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Surface-modified membranes as a matrix for protein purification

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ABSTRACT

Recently introduced membrane-based chromatographic supports for protein separation are available either with a coupled ligand, *e.g.*, protein A, protein G or ion-exchange groups, or as activated matrices for coupling a desired ligand. The coupling conditions for protein A and immunoglobulin G to an epoxy-activated membrane were determined. The performance of the prepared affinity membranes was investigated using pure rabbit immunoglobulin G and protein A as a model system. For practical application monoclonal antibodies from cell culture supernatant were purified with a prepared protein A membrane and for comparison with a sulphonic acid ion exchange membrane.

INTRODUCTION

Affinity chromatography using particulate materials is a highly developed method for the purification of biomolecules [1]. It is commonly used in the final steps of purification procedures. However, there are some drawbacks for large-scale application even for the commonly used soft gels. The compressibility of the gels and pore diffusion limit the flow-rates. To overcome these disadvantages the particle diameter has been reduced and more rigid materials, *e.g.*, synthetic polymers or silica-based particles, have been introduced [2], but such supports require high-pressure equipment and silica-based particles are not stable at pH >8.

Alternatively, the use of membranes as chromatographic matrices has been proposed. In recent years microporous membranes, generally used for separation of cells and whole-broth clarification, were successfully modified for coupling ligands covalently. These new supports are rigid, pore diffusion is negligible (mass transfer is governed mainly by forced convection) and high-pressure equipment is not necessary [3].

Affinity membranes promise some advantages over common particulate materials, and several applications have already been published: fibronectin was purified using gelatin hollow fibres [3]; a *p*-benzamidine membrane was used for removal of thrombin and kallikrein from blood [4]; Hou and Zaniewski [5] isolated urokinase by means of metal chelate affinity membranes; Cibacron Blue membranes were useful for isolating microbial enzymes [6,7]; and protein A cartridges were tested for binding immunoglobulin G (IgG) from serum [8]. In combination with common particulate chromatographic materials, ion-exchange membrane-based cartridges gave good results in purifying recombinant interleukin [9], recombinant tissue plasminogen activator [10], β-1,4-xylanase [11] and antibodies [12].

The scope of this work was to study the coupling conditions for an epoxy-activated membrane and to determine the amount of protein coupled covalently. Further, the protein-binding capacity and stability of the prepared affinity membranes were investigated by using pure rabbit IgG and protein A. For practical application monoclonal antibodies from cell culture supernatant were purified. Alternatively, a monoclonal antibody reacting poorly with pro-

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tein A was isolated with a sulphonic acid ion-exchange membrane.

EXPERIMENTAL

Materials

Protein A (from a *Staphylococcus aureus* mutant secreting protein A), rabbit IgG and soybean trypsin inhibitor were purchased from Sigma and bovine serum albumin from Serva.

Cell culture supernatant from murine hybridomas containing a monoclonal antibody (mouse IgG_{2a} or mouse IgG_1) were kind gifts from Dr. U. Marx (Department of Medical Immunology, Medical School, Humboldt University, Berlin, Germany) and Dr. Wagner (Zellkulturtechnik GBF, Braunschweig, Germany), respectively. The cells were grown in a serum-free medium supplemented with BSA, transferrin, insulin and some minor additives as described previously [13].

The studies were performed with an epoxy-activated polymeric composite membrane (Sartobind Epoxy, pore size 0.2 μm) and a sulphonic acid ion-exchange membrane (Sartobind S, pore size 0.45 μm), which were kind gifts from Sartorius (Göttingen, Germany).

All solutions applied to the membranes were prefiltered using a 0.2-μm sterile filter. In order to achieve a uniform flow distribution, single membrane sheets were placed in an ultrafiltration cell (Amicon type 8050, 13.4 cm^2 membrane area, and Amicon type 8400, 42 cm^2 membrane area), without stirring and pressure equipment. Constant flow-rates were maintained by means of a peristaltic pump on the filtrate line (Pharmacia P-1).

Protein was determined according to the method of Lowry *et al.* [14] with BSA as standard, if not indicated otherwise. All investigations were carried out at room temperature.

Coupling of proteins to Sartobind Epoxy

Proteins were dissolved in appropriate coupling buffer (0.5 *M* phosphate, pH 5, 7 and 8, or 0.5 *M* carbonate, pH 9), resulting in concentrations of 0.9–1 mg/ml. The protein solutions were circulated through the membranes for 2–26 h at a flow-rate of 1 ml/min using a peristaltic pump (Pharmacia P-1).

Membranes to be used for chromatography were coupled for 16 h and treated with 2% ethyl glycinate in 0.1 *M* borate buffer (pH 8.3) for 2–3 h to block remaining reactive groups. Subsequently the membranes were washed three times with coupling buffer followed by 0.1 *M* citric acid (pH 2.5).

Membranes to be assayed for covalently coupled protein were washed with coupling buffer, 0.1 *M* citric acid (pH 2.5), 10% sodium dodecyl sulphate (SDS), 6 *M* urea and water.

Determination of protein bound covalently to Sartobind Epoxy

Method I. The amount of protein coupled covalenty was determined by amino acid analysis (similarly to the previous method for gel materials [15]). Membranes were hydrolysed in 6 *M* HCl–0.1% phenol in evacuated tubes for 24 h at 110°C. The HCl was evaporated and the remaining residue dissolved in 0.1 *M* sodium citrate buffer (pH 2.2), centrifuged and aliquots of the supernatant were applied to a Biotronic LC 5001 amino acid analyser. The separation was effected on a BTC 2710 cation-exchange resin column (210 × 3.2 mm I.D.) with step gradients, including pH, temperature and salt concentration changes. After reaction with ninhydrin, detection was applied at 440 and 570 nm. Calibration was performed using a Pierce amino acid standard containing 1 nmol of each amino acid. Membranes without protein were treated in the same way as a control.

Method II. Membranes were hydrolysed in 6 *M* HCl for 24 h at 37°C. After neutralization with 6 *M* NaOH, the solution was centrifuged and the supernatant analysed according to the method of Lowry *et al.* [14]. Calibration graphs were obtained by hydrolysing known amounts of the appropriate protein in the presence of the starting membrane under the same conditions (similarly to the previous method for gel materials [16]).

Determination of protein-binding capacity of protein A and IgG membranes

The adsorption characteristics of the prepared affinity membranes were investigated by passing solutions of different concentrations of protein A or rabbit IgG in 50 m*M* phosphate (pH 7.5) through the corresponding membrane. Protein solutions were applied until the concentration in the outlet was nearly the same as that in the starting solution.

Unbound protein was washed out with 50 m*M*

phosphate (pH 7.5) and desorption was effected with 0.1 *M* citric acid (pH 2.5). The area of the protein A membrane was 13.4 cm^2 and that of the IgG membrane 42 cm^2.

Affinity chromatography

Cell culture supernatant containing mouse IgG_{2a} antibody was concentrated by ultrafiltration and applied to a protein A membrane (13.4 cm^2) previously equilibrated with 0.1 *M* phosphate (pH 8.3) at a flow-rate of 1 ml/min. Washing was performed with 0.1 *M* phosphate–2 *M* NaCl and elution with 0.1 *M* citric acid (pH 3.5). The antibody concentration was determined by enzyme-linked immunosorbent assay (ELISA).

Ion-exchange chromatography

Cell culture supernatant containing mouse IgG_1 monoclonal antibody (conductivity 12 mS, pH 7.8, protein content 1 mg/ml, mainly BSA, antibody concentration 120 μg/ml) was diluted with 20 m*M* citrate (pH 5.5) and the pH adjusted, resulting in a conductivity of 4.5 mS (pH 5.5) a protein content of 0.25 mg/ml and an antibody concentration of 30 μg/ml.

The prefiltered supernatant was applied to a sulphonic acid ion-exchange membrane (42 cm^2), previously equilibrated with 20 m*M* citrate (pH 5.5). Washing was performed with equilibration buffer and elution with equilibration buffer supplemented with 140 m*M* and 1 *M* NaCl, respectively. The flow-rate throughout the whole process was 5 ml/min.

Electrophoresis

SDS–polyacrylamide gel electrophoretic (SDS-PAGE) analysis was done using Pharmacia Phast Gels (8–25% polyacrylamide–SDS) and the silver staining method according to Butcher and Tomkins [17].

RESULTS AND DISCUSSION

Preparation and characterization

Coupling of proteins to Sartobind Epoxy. We chose the direct determination of covalently coupled protein by hydrolysis of the membranes, as this method has proved to work well for gel materials [15,16]. The indirect method often used for measuring the difference of protein concentration before and after coupling needs a large amount of material for analysis to give reasonable values and reduced errors.

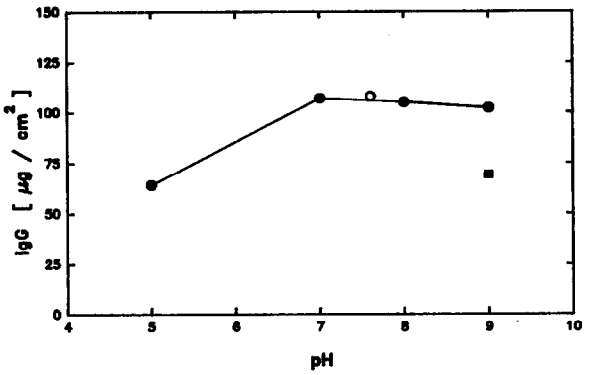

Fig. 1. Coupling of rabbit IgG to epoxy-activated membrane. Coupling buffer: 0.5 *M* phosphate (pH 5–8) or 0.5 *M* carbonate (pH 9); values obtained by amino acid analysis. Coupling time: ■ = 5 h; ● = 16.5 h; ○ = 26 h.

Fig. 1 shows the results for coupling rabbit IgG at different pH values. At pH 7–9 the amount of coupled protein was 107 μg/cm^2 (=0.67 nmol, IgG MW = 160 000 dalton), whereas only 60% of the maximum value was obtained at pH 5. This value is in good agreement with the value stated by the producer obtained by radioactive assay (100 μg/cm^2). Using the volume conversion factor for the membrane, 45 cm^2 ≙ 1 ml ≙ 265 mg, 4.8 mg protein was bound per ml membrane or 18.1 mg protein per g membrane.

Coupling was performed overnight for 16.5 h. A prolonged incubation time in the optimum pH range did not result in a higher coupling yield, whereas a shorter time (5 h) reduced the amount of IgG coupled covalently to 64%.

Protein A was coupled at pH 8 for various incubation times (Fig. 2). The maximum capacity was reached after 16 h, corresponding to 35 μg/cm^2 or 0.83 nmol/cm^2, but in contrast to IgG shorter incubation times decreased the total amount only to 85%, which might be due to the lower molecular weigth of protein A (42 000 dalton), leading to better access to the activated groups. For comparison, with an even smaller protein, soybean trypsin inhibitor (MW=23 000 dalton), the value obtained for coupling after 16 h at pH 8 was 20 μg/cm^2 or 0.87 nmol/cm^2.

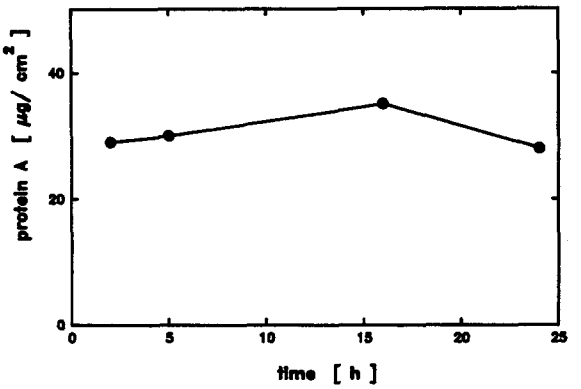

Fig. 2. Protein A bound covalently to epoxy-activated membrane. Protein A in 0.5 *M* phosphate (pH 8) was circulated for 2–24 h. Subsequently, the membranes were washed, hydrolysed and assayed by method II.

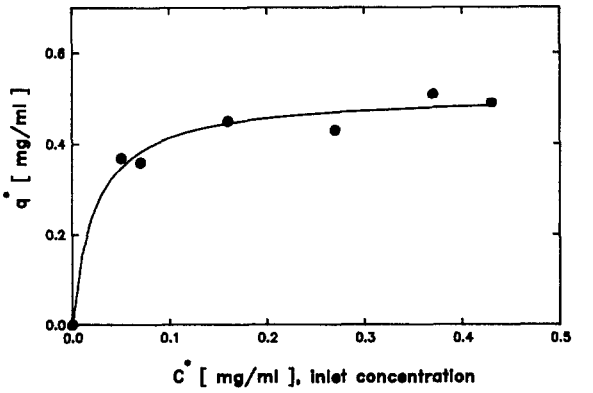

Fig. 3. Adsorption isotherm of protein A membrane. Pure rabbit IgG in 50 m*M* phosphate (pH 7.5) was applied until the outlet concentration was identical with the inlet concentration. After washing with 50 m*M* phosphate (pH 7.5), bound IgG was eluted with 0.1 *M* citric acid (pH 2.5). Flow-rate, 3 ml/min.

Protein-binding capacity of protein A and IgG membranes. Figs. 3 and 4 show the adsorption isotherms for the protein A membrane and IgG membrane, respectively. The shapes of the isotherms indicate Langmuir-type adsorption, which can be described by the equation

$$dq/dt = k_1C(q_m - q) - k_2q \qquad (1)$$

where C is the concentration of adsorbate in solution, q the solid-phase concentration of adsorbed molecules and q_m the maximum capacity of the adsorbent. At equilibrium, eqn. 1 leads to

$$q^* = \frac{q_mC^*}{K_d + C^*} \qquad (2)$$

where $K_d = k_2/k_1$ is the dissociation constant of the system. The following values were obtained: K_d = 0.047 mg/ml ($2.9 \cdot 10^{-7}$ *M*) and q_m = 4.74 mg/ml for the system protein A membrane–rabbit IgG, and K_d = 0.023 mg/ml ($5.5 \cdot 10^{-7}$ *M*) and q_m = 0.51 mg/ml for the system IgG membrane–protein A.

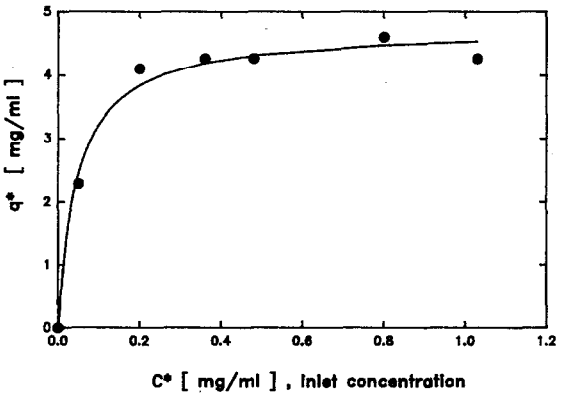

Fig. 4. Adsorption isotherm of IgG membrane. Pure protein A in 50 m*M* (pH 7.5) was applied until the outlet concentration was identical with the inlet concentration. Washing was done using 50 m*M* phosphate (pH 7.5) and elution with 0.1 *M* citric acid (pH 2.5). Flow-rate, 2 ml/min.

A binding ratio of more than one bound IgG molecule for each protein A molecule (1.3 nmol IgG/nmol protein A) was found for the protein A membrane by using the maximum amount of IgG bound obtained from the isotherm and the amount of protein A coupled covalently to the membrane determined as described. For the system IgG membrane–protein A the binding ratio was calculated to be 2.5 nmol IgG/nmol protein A. These values are in agreement with findings for particulate materials [18] and are reasonable as one protein A molecule was found to bind two molecules of IgG [19].

The lower binding ratio obtained in the system protein A membrane–IgG might be due to multipoint attachment of protein A. Shorter coupling times may be useful in preventing this phenomenon. Another point to be considered is the steric hindrance of the large IgG molecule.

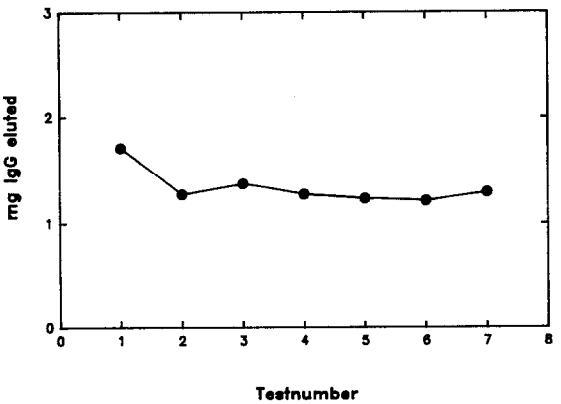

Fig. 5. Repeated use of protein A membrane. Saturating concentrations of IgG in 50 m*M* phosphate (pH 7.5) were applied. After washing with 50 m*M* phosphate (pH 7.5) and elution with 0.1 *M* citric acid (pH 2.5) the membrane was re-equilibrated and used again.

Stability of protein A membrane. For practical applications, the stability of the prepared affinity membranes is very important. The results of repeated use of the protein A membrane are shown in Fig. 5. After the first run a decrease of *ca.* 20% in IgG capacity was observed, and this value remained stable in subsequent runs. This result is similar to those of other workers for protein A columns, and is probably due to non-covalently bound protein being washed out after the first run rather than to ligand leakage [20]. The protein A membrane used for chromatography was washed under mild conditions compared with the membranes needed for amino acid analysis and some non-covalently bound protein A may have been retained on the matrix. From the difference in IgG adsorption in the first and the following runs and the binding ratio of 1.3 nmol IgG/nmol protein A, about 10 μg/cm^2 non-covalently bound protein A were retained on the matrix material after coupling and washing.

The membrane was re-used about 30 times, including runs with pure rabbit IgG and crude cell culture supernatant. During this period a decrease in capacity of 30% was observed. Cleaning with 6 *M* urea was done occasionally, but cleaning procedures for matrices containing proteins, which cannot be cleaned with NaOH and used with high protein concentrations and crude solutions, still have to be optimized.

Resolution. When considering the chromatographic feature of the membranes, the resolution of such matrices is of concern. Generally, high resolution could be expected. Plate heights can be predicted from the theoretical relation of 2–5 d_p, where d_p is the nominal pore size of the membrane, resulting in values of 0.4–2 μm for the present membranes. From the experiments performed (*e.g.*, see Fig. 6), values of about 3–7 μm can be calculated for flow-rates of 1–3 ml/min (bed height 200 μm), which are larger than expected but in agreement with earlier findings [21]. The main reason for this discrepancy is probably the unfavourable ratio of the dead volume of the filter device to the membrane bed volume, rather than axial dispersion in the membrane, which may lead to some back-mixing effects and result in peak broadening. This is supported by other workers [22]. Nevertheless, a resolution as high as for very small particulate high-performance liquid chromatographic materials (1–5 μm) can be assumed for the membrane matrices [21].

Applications

Affinity chromatography. Fig. 6 shows the purification of a monoclonal antibody (mouse IgG_2) from cell culture supernatant. The cell-free supernatant was concentrated by ultrafiltration using an Amicon PM 30 membrane. A 5-ml volume of the supernatant, containing 13.2 mg/ml of protein (mainly BSA) and 300 μg/ml of antibody, was applied to a protein A membrane (13.4 cm^2) previously equilibrated with 0.1 *M* phosphate (pH 8.3). Washing was done with 20 ml of equilibration buffer suplemented with 2 *M* NaCl and desorption was carried out using 0.1 *M* citric acid (pH 3.5). During the whole process the flow-rate was maintained constant at 1 ml/min. The first peak represents the unbound fraction and the second the eluted antibody. The recovery of the applied amount of antibody was 93% (Table I). As judged by SDS-PAGE the antibody was pure (Fig. 7), showing two bands corresponding to the heavy and light chains of IgG.

Ion-exchange chromatography. Ion-exchange chromatography is an alternative to affinity chromatography when the antibody reaction with protein A is weak; also ion-exchange matrices might be preferred when isolating antibodies for pharmaceutical use, in order to prevent contamination with

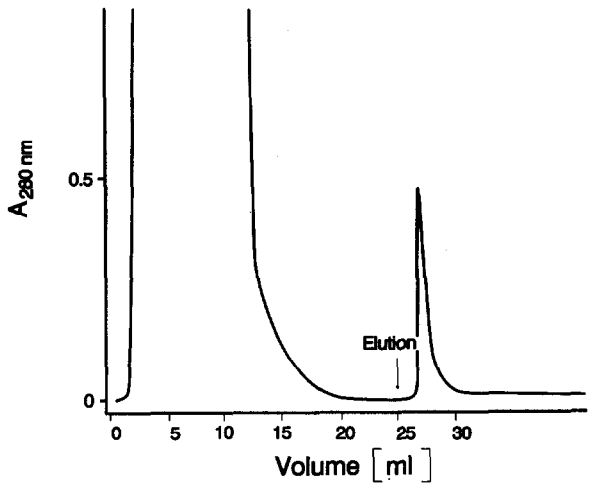

Fig. 6. Purification of monoclonal antibody from cell culture supernatant. Concentrated serum-free supernatant containing 300 μg/ml of monoclonal antibody and 13.2 mg/ml of protein was applied at a flow-rate of 1 ml/min to a protein A membrane (13.4 cm^2). Washing was performed using 0.1 *M* phosphate (pH 8.3)–2 *M* NaCl and elution with 0.1 *M* citric acid (pH 3.5). The arrow indicates the start of elution.

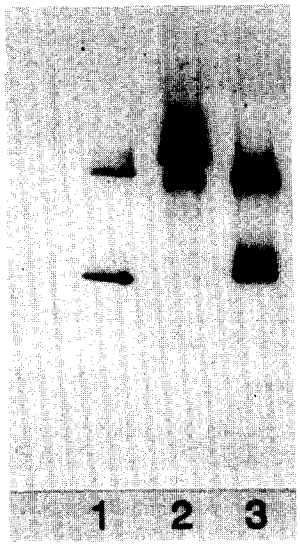

Fig. 7. SDS polyacrylamide gradient gel (8–25%) electrophoresis with silver staining. Lanes: 1 = protein A-membrane purified monoclonal antibody; 2 = crude cell culture supernatant; 3 = pure rabbit IgG.

protein A [23]. We therefore compared the results from affinity membranes with those from the purification of a mouse IgG_1 antibody by means of sulphonic acid ion-exchange membrane. From the manufacturer's information the ion-exchange membrane used has a capacity for proteins of 10–100 mg/ml (1 ml ≙ 50 cm^2), depending on the type of protein, protein concentration and buffer system.

Fig. 8 illustrates the antibody purification with an ion-exchange membrane. By dilution with the chromatographic buffer the conductivity and pH of the cell culture supernatant were decreased appropriately and applied at a flow-rate of 5 ml/min to a

TABLE I

PURIFICATION OF MONOCLONAL ANTIBODY FROM CELL CULTURE SUPERNATANT BY PROTEIN A MEMBRANE (VALUES OBTAINED BY ELISA)

Step	Antibody (μg/ml)	Volume (ml)	Total amount of antibody (mg)
Supernatant	300	5.0	1.5
Breakthrough (unbound proteins)	0.18	6.8	<0.01
Wash[a]	0	7.2	0
Elution	500	2.8	1.40

[a] Only the first fractions were assayed.

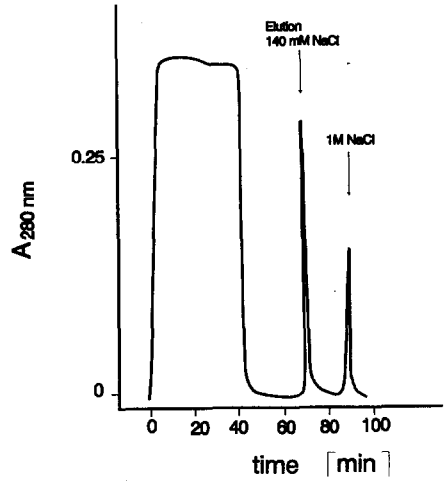

Fig. 8. Typical chromatogram for antibody isolation with the sulphonic acid ion-exchange membrane. Serum-free cell culture supernatant containing 30 μg/ml of antibody and 0.25 mg/ml of protein was applied at a flow-rate of 5 ml/min to a sulphonic acid ion-exchange membrane (42 cm^2). Elution of the antibody was performed using 50 m*M* citrate (pH 5.5)—140 m*M* NaCl. Additional cleaning was effected with 1 *M* NaCl.

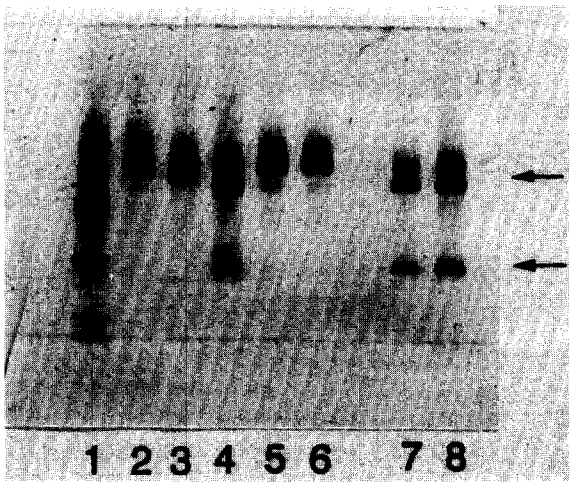

Fig. 9. SDS polyacrylamide gradient gel (8–25%) electrophoresis with silver staining; isolation of monoclonal antibodies with sulphonic acid ion-exchange membrane (fractions from two runs). Lanes: 1 = Pharmacia molecular weight markers (78 000, 66 250, 45 000, 30 000, 17 200, 12 300 dalton); 2 = cell culture supernatant; 3 = breakthrough; 4 = monoclonal antibody eluted with 140 m*M* NaCl; 5 = cell culture supernatant; 6 = breakthrough; 7 and 8 = monoclonal antibody eluted with 140 m*M* NaCl. Arrows indicate the light and heavy chains.

sulphonic acid ion-exchange membrane (42 cm^2). Elution (second peak in Fig. 8) with 140 *M* NaCl resulted in 1.63 mg of antibody in 7.7 ml; no more antibody was eluted with 1 *M* NaCl. The antibody obtained was slightly contaminated with BSA as judged by SDS-PAGE (Fig. 9) and required an additional purification step by gel filtration.

CONCLUSION

Affinity and ion-exchange membranes are useful matrices for purifying monoclonal antibodies. They are simple to handle, as bed packing procedures are not necessary, and scale-up can be performed easily. Cross-flow filtration experiments are under investigation and seem to be even more promising than dead-end filtration, because crude homogenates and cell culture supernatants can be applied without prior clarification.

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