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Application of high-performance membrane chromatography for separation of annexins from the plasma membranes of liver and isolation of monospecific polyclonal antibodies

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Abstract

The separation of annexins, calcium-binding plasma membrane-associated proteins from rat liver and Morris hepatoma 7777 by high-performance membrane chromatography (HPMC) is described. The annexins with low molecular masses, CBP 33 and CBP 35, and the annexin with a high molecular mass, CBP 65/67, can be separated within 10 min from one another by anion-exchange HPMC under non-denaturing conditions. The separation devices used consist of compact, porous disks (QuickDisk) on the one hand and of bundled membranes made of cellulose fibers (MemSep) on the other. Both have been found to be equally well suited for this separation. The annexins obtained in this way are subsequently bound to epoxy-activated porons disks and used for the separation of monospecific polyclonal antibodies against the annexin CBP 65/67.

1. Introduction

Calcium plays a central role in many physiological processes, e.g. muscle contraction, nervous stimulation and blood coagulation. Another important role of calcium is its function as second messenger in stimulus–response coupling. The largest single category of calcium-binding proteins bind calcium through a simple helix–loop–helix structure known as "E–F" hand [1]. To this category belong, e.g. calmodulin and troponin C. Another major class of calcium-binding proteins, called annexins, bind calcium through an alternative, more complex structure. A further characteristic of annexins is their phospholipid binding ability [2].

Although annexins were discovered *ca.* ten years ago and despite the fact that some of them have been characterized by molecular cloning, the biological function of annexins is still not well understood. These proteins have been discovered in mammals and other vertebrates [2–4], but also in plants and lower organisms, such as dictyostelium, hydra and drosophila [2,5–7].

In mammals, annexins have been found in almost all tissues [8]. Some of their functions, as discussed in the literature, play a role in phospholipase-A_2 inhibition, membrane fusion, Ca^{2+}-channel activity and blood coagulation [2,9–13]. Besides, recent findings suggest that

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some annexins or at least annexin VI interact with the intracellular domain, e.g. cell surface receptors such as EBV/C3d (CR2), in lymphocytes [14] and with cell-CAM, a receptor involved in cell-to-cell interaction in hepatocytes [15].

Annexins are present in rat liver in relatively high concentrations. At least a part of these proteins are localized in liver and hepatoma plasma membranes, where they can be detected by immunofluorescence microscopy, using monoclonal and polyclonal antibodies [15]. In both liver and Morris hepatoma cells three groups of annexins with apparent molecular masses in SDS-PAGE of 33 000 (CBP 33), 35 000 (CBP 35) and 65 000–67 000 (CBP 65/67) can be detected. With higher de-differentiation of the hepatomas the content of annexins with lower molecular masses, CBP 33 and CBP 35, increases relatively to the content of CBP 65/67 [8]. All three annexins bind strongly to plasma membranes and can be highly enriched by EDTA extraction during their selective solubilization [16].

All annexins are very similar in their amino acid sequence and also in their chromatographic behavior. They contain a highly conserved 70 amino acid domain, which is repeated either four or eight times in the overall structure [2]. This repeating sequence is the main reason why all annexins behave in an almost identical way during interaction with most chromatographic supports [17].

All polyclonal antisera against one annexin invariably react with all other annexins of the same species, and often with annexins of other species [18]. Such cross-reacting is observed even in the case of some monoclonal antibodies [19].

The water solubility of annexins is influenced by the presence of calcium ions and phospholipids. All annexins bind these components with very high affinity. After the binding of calcium the hydrophobic domains on the surface of the molecule are exposed. They are otherwise inaccessible inside the molecule. However, if calcium is complexed with EDTA or EGTA, the proteins can again be solubilized [16,20]. The ability of the annexins to interact with hydrophobic as well as with hydrophilic components (Janus-faced proteins) [21], depending on the presence of particular ligands such as calcium, is exploited for their solubilization and enrichment from plasma membranes. This characteristic is also useful for isolation and separation of annexins by calcium-dependent hydrophobic interaction chromatography (HIC) [17,20]. However, the hydrophobicity, which is hard to control, is one of the reasons why the quantitative recovery of a native annexin is hard to achieve by means of a chromatographic separation. This is mainly due to possible non-specific interactions with the support. Denaturing reagents or detergents have to be used frequently to suppress these phenomena [22,23].

High-performance membrane chromatography (HPMC) is a method for separating biopolymers. The characteristics of the support required for this method and the geometry of its separation unit offer good conditions for the separation of highly hydrophobic proteins and therefore of annexins [24,25]. The danger of non-specific interaction is diminished by the surface of the support, which is hydrophilic and the area of which is reduced to a minimum. Diffusion in a membrane takes place within a very small space, hardly any distances have to be overcome. This favors mass transfer, allowing fast separation [26,27].

This paper describes the isolation of three annexins from rat liver and Morris hepatomas by means of HPMC. Each separated annexin was subsequently immobilized and used for the production of monospecific, polyclonal antibodies against the annexin CBP 65/67.

2. Experimental

2.1. Animals and chemicals

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

many), Serva (Heidelberg, Germany) or Sigma (München, Germany).

2.2. Plasma membranes and polyclonal antibodies

Liver and Morris hepatoma 7777 plasma membranes were isolated by zonal centrifugation using a Kontron centrifuge (Kontron Analytik, Munich, Germany) as described elsewhere [28]. Membrane purity was routinely checked by electron microscopy and by assaying of marker enzymes as described by Tauber and Reutter [28]. Annexins were isolated by stepwise extraction of liver and Morris hepatoma 7777 plasma membranes according to the already published protocol [22]. Polyclonal antibodies were produced by injecting rabbits with annexins isolated by preparative SDS-PAGE or reversed-phase (RP) HPLC [23]. Protein content of membrane and serum samples was determined according to the procedure of Lowry et al. [29].

2.3. HPLC

The HPLC system consisted of two pumps, a programmer, a spectral photometer with a deuterium lamp and a Knauer loop injection valve (all from Knauer Gerätebau, Berlin) and a fraction collector (BioRad, Munich, Germany). In the case of ion-exchange and hydrophobic-interaction chromatography the salt gradient was controlled by measuring the osmotic pressure (Halbmikro-Osmometer, Typ Dig. L., Knauer Gerätebau).

2.4. Separation devices

The poly(glycidyl-methacrylate) disks (Quick-Disk) were purchased from Säulentechnik Knauer (Berlin, Germany). The thickness of the disk layers was 3 and 4 mm respectively, the diameter was 25 mm. They were cut in disk-shape. When the separation disks were used for ion-exchange or hydrophobic-interaction HPMC, the chosen ligands were previously bound synthetically [24]. In the case of affinity chromatography the ligand was positioned on the membrane in epoxy form "in situ", according to the following protocol: after installing the disk in the appropriate cartridge, any remaining non-polymerized components were washed out with 20 ml of methanol. The disk was then rinsed with 40 ml of bidistilled water and 40 ml of 0.1 *M* sodium boronate buffer, pH 8.0. The ligand, in this case one of isolated annexins, solved in boronate buffer, was added in quantities of *ca.* 2–10 mg per g of support. In the case of a 25 mm disk, thickness 3 mm (*ca.* 0.6 g of support), 2 mg of isolated annexin in 10 ml of 0.1 *M* sodium boronate buffer, pH 8.0 (binding buffer) were pumped through the disk at a flow-rate of 1.0 ml/min. The solution is left to circulate on the disk for at least 2 h. Subsequently the disk is rinsed with 50 ml of binding buffer. Any remaining free epoxy groups are blocked with 0.2 *M* Tris-HCl buffer, pH 8.0. The disk is then rinsed with boronate buffer or Tris-buffered saline (TBS), pH 7.4, and stored at 4°C for further usage. The immobilization of protein A has been described elsewhere [24].

The MemSep separation unit (Millipore, Vienna, Austria) contains either DEAE or quaternary ammonium groups as ligands (MemSep DEAE or MemSep Q). The inner cartridge diameter was 20 mm, the thickness of the layers was *ca.* 10 mm. These units were used for anion-exchange (AE) HPMC.

2.5. Buffers

The buffers used for anion-exchange HPMC were buffer A, 10 m*M* Tris-HCl, pH 7.8, and buffer B which was the same as buffer A, but containing 1 *M* sodium chloride. Both buffers contained 1 m*M* EDTA.

For the isolation of antibodies, buffer A (application buffer) was 50 m*M* sodium phosphate, pH 8.0, with 0.15–0.20 *M* sodium chloride. The buffer used to wash the column was buffer A with 1% (v/v) Triton X-100. The adsorbed antibodies were eluted with 0.1 *M* sodium citrate, pH 2.4 (buffer B).

For affinity chromatography with immobilized annexins a buffer was used, which contained 10 m*M* Tris-HCl, pH 8.0, with 0.155 *M* sodium

chloride, 1 m*M* EDTA, 0.1% (v/v) reduced Triton X-100. The bound monospecific antibodies were eluted with 0.1 *M* sodium citrate, pH 2.4.

2.6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The 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. In other experiments, 5–15 μl of sample were taken from the collected fractions after HPMC separation and mixed with buffer containing five times higher concentrations of the above-mentioned substances. The amount of buffer taken for the experiments was such that the original concentration was obtained after dilution by the sample. SDS-PAGE was carried out by the Laemmli method [30], using a mini system (Protean, Bio Rad, Munich, Germany). The amount of applied protein was between 0.5 and 2 μg per line.

Immunoblotting of electrophoretically separated proteins was performed according to Towbin et al. [31]. Detection of proteins was achieved by luminography. The luminescent blots were covered by a translucent polyethylene film and exposed to an X-ray film (X-omat S, Kodak, Stuttgart, Germany).

3. Results

3.1. Separation of annexins

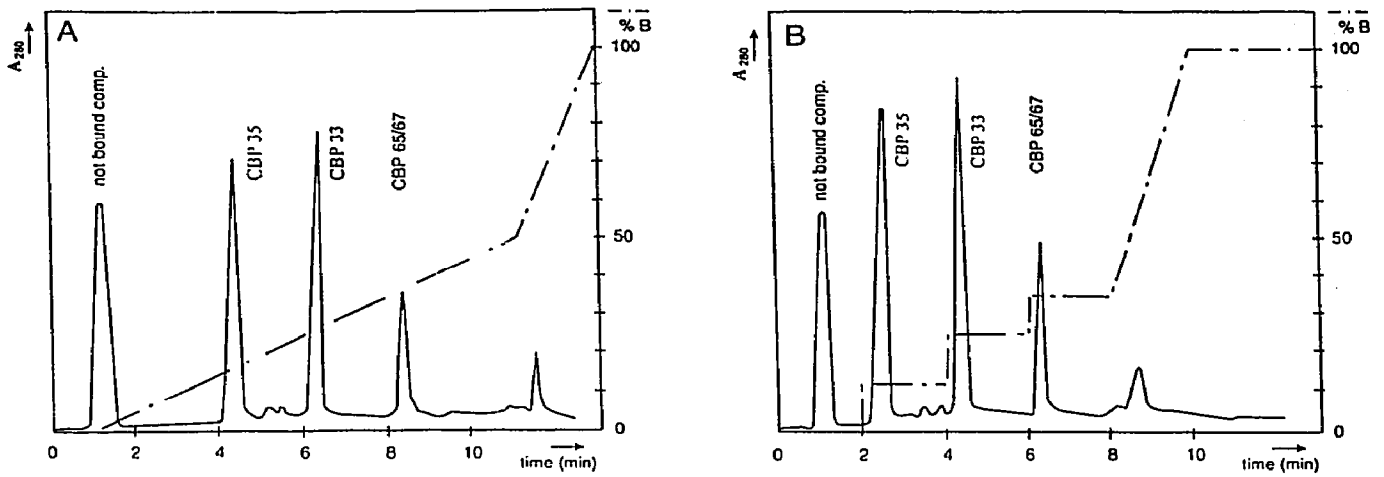
Fig. 1A shows the separation of annexins which were solubilized with EDTA from the plasma membranes of Morris hepatoma 7777. In this highly malignant and weakly differentiated hepatoma all three annexins, CBP 33, CBP 35 and CBP 65/67 occur in almost equal quantities, each annexin amounting to about one third of the whole protein amount in this extract [8,16]. As can be seen in Fig. 1B, separation can be also performed by using a step gradient. Fig. 2A shows the separation of the same sample with a MemSep DEAE cartridge. This separation unit also allows separation of single proteins by using a step gradient, similar to Fig. 1B. When MemSep is used, the individual annexins are eluted in the order CBP 33 (peak 1, Figs. 2A and 2B), CBP 35 (peak 2) and CBP 65/67 (peak 3). However, when a QuickDisk DEAE unit is used, CBP 35 (peak 1) is eluted before both CBP 33 (peak 2) and CBP 65/67 (peak 3). This order cannot be changed by adding higher amounts of EDTA or EGTA to the separation buffer. The use of stronger anion-exchangers such as quaternary annexins did not influence the separation behavior of these proteins, neither with QuickDisk Q nor with MemSep Q.

As can be seen in Fig. 3A, CBP 33 and CBP 35 are present also in the extracts from the plasma membranes of rat liver. The low-molecular-mass annexins are hardly visible in SDS-PAGE, when compared to the amount of CBP 65/67 in this sample (Fig. 3B). In the extracts from plasma membranes of the liver, the order of the peaks resulting from elution of CBP 33 and 35 was also different, depending on the use of either MemSep or QuickDisk (the chromatogram resulting from the MemSep unit is not shown here).

3.2. Membrane affinity chromatography and isolation of monospecific polyclonal antibodies

The application of protein A or protein G, immobilized either to QuickDisk or membranes, allows the isolation of immunoglobulins from rabbit serum in less than 15 min. SDS-PAGE of immunoglobulins isolated with QuickDisk is shown in Fig. 4. Diluted rabbit serum was applied to the separation unit, which contained immobilized protein A. Non-specifically bound components were washed out with PBS and 1% Triton X-100. The bound immunoglobulins were subsequently eluted at low pH (see Experimental).

The isolated polyclonal antibodies react against all three annexins, CBP 33, CBP 35 and CBP 65/67. Therefore, monospecific polyclonal anti-CBP 65/67 antibodies were separated through a kind of chromatographic depletion. The isolation scheme is shown in Fig. 5. The resulting monospecific, polyclonal antibodies react against the annexin CBP 65/67 only. The



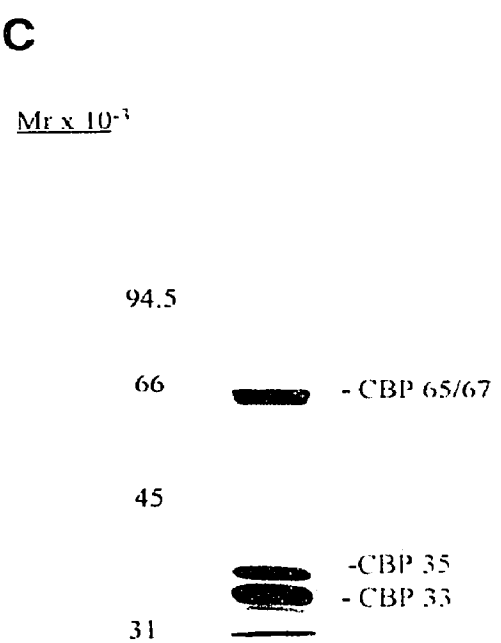


Fig. 1. High-performance membrane chromatography of annexins extracted from Morris hepatoma 7777 plasma membranes (cf. Ref. [16]). About 2 mg of protein in 2 ml of buffer A were applied. Separation unit: compact disk QuickDisk DEAE, diameter 25 mm, height 3 mm. Separation conditions: for buffer see Experimental, flow-rate 3 ml/min, pressure 0.2 mPa, room temperature. (A) Chromatogram with a linear gradient, (B) chromatogram with a step gradient. The gradients are shown in the figure. (C) SDS-PAGE of the applied sample.

antibodies which cross-react with CBP 33 and CBP 35 were bound to previously applied QuickDisk separation units (see Figs. 5 and 6). The result of such a separation is shown in the immunoblot in Fig. 6. The starting antiserum reacts with all three annexins from the plasma

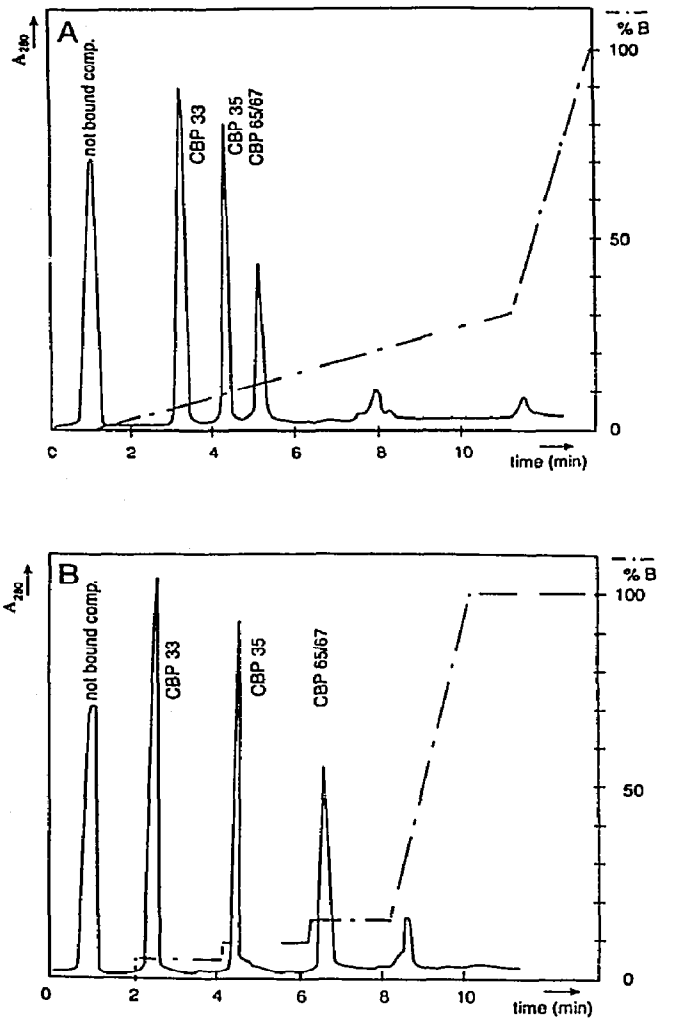


Fig. 2. High-performance membrane chromatography of annexins from Morris hepatoma with a MemSep separation unit. Separation unit: bundled membranes, MemSep 1000 DEAE, diameter *ca.* 20 mm, height 10 mm, sample as in Fig. 1. Separation conditions: for buffer see Experimental, flow-rate 3 ml/min, pressure 0.1 to 0.2 mPa, room temperature. (A) Linear gradient, (B) step gradient. The gradients are shown in the figure.

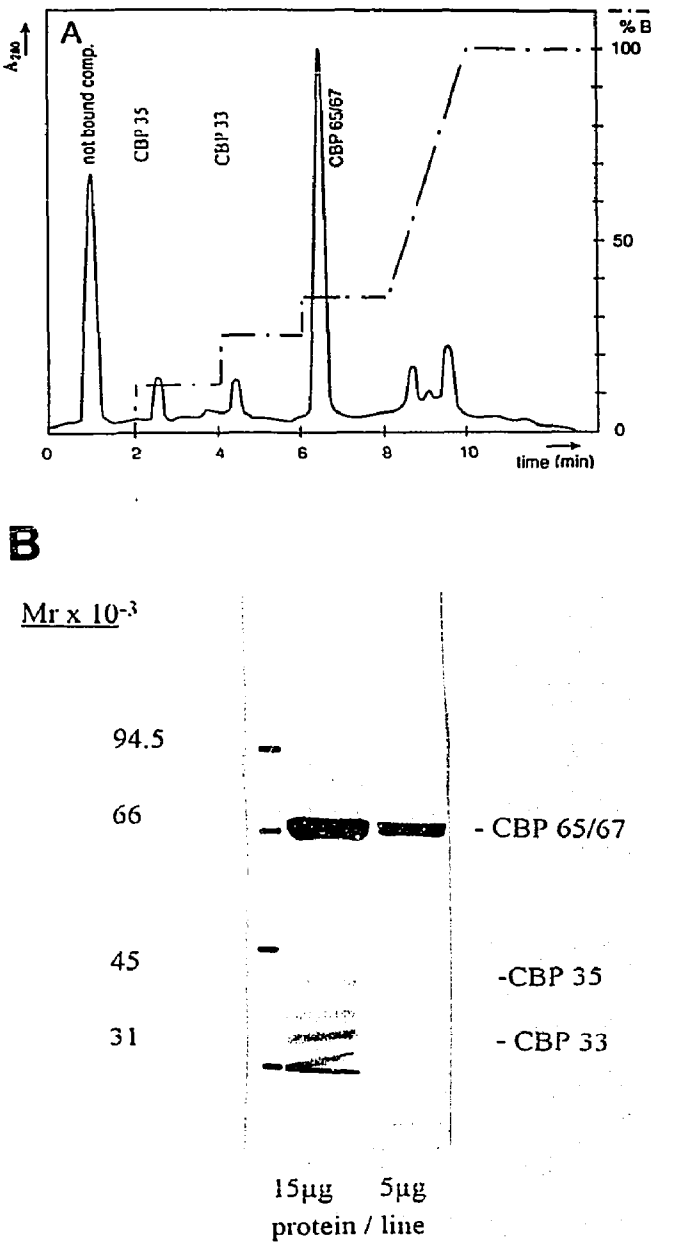


Fig. 3. Separation of annexins from the plasma membranes of liver by HPMC. Sample: EDTA extract from the plasma membranes of rat liver (cf. Ref. [16]). About 1 mg of protein in 2 ml of buffer A was applied. Separation unit: QuickDisk DEAE, diameter 25 mm, height 3 mm; for other conditions see Fig. 1A. (A) Chromatogram, (B) SDS-PAGE of the sample.

membrane extract of Morris hepatoma 7777 (left), whereas the purified antibodies react specifically only with the 65/67 band (right).

4. Discussion

The most widely used media for protein separations are still soft gels on an agarose basis. These tools have recently seen steady improvements, as they were comparable with so-called high-performance separation media. Through

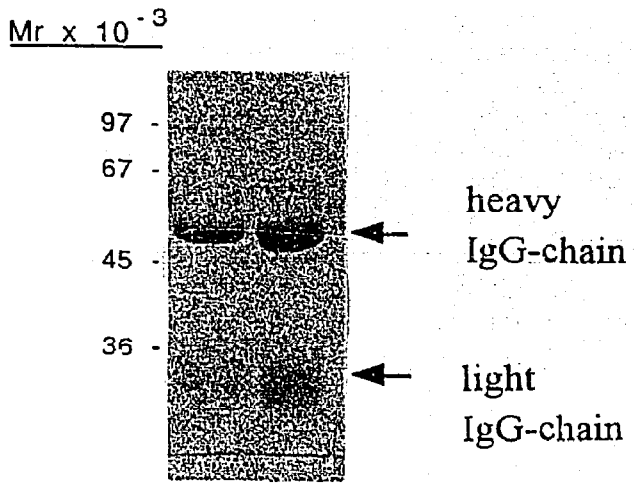


Fig. 4. SDS-PAGE of polyclonal antibodies isolated by protein A high-performance membrane affinity chromatography. Separation unit: QuickDisk protein A, diameter 25 mm, height 3 mm, with 3 mg of bound protein A. Flow-rate, 1 ml/min during application, 3 ml/min during rinsing and elution; pressure: 0.05 to 0.2 mPa; for buffer see Experimental. The chromatogram is not shown, only the eluted IgG is analysed by SDS-PAGE (cf. Ref. [24]).

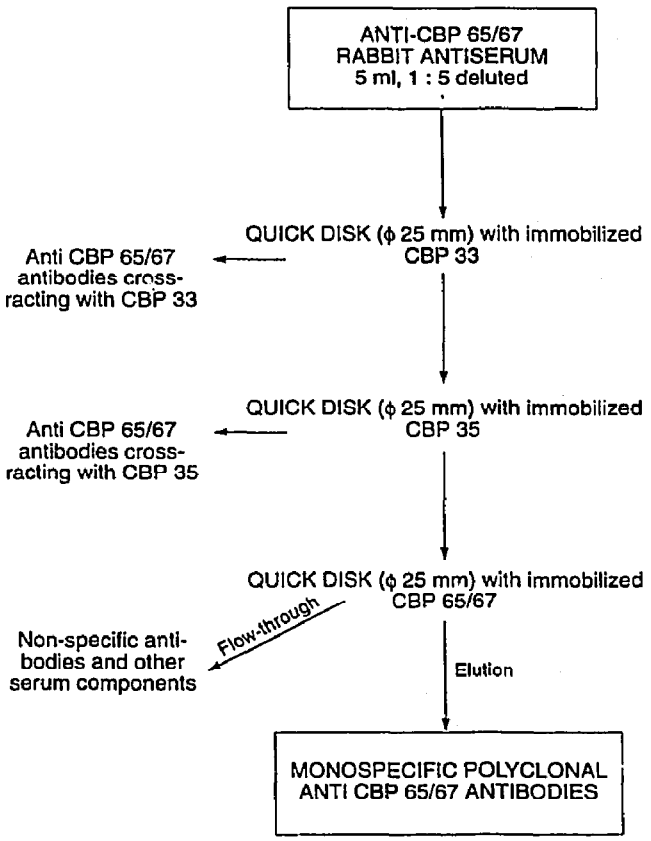


Fig. 5. Isolation schema for the preparation of monospecific, polyclonal antibodies against annexin CBP 65/67 from rat liver.

enhanced separation performance and higher pressure resistance the soft gels have reached a remarkably good position. In terms of non-specific binding and chemical stability, agarose and similar media on a polysaccharide basis are still superior to substances made of synthetic polymers and silicagel. Despite their high pressure resistance and good separation performance, the acceptance of silica gel-based media in biochemistry is making only little progress, especially in the case of preparative separations of proteins. The most important problem with the use of silica gels is their poor stability at high pH and the high degree of non-specific binding especially for hydrophobic biopolymers. Polymer packings for HPLC of biopolymers have been widely used for several years. The best known types are MonoQ, MonoS and TSK beads. Other polymers with ligands for ion-exchange, hydrophobic-interaction or affinity HPLC have also been applied [32,33]. Polymer supports have a high pH-stability, and non-specific binding is easier to control.

The key problems that occur in the separation of biopolymers on bulk packings are firstly mass transfer, caused by the slow diffusion into the pores of the support, and so-called nonspecific binding to the surface of the support. In such chromatographic separations, membranes and porous disks have some advantages over bulk packings. They are almost completely lacking pore diffusion, due to the 10- to 100-fold smaller surface. Because of the construction, their pressure drop is extremely low. The ligand is not located within the pores, as is the case with porous bulk supports, but on the surface [24,26,27]. Therefore diffusion is practically nonexistent, and the mass transfer is correspondingly fast. Unarska et al. [26] have shown

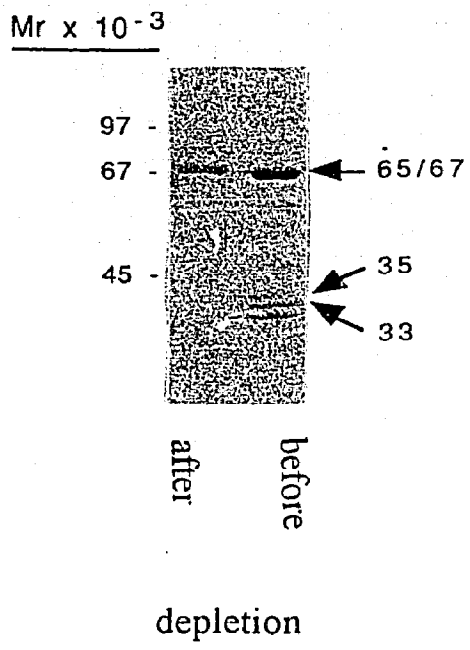


Fig. 6. Control of specificity of the depleted anti CBP 65/67 antibodies, isolated according to the schema shown in Fig. 5. Immunoblot of plasma membranes from Morris hepatoma 7777, extracted by EDTA (cf. Fig. 1 and Ref. [16]). (Left) With the original antiserum, (right) with monospecific polyclonal anti CBF 65/67 antibodies. After depletion of the antibodies which cross-react with CBP 33 and CBP 35 only the CBP 65/67 band is visible [24].

that the interaction between IgG and immobilized protein A is up to 200 times faster, as compared to protein A agarose, when the protein A is immobilized on membranes made of hollow fibres. This rapid interaction occurs because of improved mass transfer, due to the lack of diffusion inside the membranes, by which the exchange would otherwise be impaired.

The supports used for HPMC are made of synthetic polymer in the case of QuickDisk, namely poly(glycidyl-methacrylate) [24]. The MemSep separation units consist of bundled membranes made of cellulose fibres [25]. Both supports have proved to be useful for the separation of annexins.

Former attempts to separate the three annexins CBP 33, CBP 35 and CBP 65/67 by anion-exchange HPLC under non-denaturing conditions were not successful, neither with columns on a silica gel basis nor with the above-mentioned polymer-based columns such as MonoQ and DEAE TSK 5PW. Under non-denaturing conditions the proteins could be separated on HPLC columns only when special supports were used, e.g. crown ether, collagen or hydroxylapatite [17,24].

The three annexins can be separated by size-exclusion or reversed-phase HPLC. However, in order to suppress non-specific bindings of these proteins to the support and to achieve quantitative recovery from the column, such separations have to be carried out in the presence of denaturing reagents such as detergents and formic acid [17,23].

Membrane chromatography allows the separation of annexins from one another on anion-exchange units. The relatively small surface of the support as well as the almost complete lack of diffusion allow fast isolation, high recovery rates and minimal non-specific binding.

The rather low capacity of MemSep membranes compared to QuickDisk (Josic and Štrancar, unpublished observation) is here insignificant, as the amount of separated protein was 3 to 4 mg per run and therefore below the maximum capacity of single separation units, namely 6 to 7 mg per cartridge in the case of MemSep and *ca.* 30 to 40 mg with QuickDisk. The reversed order in which CBP 33 and CBP 35 are eluted with MemSep and QuickDisk respectively, may be caused, among other factors, by certain non-specific (non-ionic) interactions with the support. It is hard to find out, whether the non-specific interaction takes place on the cellulose membrane or on the poly(glycidyl methacrylate) membrane, as the phenomenon was not further investigated. It is known that MemSep membranes have a rather low ligand density inherent of the way they are currently produced.

The observation made by Josic et al. [34] and Tennikova and Svec [35], that a step gradient can be applied in separations with QuickDisk units was also confirmed here for the use of MemSep HPMC-units [25]. As can be seen in Figs. 1 and 2, the gap between single peaks can be determined by the shape of the step gradient. The protein requires a particular, minimal salt

concentration, at which it is eluted under chromatographic conditions using a linear gradient. Rinsing or eluting, respectively, of a peak can be carried out for a long period of time without adversely affecting the subsequently eluting components. This kind of behavior was observed for both compact disks (QuickDisk) and membranes (MemSep) and may be of considerable interest when these separation techniques are used on a preparative scale.

Short separation times and low non-specific binding are excellent conditions for the use of membranes in affinity chromatographic separations. Preliminary experiments revealed that MemSep cartridges with activated groups allow a rather low ligand density in immobilization. This is the reason why epoxy-activated disks are used for the immobilization of proteins and for affinity chromatography. When antibodies were isolated from rabbit antiserum (Fig. 4), *ca.* 4 to 5 mg of IgG could be isolated from 1 ml of serum. This agrees well with the results obtained for a protein A Sepharose column with a column volume of *ca.* 0.5 ml.

The use of membrane chromatography for the isolation of monospecific, polyclonal antibodies by depletion of cross-reacting antibodies from the antiserum demonstrates that this method is an adequate tool for solving special, complex problems. In a single run, shown in Figs. 5 and 6, *ca.* 100 μg of monospecific anti-CBP 65/67 polyclonal antibodies were produced. They were subsequently used for successfully cloning and sequencing of cDNA of this protein. Based on the similarity of the sequences it was finally confirmed that CBP 65/67 from rat liver is a homologue of the human annexin VI [2,32,36].

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