduced) was added to both buffers.

Buffer A for heparin and collagen HPMAC was 5 m*M* Tris–HCl (pH 8.0) and buffer B was buffer A containing 500 m*M* sodium chloride. In some experiments 0.25% (v/v) of Triton X-100 (reduced) or another detergent was added to both buffers.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The dialysed and freeze-dried samples were dissolved in 62.5 m*M* Tris–HCl buffer (pH 6.8) containing 3% (w/v) of SDS, 5% (v/v) of mercaptoethanol, 10% (v/v) of glycerol and 0.001% (w/v) of bromophenol blue. In other experiments, 10–30 μl of sample were taken from the collected fractions after HPMC or HPLC separation and mixed with buffer containing five times higher concentrations of the above-mentioned substances. The amount of the buffer taken for the experiments was measured in such a way as to yield the original concentration after dilution with the sample. SDS-PAGE was carried out by the Laemmli method [14], using a mini system (Protean; Bio-Rad). The amount of applied protein was between 5 and 20 μg per line.

RESULTS AND DISCUSSION

Construction of the separation unit

Sample distribution is one of the main problems when wide but thin discs are used for separation. Tennikova *et al.* [1] overcame these difficulties by installing the chromatographic unit, that is, the disc, at the bottom of a filtration device. The sample or, during rinsing and elution, the mobile phase, is added and pumped through the porous disc. A disadvantage of this method is the very high dead volume accumulating before the separation unit, the

disc or the membrane. If the sample is applied by an HPLC pump, its dilution through the following mobile phase is inevitable. The use of an Amicon filtration device for concentration of protein solutions avoids these consequences [4]. The chromatographic disc is installed instead of an inert, porous membrane at the bottom of the filtration device. The sample is transported through the porous disc by means of compressed air. The container is subsequently connected with the HPLC pump and filled with buffer A. In the next step the elution buffer is pumped into the container and stirred. This results in a buffer gradient with an exponential slope. A chromatogram obtained through this method, using a DEAE disc, is shown in Fig. 1.

The disadvantage of this method lies in the complicated sample application. When rinsing the disc and during subsequent elution, the container has to be switched from the compressed air pipe to a chromatographic pump. During this procedure it is also difficult to reproduce the gradient. It is therefore advisable to control the salt concentration, *e.g.*, by measuring the osmotic pressure in the collected fractions (see Fig. 1).

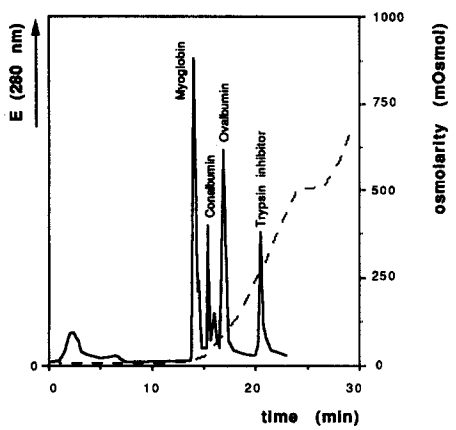

Fig. 1. Anion-exchange HPMC of standard proteins. The chromatographic unit, with a DEAE poly(glycidyl methacrylate) disc was installed at the bottom of an Amicon filtration device. The sample was applied by means of compressed air. The container was subsequently filled with buffer A and proteins were eluted by an exponential salt gradient (see also text and ref. 2). Chromatographic conditions: separation unit, DEAE poly(glycidyl methacrylate) disc, diameter $d = 40$ mm, thickness $h = 2$ mm; buffer A, 10 m*M* Tris–HCl (pH 7.6); buffer B, buffer A containing 1 *M* NaCl; flow-rate 1.5 ml/min; pressure, 1–2 bar; room temperature. The slope of the exponential gradient was controlled by measuring the osmotic pressure (dashed line).

The advantage of this method is that it allows fairly large amounts of diluted sample to be applied. Only one pump is required for gradient elution. Continuous stirring, which takes place before the separation unit, provides good sample distribution, thereby making use of the entire disc surface. This kind of sample application is therefore recommended in preparative chromatography with its large sample volumes. It is also useful in affinity chromatography, where elution is usually carried out by a step gradient. When discs are used for enzyme immobilization, this strategy is the method of choice for the enzymatic conversion of the substrate [4].

Another option for solving the sample distribution problem is the construction of holders with large dead volumes before the separation membrane or disc. Such systems have fairly large capacities. However, their use in high-performance chromatographic separations is limited, as peak broadening is considerable, as shown in Fig. 2. The application of such units for the concentration of certain components in the sample preparation procedure, or for the removal of components, has proved very effective.

With the MemSep cartridges, and partly with the ZetaPrep and FMC systems, the ratio between the width of the disc and its thickness is much more favourable, owing to bundling of single membranes. This eases the problem of sample distribution, but leads to difficulties through leakages. The

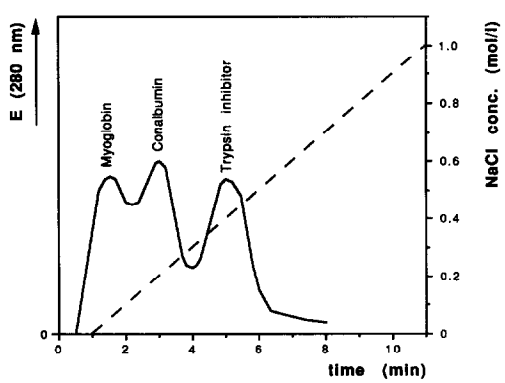

Fig. 2. Anion-exchange HPMC of standard proteins by using the chromatographic disc embedded in a filtration unit. Chromatographic conditions: separation unit, Acti-Disk (FMC), $d = 40$ mm, $h = 2$ mm; separation buffers as in Fig. 1; flow-rate, 5 ml/min; pressure, 1 bar; room temperature. The gradient is shown (dashed line).

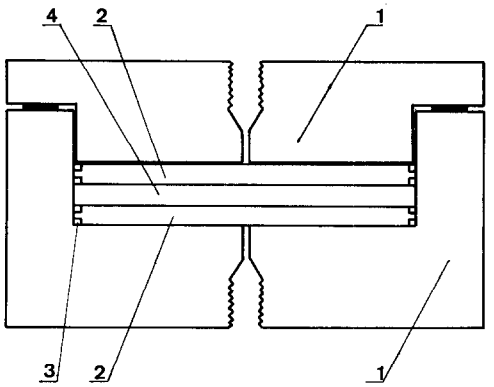

Fig. 3. Construction scheme of the disc cartridge for HPMC. The diameters of the discs used in the experiments were 10, 25 and 50 mm. 1 = Holder; 2 = distribution plates; 3 = O-rings; 4 = disc made of poly(glycidyl methacrylate).

seals have to be installed in such a way that the sample is pumped through the membrane and not through the hollow space between the membrane and the cartridge. After initial difficulties, the problem was solved for MemSep cartridges, for instance, by positioning appropriate seals between single membranes. The problem of inadequate pressure resistance of such discs has not yet been solved satisfactorily. So far the cartridges can only be operated at pressures up to about 10 bar.

When compact discs made of porous, synthetic materials are used, bundling is not so favourable. Polymerization allows the direct production of thicker layers. Leakage problems are easily avoided through O-rings. The construction scheme of the type of disc cartridge used in the experiments is shown in Fig. 3. As mentioned before, the problem of sample distribution is most acute when relatively thin but wide discs are used. Experiments have shown that only a small portion of the disc, usually in its centre, is used for the binding or separation of the sample, when distribution plates are taken, which are in common use for HPLC columns. The jet of liquid coming out of a capillary with a maximum diameter of 1 mm could not be distributed adequately on a surface of 25 mm diameter. Apart from poor distribution, another problem arose. The extreme pressure building up in the centre of the disc shortened the life of the separation device considerably. Therefore, appropriate distribution plates had to be installed before and behind the separation device. The patterns in Fig. 4. give an idea of the construction of the distribution plates.

In Fig. 4 the sample distribution using different types of plates is shown. By using a disc with active epoxy groups, the distribution of a sample could be monitored by injecting a ferritin solution. The protein ferritin with its iron content and reddish colour reacts on reaching the disc with its reactive epoxy groups and is immobilized. This results in an illustration of the distribution pattern of the sample. In all twenty plates were investigated, and only a few typical patterns are shown in Fig. 4. As can be seen, the best distribution is achieved with plate 3. With

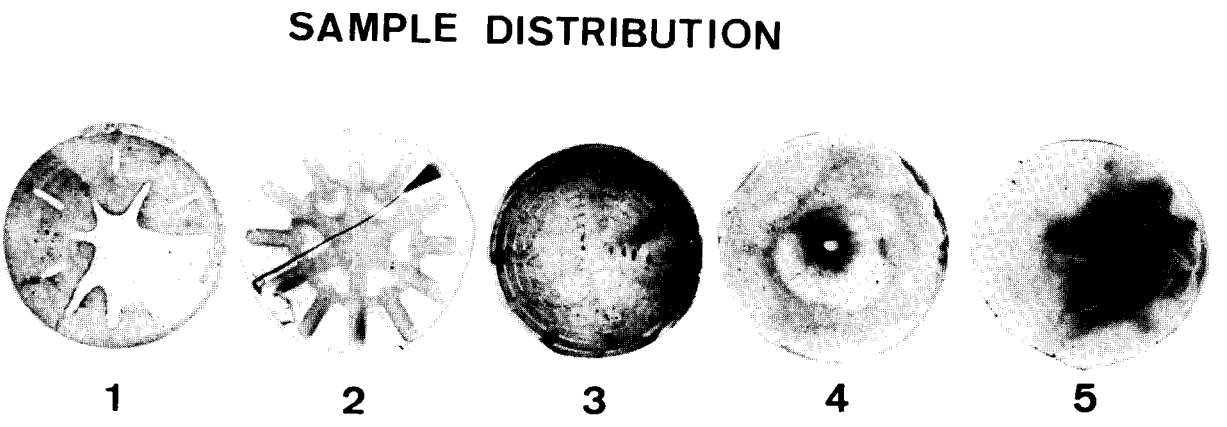

Fig. 4. Sample distribution on the surface of an HPMC disc using different types of distribution plates. The distribution patterns were made by immobilization of ferritin on an epoxy-activated poly(glycidyl methacrylate) disc of 25 mm diameter and 2 mm thickness. For more details see text.

plate 1, for example, only about 60% of the available disc surface was used owing to poor sample distribution. With chromatographic columns, and even with those separation devices which contain bundled membranes, the ratio between layer thickness and diameter is much better. Poor distribution on the surface is compensated for through subsequent spreading in the first few millimetres of the separation layer. Such compensation is impossible when the layer of a disc with a large diameter is very thin, invariably resulting in lower capacity and poor separation. Fig. 5. shows the chromatograms on a DEAE disc of 25 mm diameter and 2 mm thickness. A comparison was made between a separation carried out with an optimized distributor and one carried out without such optimization. Fig. 5 also shows a MemSep disc chromatogram. This disc consists of a large number of bundled membranes. Finally, a chromatogram is shown (Fig. 5D) which was obtained on an HPLC column using the same standard proteins.

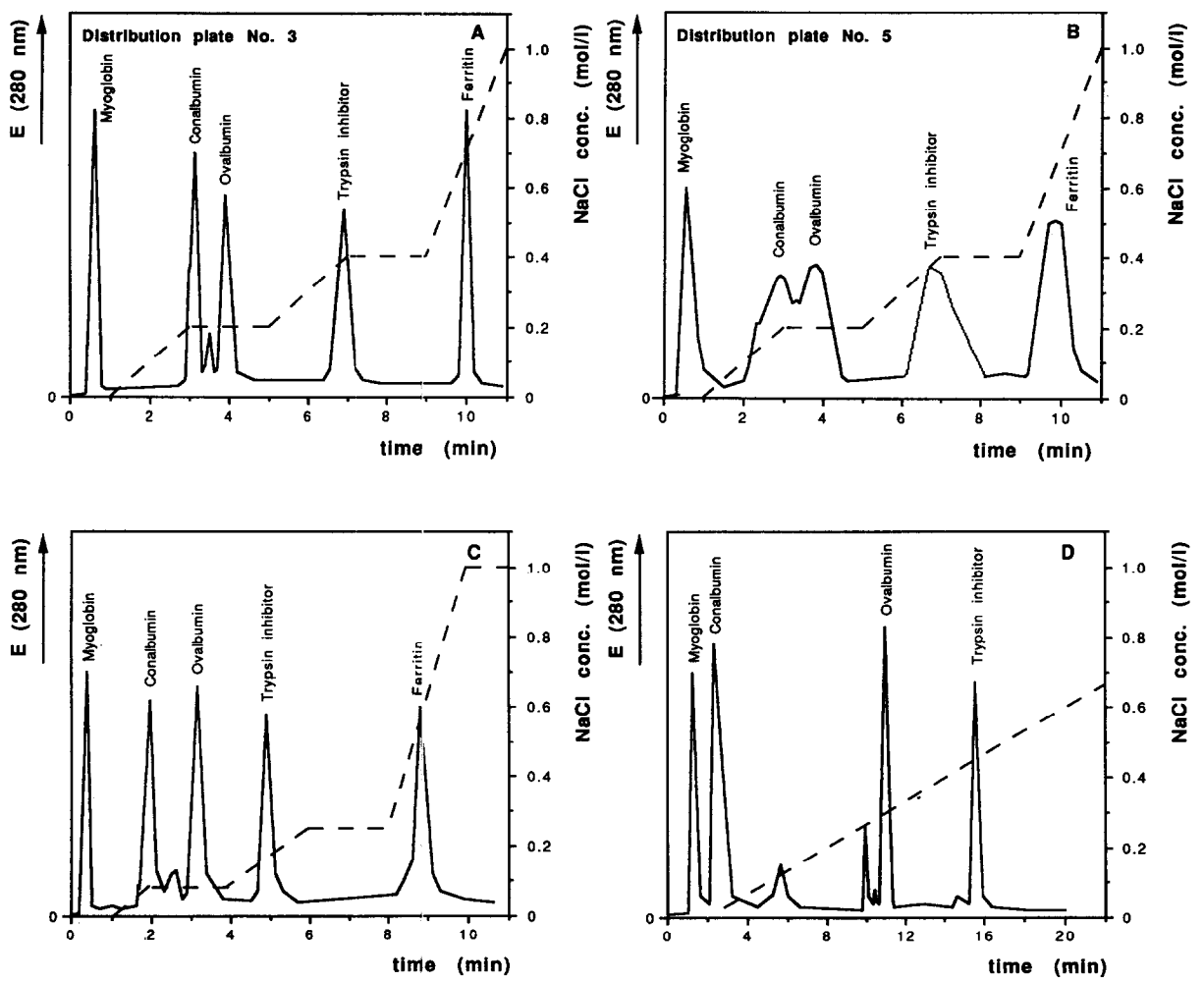

Fig. 5. Anion-exchange HPMC of standard proteins on two different types of discs and cartridges in comparison with the separation on a HPLC column. (A and B) a DEAE poly(glycidyl methacrylate) disc, $d = 25$ mm, $h = 2$ mm, was used, (A) with optimized distribution; (C) separation performed on a MemSep disc consisting of a large number of bundled membranes; (D) separation with a WEAX HPLC column (see also Experimental). Chromatographic conditions: flow-rates on discs, 3 ml/min; pressure, 1–4 bar; the flow-rate on HPLC column, 1 ml/min; pressure, 10–12 bar. Buffers and other conditions as in Figs. 1 and 2.

Optimization of separation

According to investigations by Unarska *et al.* [6], reaction rates in membranes made of nylon fibres are several hundred times faster than those in the corresponding low-pressure columns with agarose beads. In the experiments a system with protein A as ligand and IgG as ligate was used. The much improved reaction kinetics with a disc made of porous poly(glycidyl methacrylate) with the immobilized enzyme carbonic anhydrase also explain the at first puzzling phenomenon of increased enzymatic activity at higher flow-rates [4]. We therefore decided to investigate the influence of the flow-rate on chromatographic separations with poly(glycidyl methacrylate) discs. The results of such an experiment are shown in Fig. 6. It can be seen that a separation of three standard proteins can be achieved within 10 min or less. The optimal flow-rate for discs of 25 mm diameter and 2 mm height is between 3 and 5 ml/min.

In Fig. 7, the influence of disc thickness on separation performance is shown. With increasing thickness better separation is achieved. At present the maximum thickness that is technically possible is 10 mm. However, thicker layers may lead to further improvements in performance, once they can be produced and installed in holders through appropriate technology. Similar results were achieved

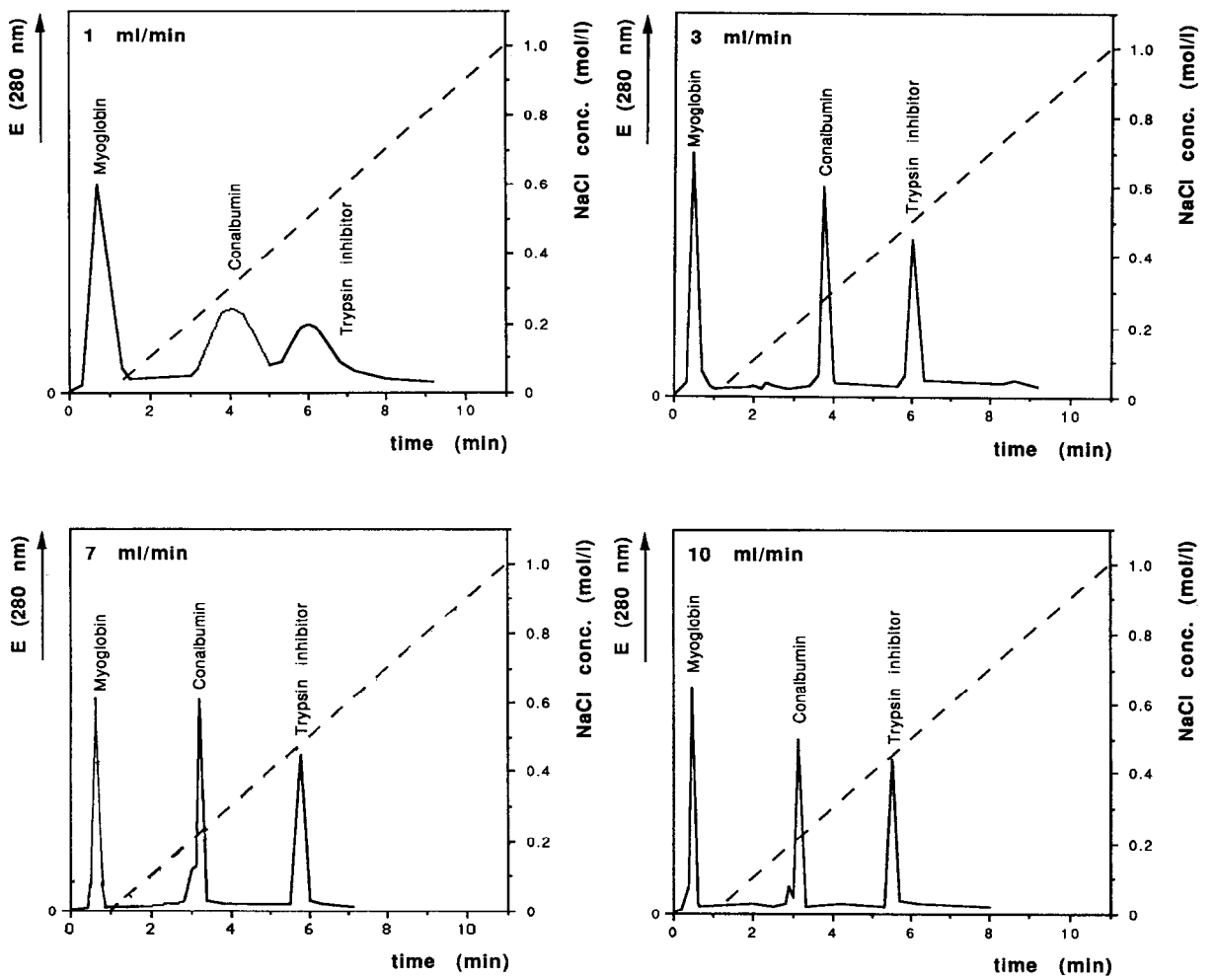

Fig. 6. Influence of flow-rate on chromatographic separation using a DEAE poly(glycidyl methacrylate) disc. Discs of 25 mm diameter and 2 mm thickness were used. Other chromatographic conditions as in Figs. 1 and 2.

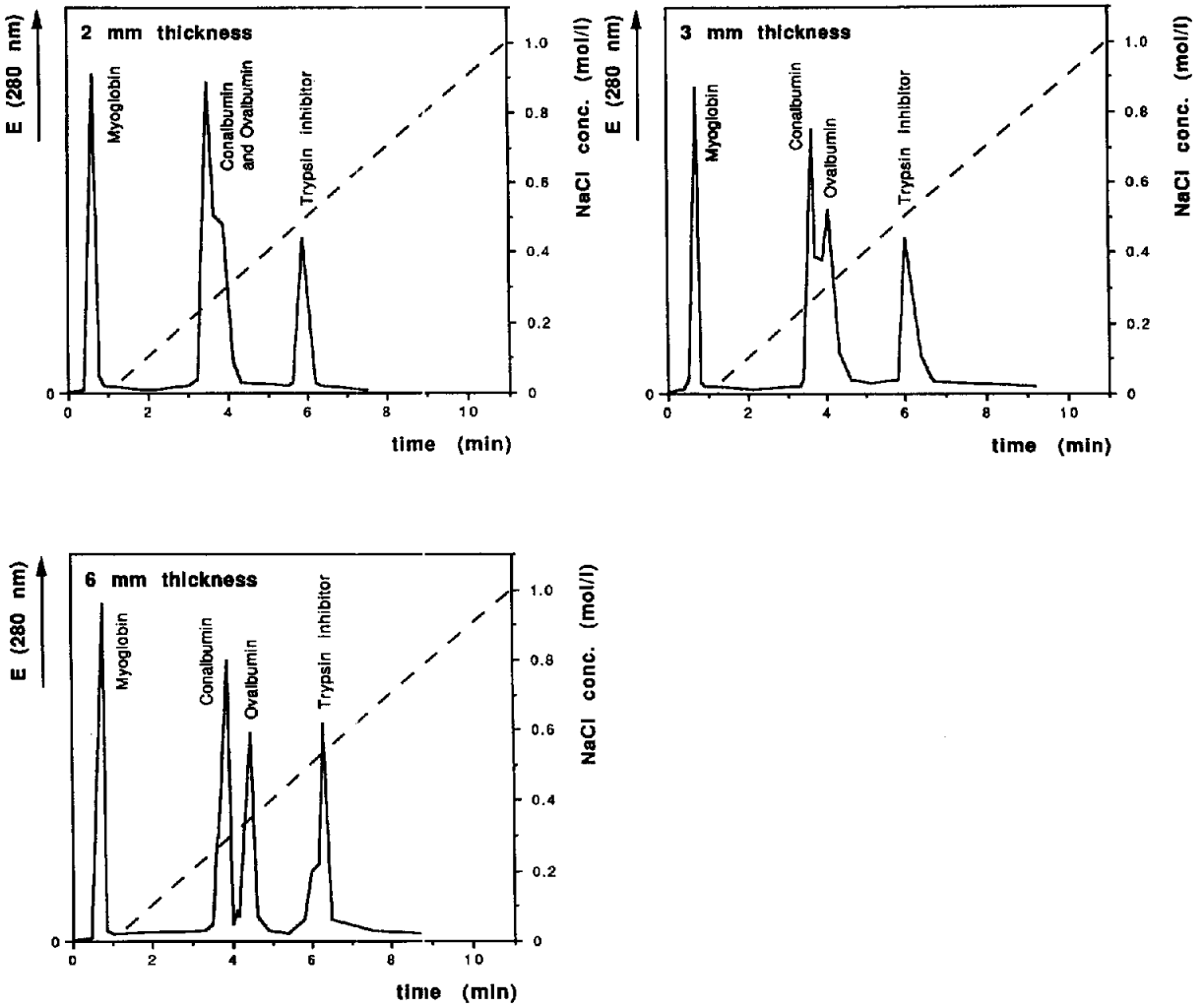

Fig. 7. Influence of disc thickness on separation performance. Several DEAE poly(glycidyl methacrylate) discs of 25 mm diameter but different thicknesses were used. Chromatographic conditions as in Fig. 2.

with discs of 10 mm diameter (not shown here). Apart from the direct production of discs with different thicknesses, higher capacity and improved separation performance can be achieved through the bundling of thin discs. The MemSep and ZetaPrep discs, among others, adopt this approach. However, in these two instances very thin, elastic membranes made of hollow fibres are bundled. When porous poly(glycidyl methacrylate) discs are used instead, the options of bundling are limited. The compact disc is thicker and less flexible than fibre membranes. In such systems it is therefore more difficult to avoid leakages. Only when the disc diameter did not exceed 10 mm, could bundling be carried out without further complications. Separations with such a system are shown in Fig. 8. Here the difficulties can be overcome simply by producing thicker discs, so far up to 10 mm (see above). The results shown here are in some contrast to those obtained by Tennikova *et al.* [1], who did not observe any improvement in the separation performance when the flow-rate was increased or thicker discs were used. They carried out their experiments with discs whose chemical composition was identi-

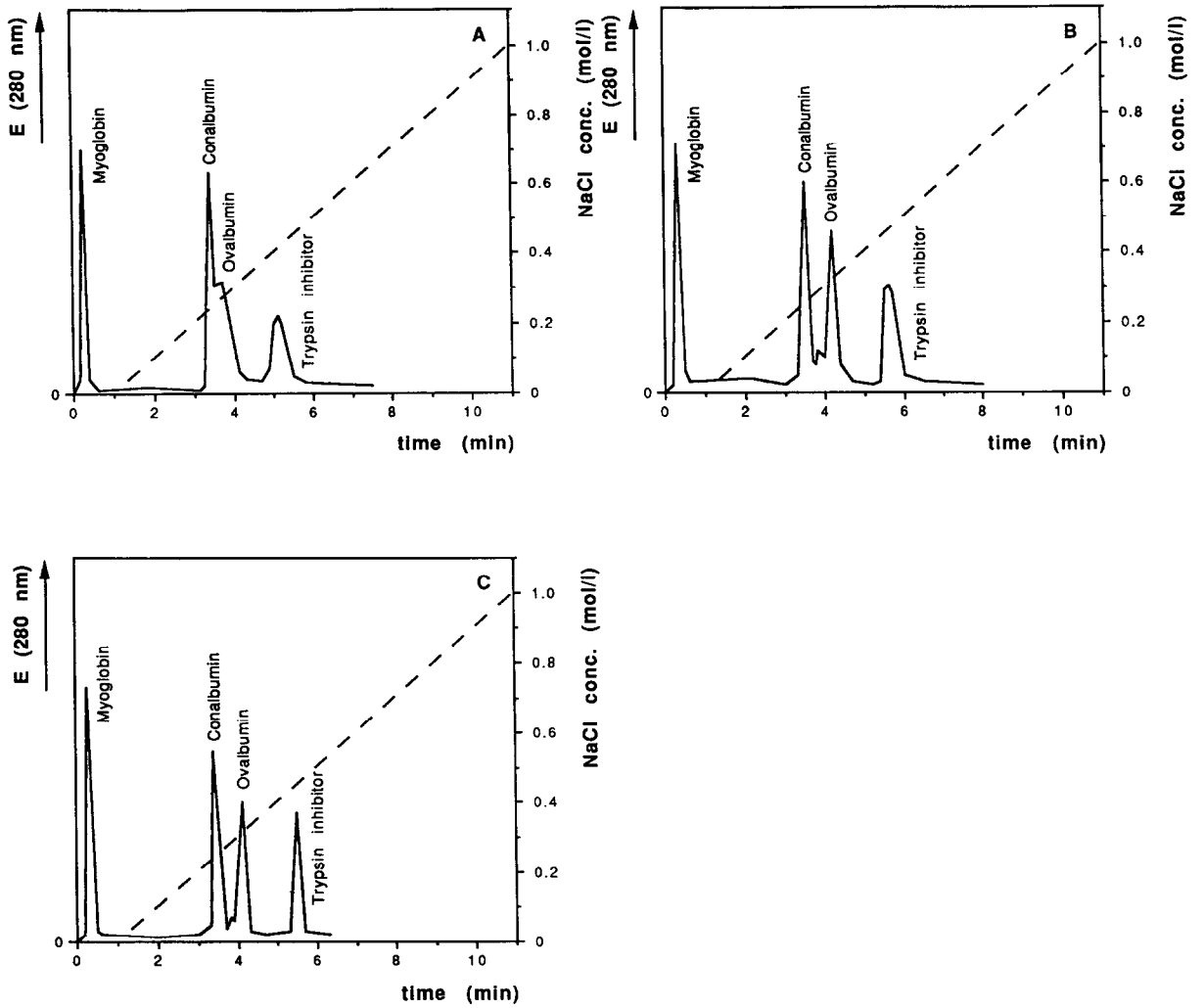

Fig. 8. Influence of disc thickness on separation performance. Comparison between separation with a compact disc and a separation unit constructed of several thin discs piled up. DEAE poly(glycidyl methacrylate) discs of 10 mm diameter were used. (A) Disc thickness 2 mm; (B) three discs were piled up, total thickness 6 mm; (C) separation on a compact disc of 6 mm thickness. Chromatographic conditions as in Fig. 2.

cal with those used here. However, the mechanical structure of their separation device was fundamentally different from the construction of the cartridge shown above.

Influence of detergents on separations

When poly(glycidyl methacrylate) discs are used for the separation of hydrophobic proteins, for their purification and possibly virus inactivation in the sample, it is important to check the influence of detergents on the separation and on the stability of the equipment. We investigated the non-ionic detergents Triton X-100 (reduced), Triton X-114 (reduced), octyl glucoside and decanoyl- and octanoyl-N-methylglucamide (MEGA-10 and MEGA-8), and also the zwitterionic detergent 3-[(3-chloro)dimethylammonio]-1-propane sulphonate (CHAPS) and the anionic detergent SDS.

As the support is strongly hydrophilic, one would expect that the use of non-ionic detergents would

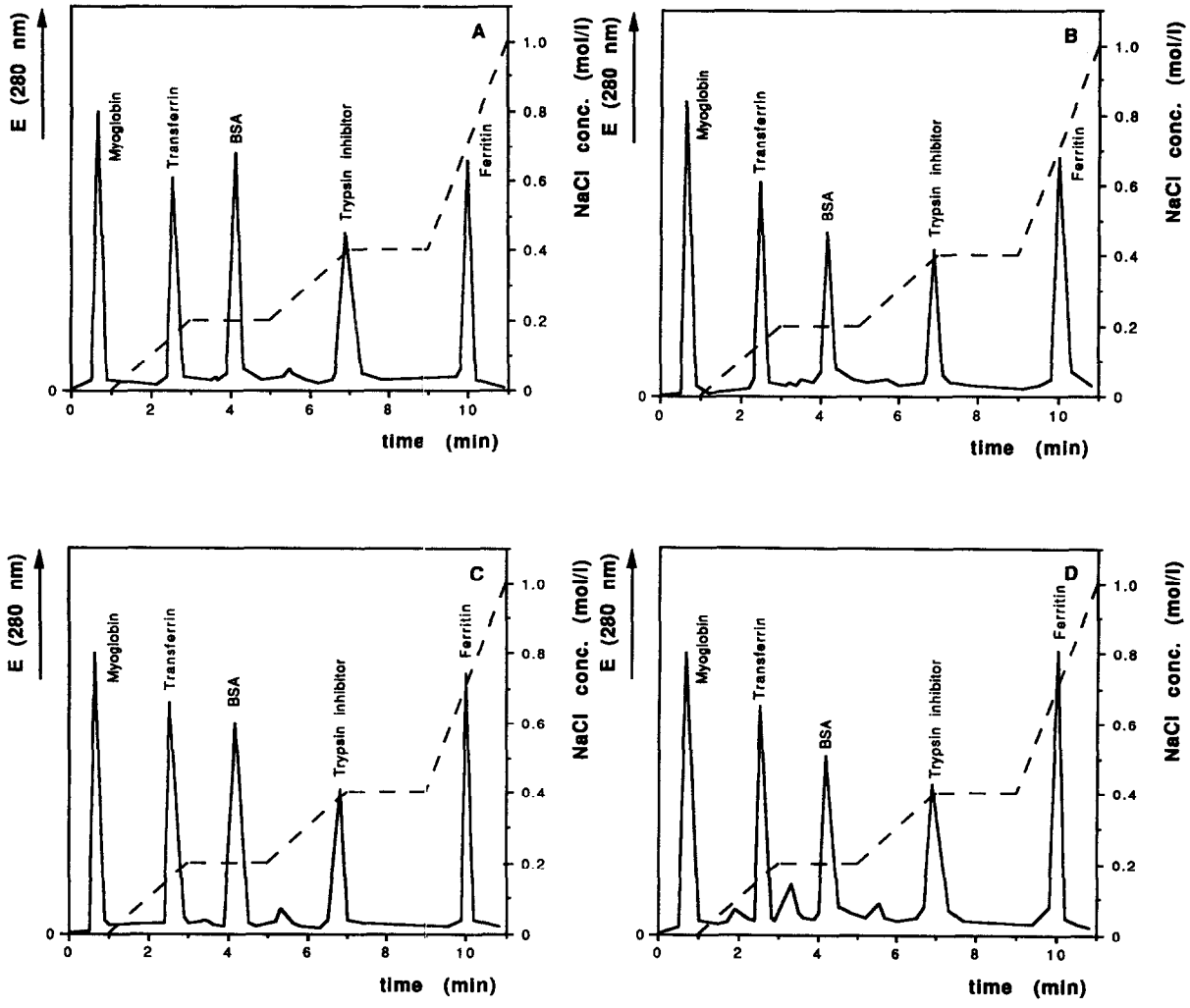

Fig. 9. Influence of non-ionic and zwitterionic detergents on anion-exchange HPMC. Detergents added to both buffers: (A) Triton X-100 (reduced), 0.25% (v/v); (B) octyl glucoside, 0.25% (w/v); (C) CHAPS, 0.25% (w/v). (D) Separation of standard proteins without addition of detergent, after more than 100 runs. In some of the previous runs ionic or non-ionic detergents were used. Chromatographic conditions as in Fig. 2.

have no negative influence on the separation [1,13]. Fig. 9 shows separations of standard proteins without and with detergent, here the non-ionic detergent Triton X-100 (reduced), the non-ionic detergent octyl glucoside and the zwitterionic detergent CHAPS. The separation can be carried out with 0.25% Triton X-100 (reduced). At concentrations of Triton X-100 (reduced) higher than 0.25%, several technical problems arise. For instance, increased amounts of foam impaired optical detection. Also, the solubility of the detergent is poor at high salt concentrations, which can lead to the clouding of buffer B. With the use of Triton X-114 (reduced), the solubility problems in buffer B are even more acute. For example, in affinity chromatography, where the salt concentrations in the buffers are lower and where the separation is monitored by SDS-PAGE instead of optical detection, higher concentrations of Triton detergents can be used [4]. Other detergents, such as octyl glucoside,

MEGA-10 and CHAPS, can be used in higher concentrations, up to about 1%, without adversely affecting the separation. Octyl glucoside was tested up to a concentration of 2%.

Although some detergents were used at concentrations that exceeded their respective critical micelle concentrations, no difficulties were observed in the experiments. With Triton X-100 and especially Triton X-114, high salt concentrations of 1 *M* NaCl in buffer B together with a detergent concentration of 0.25% (v/v) or more, clouding can occur. However, this impaired the optical detection only and left the chromatographic separation unaffected. Consequently, a concentration of about 0.25% (v/v) of Triton X-100 (reduced) and 0.1% (v/v) of Triton X-114 (reduced) have to be considered as the maxima for optical detection. As had to be expected, no separation was achieved when SDS was added. This detergent binds to the proteins and consequently they all have uniform negative charges, so they can no longer be separated through different charges on the molecule surfaces (the chromatogram is not shown). Treatment of the discs with SDS can be essential for removing hydrophobic substances during the purification procedures. The experiments with this detergent were carried out in order to verify the possibility of removing hydrophobic substances and reusing the disc after its purification.

All the detergents, including SDS, could be washed out successfully after application. Separation without detergents was possible even after SDS had been used in previous separations and washed out. Neither the quality of the separation nor the recovery suffered any decline (Fig. 9D). For purification of the discs the use of SDS or the other ionic or non-ionic detergents that we investigated can be recommended. The separation performance is not impaired after rinsing the disc. Before the separation shown in the lower half of Fig. 9 was carried out, different detergents had been applied and washed out several times, using the same disc, namely SDS, Triton X-100 (reduced), octyl glucoside and CHAPS. The disc showed the same separation performance after this treatment and after more than 100 separations.

TABLE I

CAPACITY OF DEAE POLY(GLYCIDYL METHACRYLATE) DISCS FOR BSA IN RELATION TO DISC DIAMETER AND LAYER THICKNESS

Layer thickness (mm)	Diameter (mm)	Capacity (mg BSA)
2	10	4
	25	25
	50	150
4	10	10
	25	60
	50	300

Scaling up

The poly(glycidyl methacrylate) discs used in the experiments had diameters between 10 and 50 mm. The results of separations using DEAE discs with diameters of 10, 25 and 50 mm are shown in Fig. 10. It can be seen that the results obtained with a 10 mm diameter disc can also be achieved with a 50 mm diameter disc. The DEAE disc of 50 mm diameter and 4 mm thickness had a capacity of as much as 300 mg of serum albumin. A further improvement in capacity and separation performance is possible through a larger surface and thicker layers (see Table I and Figs. 7 and 8).

Separation experiments with membrane and serum proteins

In order to test the discs in experiments with biological samples, serum and membrane proteins with different hydrophobic properties were chosen. The separations were carried out in anion-exchange and affinity modes. Apart from separation performance, protein recovery and of enzymatic activity were determined.

Anion-exchange HPMC. Fig. 11 shows the separation of serum proteins by anion-exchange HPMC. A compact disc made of poly(glycidyl methacrylate) and a MemSep disc consisting of several bundled membranes (see above) were used. Although a direct comparison of the separation results is not possible owing to the different geometries of the separation devices [the MemSep disc has a diameter of 20 mm and is 10 mm thick, whereas the poly(glycidyl methacrylate) disc has dimensions of 25 × 2 mm], it can be said that the results are fundamentally the same. With the DEAE poly(glycidyl methacrylate) disc, elution is carried out at

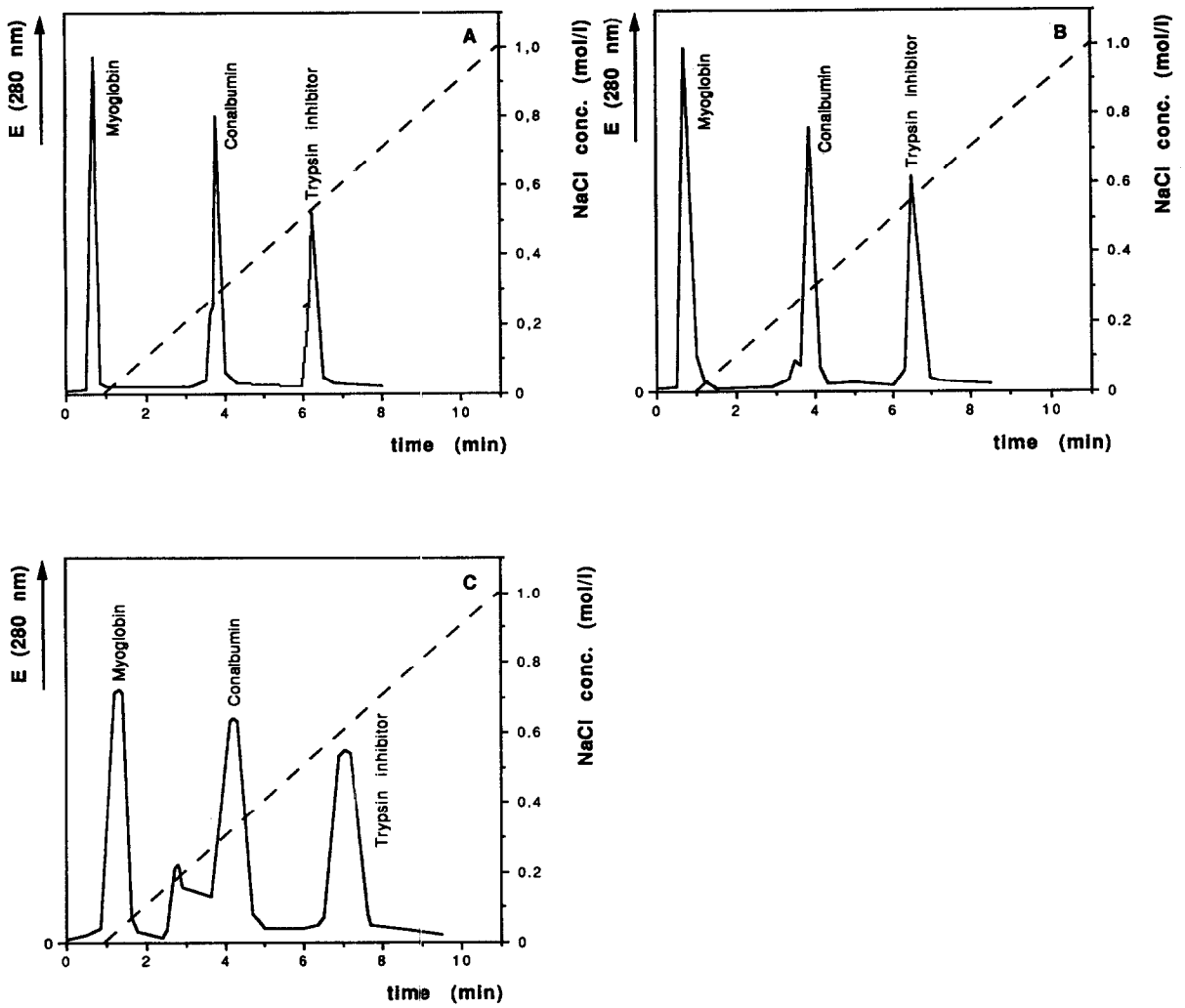

Fig. 10. Scaling-up of separation using DEAE poly(glycidyl methacrylate) discs with diameters between 10 and 50 mm. (A) A disc of diameter 10 mm and 4 mm thickness was used and 200 μg of myoglobin, 300 μg of conalbumin and soybean trypsin inhibitor were applied to the disc. (B) A disc of diameter 25 mm and 4 mm thickness was used and 1 mg of myoglobin, 2 mg of conalbumin and soybean trypsin inhibitor were applied to the disc. (C) A disc of diameter 50 mm and 4 mm thickness was used and 5 mg of myoglobin, 10 mg of conalbumin and soybean trypsin inhibitor were applied to the disc. The pressure on the discs was between 3 and 5 bar; other chromatographic conditions as in Fig. 2.

higher salt concentrations than with the MemSep disc. Consequently, the poly(glycidyl methacrylate) disc can be defined as a stronger anion exchanger than the MemSep disc. Addition of 0.25% of the non-ionic detergent Triton X-100 (reduced) was helpful in the separations with either device (see the lower part of Fig. 11). This agrees with the results obtained in previous separation experiments involving serum proteins, where it was shown that serum albumin, which constitutes a large portion of the proteins contained in the sample, has a strong tendency to aggregate with other serum proteins [15]. This is prevented by Triton X-100, and consequently the contamination of other proteins with serum albumin is much lower when the detergent was added (*cf.*, SDS-PAGE in Fig. 11). The protein recovery was above 85% in all the experiments.

In Fig. 12 the separation of membrane proteins

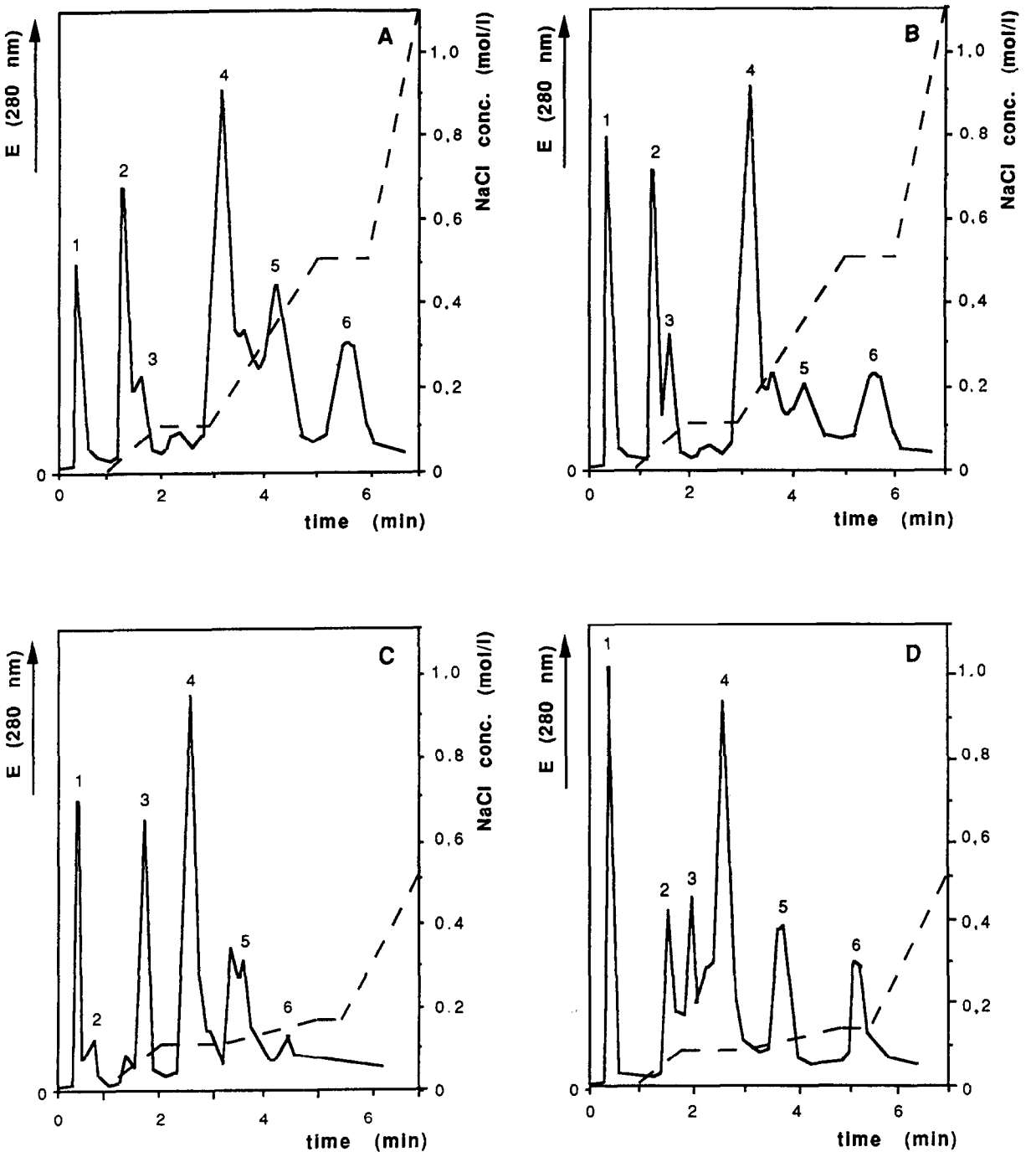

Fig. 11.

(Continued on p. 72)

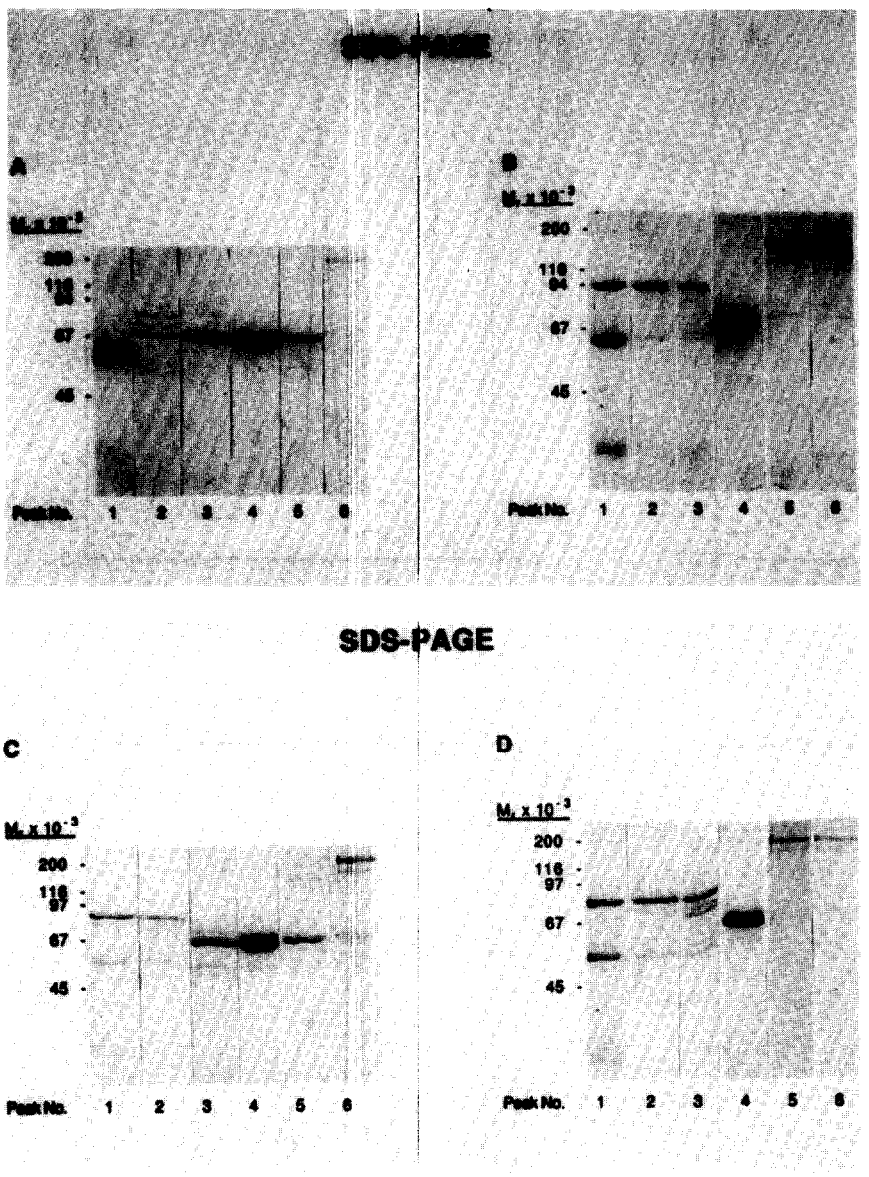

Fig. 11. Separation of rat serum proteins by anion-exchange HPMC. The DEAE poly(glycidyl methacrylate) disc had a diameter of 25 mm and a thickness of 2 mm. The chromatograms are shown on the previous page. The separations were performed (A) in the absence and (B) in the presence of 0.25% (v/v) of the detergent Triton X-100 (reduced). Separations of the same samples on a MemSep disc (C) in the absence and (D) in the presence of 0.25% (v/v) of reduced Triton X-100 (reduced) are also shown. In each experiment 100 μl serum of 8.2 mg of protein were applied. The separations were controlled by SDS-PAGE and the recovery by measuring the protein concentration in collected fractions. The recovery of proteins from the DEAE poly(glycidyl methacrylate) disc was 87% with no detergent and 93% when Triton X-100 (reduced) was used. For separation on the MemSep disc the recovery was 86% without the use of the detergent and 95% when detergent was used. Corresponding SDS-PAGE results are shown, other chromatographic conditions as in Figs. 2 and 5.

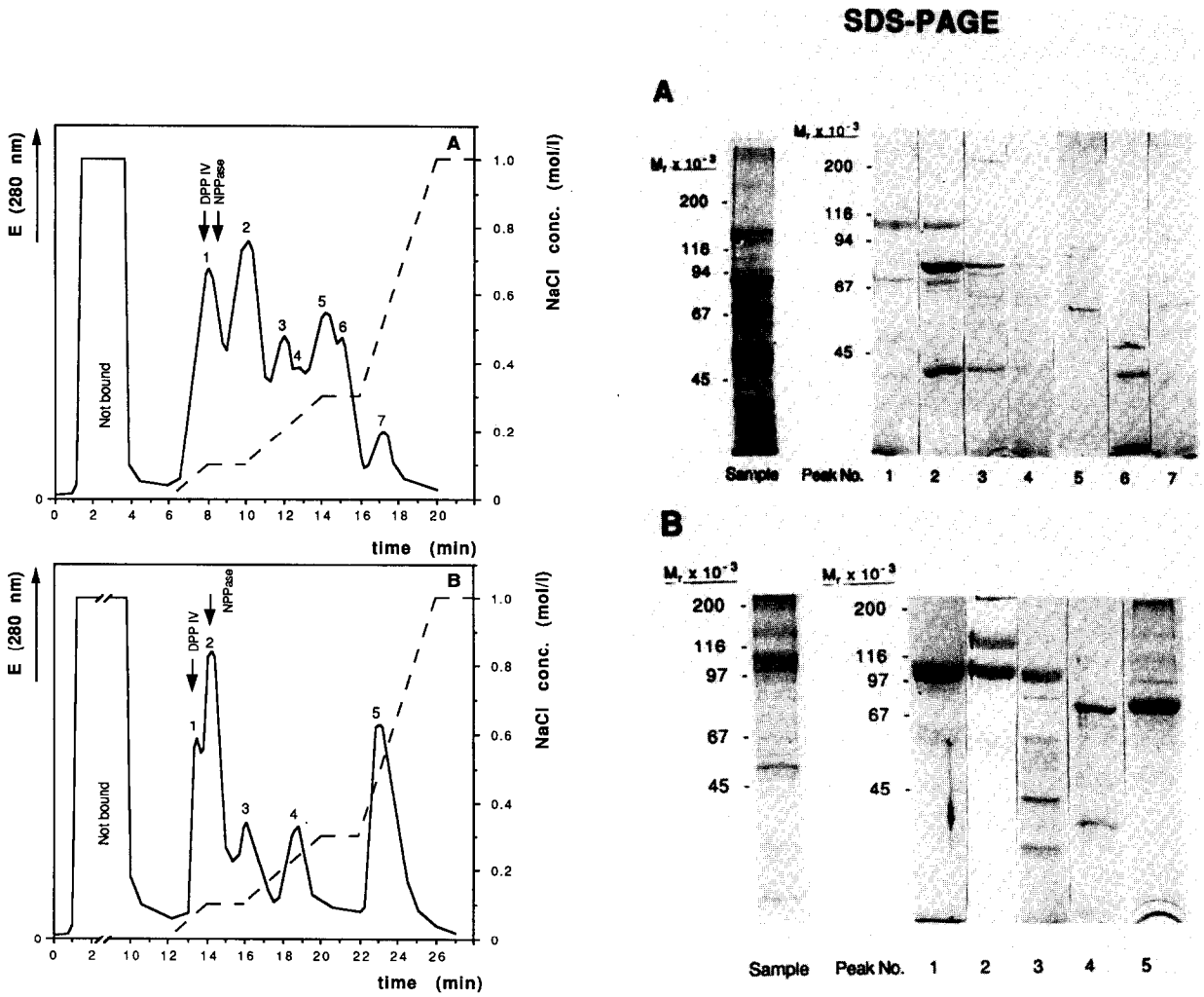

Fig. 12. Separation of kidney plasma membrane proteins with different degrees of hydrophobicity on a DEAE poly(glycidyl methacrylate) disc. (A) Extract after freezing and thawing with less hydrophobic proteins. About 7 mg of protein were used. The recovery of protein was 89% and the recovery of enzymatic activity for DPP IV was 78% and for NPPase 87%. (B) Extract after solubilization of plasma membranes with 1% (v/v) of detergent Triton X-100 (reduced). About 7 mg of protein were used. The recovery of protein was 91% and the recovery of enzymatic activity for DPP IV was 81% and for NPPase 84%. The separations were controlled by SDS-PAGE and the recovery by measuring the protein concentration and enzymatic activity of DPP IV and NPPase in the collected fractions. Corresponding SDS-PAGE results are shown. Chromatographic conditions: DEAE poly(glycidyl methacrylate) disc, 25 mm diameter, 3 mm thickness; buffer A, 10 m*M* Tris–HCl (pH 7.6); buffer B, buffer A containing 1 *M* NaCl; 0.25% (v/v) of Triton X-100 (reduced) was added to both buffers; flow-rate, 3 ml/min; pressure, 1–3 bar; temperature, 0°C (ice cooled); the gradient is shown (dashed lines).

with different degrees of hydrophobicity is shown. The separation, recovery of protein and enzymatic activity were good. The separation of less hydrophobic proteins from the extract of plasma membranes after freezing thawing [16] required less time when HPMC was used (15 min) instead of an HPLC column (45 min) [17]. Fig. 13 shows the anion-exchange HPMC of the annexin CBP 65/67 on a DEAE poly(glycidyl methacrylate) disc. Until now it was possible to separate this protein by hydroxyapatite HPLC and collagen HPAC and collagen HPMAC. The separation by reversed-phase

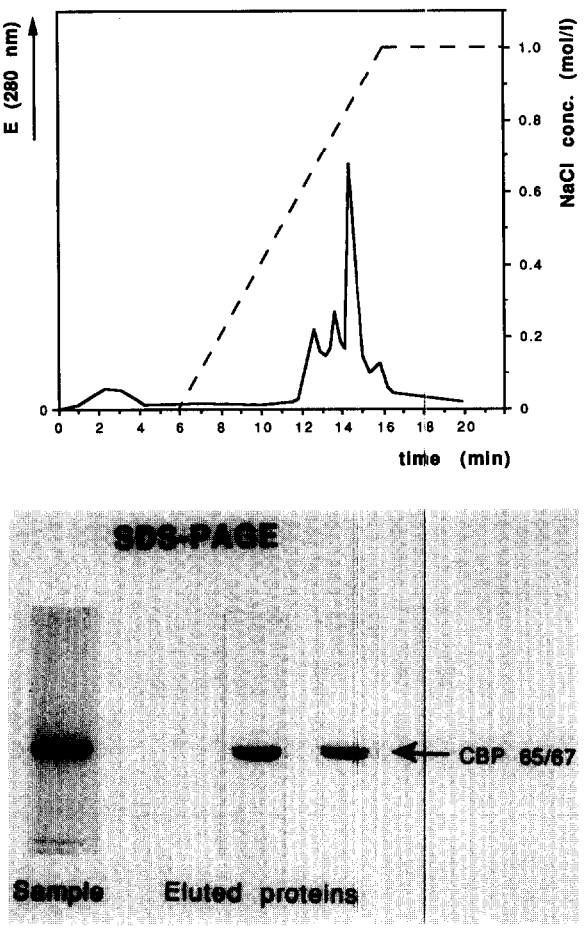

Fig. 13. Anion-exchange HPMC of annexin CBP 65/67 from rat liver plasma membranes on a DEAE poly(glycidyl methacrylate) disc. A 3-mg amount of protein was applied to the disc and 2.85 mg of protein (95%) were recovered by gradient elution. To ensure the solubility of the protein, 1 m*M* EDTA and 0.25% (w/v) of octyl glucoside were added to both buffers. Other chromatographic conditions as in Fig. 12. Eluted proteins in peaks correspond to the bands in SDS-PAGE.

HPLC required the addition of at least 50% formic acid to the eluent. Without formic acid the protein bound irreversibly to the support and could not be recovered [18]. Experiments with anion-exchange HPLC, with both silica gel-based and polymer-based supports, failed and the annexin could not be recovered from the column. The number of different peaks which can be observed in Fig. 13 is probably due to microheterogeneity of this protein [18].

Heparin and collagen HPMAC. In order to investigate the use of the separation devices for the immobilization of high-molecular-weight ligands and their chromatographic separation, heparin and collagen were immobilized on an epoxy-activated poly(glycidyl methacrylate) disc. The separation of very hydrophobic membrane proteins from plasma membranes of Morris hepatoma 7777 by heparin HPMAC is shown in Fig. 14. Although a linear gradient between 0 and 0.5 *M* NaCl was applied, all the bound proteins were eluted as one peak only. This agrees with the results obtained with plasma membrane and serum protein separations on columns with immobilized heparin [12]. Therefore, a mere step gradient is usually adequate in heparin HPAC and HPMAC for the elution of the bound proteins. In the experiment shown in Fig. 14, an important advantage of such discs for the isolation of certain substances from highly dilute solutions is shown. A disc 50 mm in diameter and 4 mm thick was used, and about 200 mg of heparin were immobilized. The membrane proteins are highly hydrophobic and easily aggregate and precipitate, even when 1% of Triton X-100 is added. It is therefore advisable to extract these proteins from dilute solutions. In this experiment 120 mg of protein were dissolved in 500 ml of buffer and applied to the disc at a flow-rate of 10 ml/min. The whole separation process (application of sample and elution) took less than 90 min. The application of 500 ml of sample on a heparin-HPAC column takes 5–10 times longer. Fig. 15 shows the separation of the annexins CBP 65/67, CBP 35 and CBP 33 on a collagen HPMAC disc. The annexins could be separated in a similar manner as on a column, but the separation time in this experiment was 12 min. An HPLC separation with immobilized collagen takes 40 min [18]. Collagen immobilization on a packed column with a support containing active groups is almost impossible and the viscous collagen solution can hardly be pumped through the column. However, the collagen could be applied without difficulty to an epoxy-activated disc because of the low pressure drop. After washing out the non-bound collagen, the pressure on the disc did not exceed 4 bar at a flow-rate of 3 ml/min.

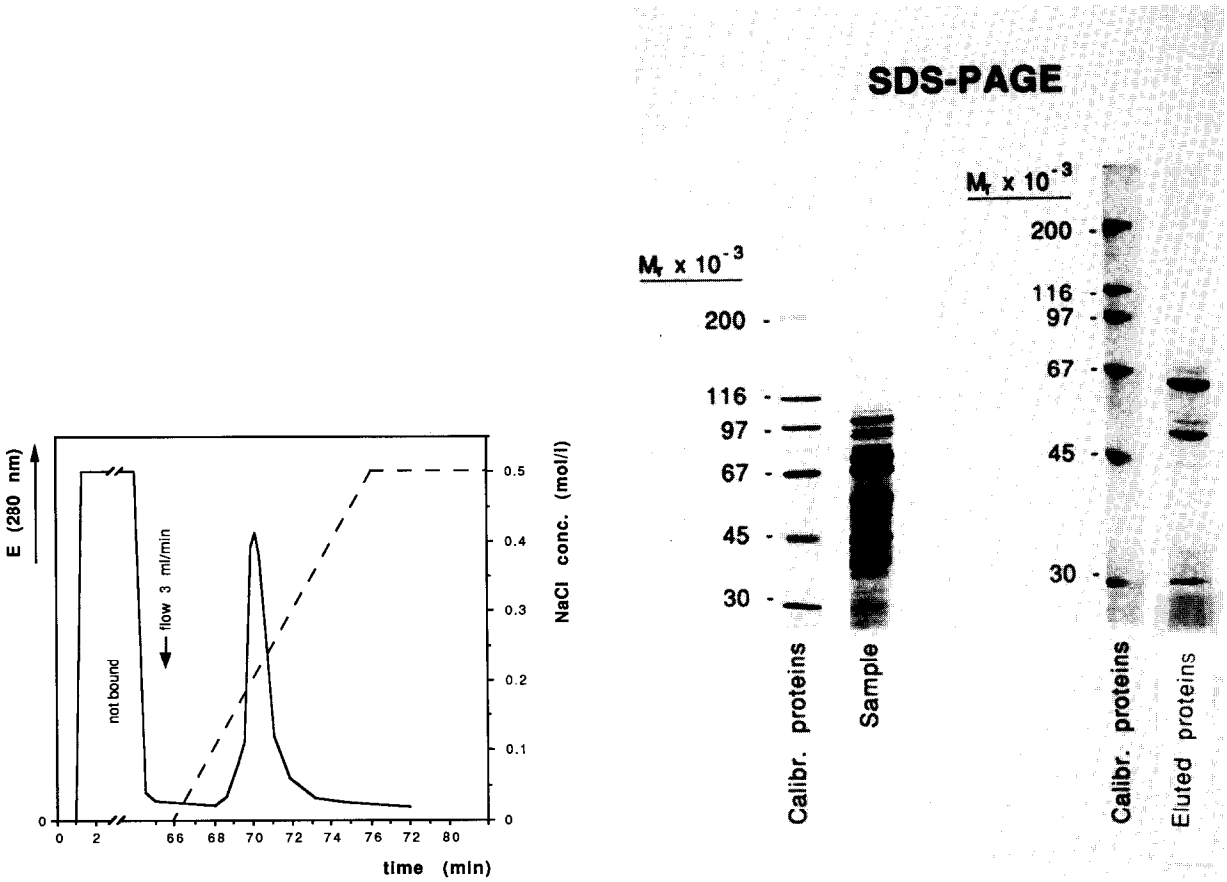

Fig. 14. Separation of hydrophobic proteins from Morris hepatoma 7777 plasma membranes by heparin HPMAC. A sample containing 120 mg of plasma proteins was extracted with 1% Triton X-100 (reduced), diluted to 500 ml with Tris-buffered saline (pH 8.0) and applied to a disc of 50 mm diameter and 4 mm thickness immobilized with 200 mg of heparin. The sample was applied at a flow-rate of 10 ml/min and the elution of bound proteins was performed at a flow-rate of 3 ml/min. An amount of 0.25% (v/v) of Triton X-100 (reduced) was added to both buffers. The pressure was 9 bar during sample application and 2–3 bar during elution. The separation was controlled by SDS-PAGE and the recovery by protein determination in the collected fractions. The recovery of proteins was 84%. The temperature was 0°C (ice cooled). The gradient is shown (broken line).

CONCLUSIONS

After solving the problems concerning sample distribution and cartridge construction, compact discs made of hydrophilic poly(glycidyl methacrylate) polymer yield results at least as good as those obtained with corresponding HPLC columns. When thicker layers are constructed, the use of poly (glycidyl methacrylate) discs does not depend on the bundling of single, thinner components. So far 10 mm thick layers have been produced through polymerization in a compact disc.

In separation systems made of compact discs, the reaction rate is better than in packed columns. Therefore, fast separations are possible at high flow-rates. The low pressure drop in these systems allows the use of low-pressure pumps. The hydrophilic support made of poly(glycidyl methacrylate) is chemically stable and heat resistant, and it can therefore be used at high pH and high temperatures. The use of detergents does not present any difficulties, allowing the separation of hydrophobic sample components. The system can be rinsed in an adequate way after each separation.

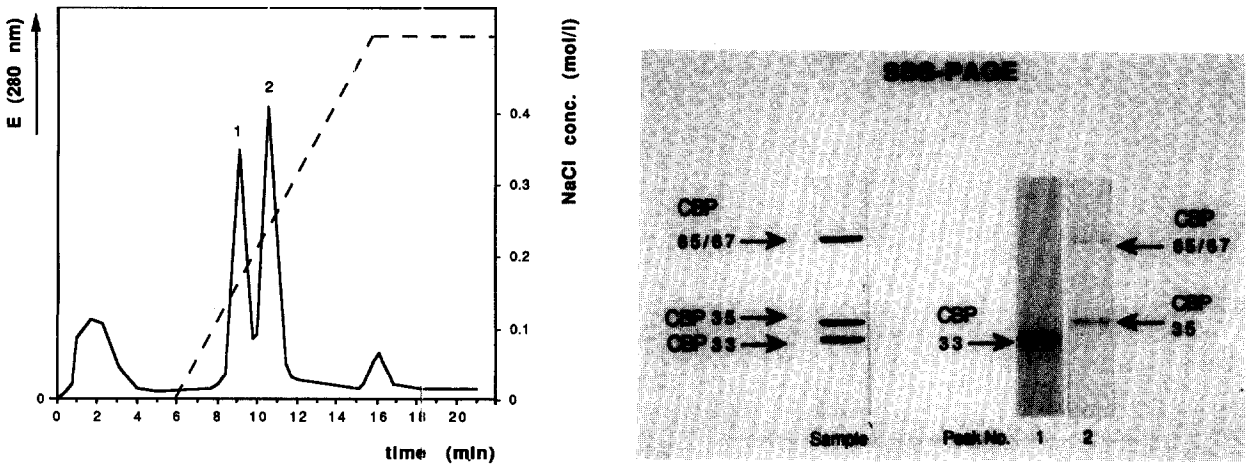

Fig. 15. HPMAC of annexins CBP 65/67, CBP 35 and CBP 33 on a poly(glycidyl methacrylate) disc with immobilized collagen. About 20 mg of collagen were immobilized on an epoxy-activated poly(glycidyl methacrylate) disc of 25 mm diameter and 2 mm thickness. The amount of protein applied to the disc was 4 mg and 3.7 mg of protein (93%) were recovered. SDS-PAGE of the collected fractions (1,2) is shown. Other chromatographic conditions: flow-rate, 2 ml/min; pressure, 2–3 bar; temperature, 0°C. The gradient is shown (dashed line).

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