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Purification of immunoglobulins G by protein A/G affinity membrane chromatography

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Abstract

An affinity membrane grafted with protein A/G or protein A was characterized for human and mouse immunoglobulins G purification. Breakthrough curves up to ligand saturation were measured and used to study the effects of flow velocities, feed solution concentrations and protein A/G versus protein A membranes. Increased flow-rate did not decrease the amount of IgG bound to the membranes. Increased feed solution concentration allowed more IgG to bind prior to breakthrough. Kinetic parameters for immunoglobulins G sorption to immobilized protein A were measured in batch experiments. The static binding capacity was determined to be 6.6 mg ml^{-1} membrane volume. Finally, this affinity membrane was used to purify IgG from cell culture supernatant. The electrophoresis of the purified IgG fractions did not show any contaminant. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Membrane chromatography; Immunoglobulins G; Protein A/G

1. Introduction

Affinity chromatography is a purification method that exploits the biospecific interactions between a protein and a ligand to economically purify proteins present at very low concentrations in complex solutions instead conventional methods [1]. Affinity membrane chromatography was designed to bypass the limitations of conventional affinity gel chromatography: slow intraparticle diffusion in large beads, or low flow-rates due to high-pressure drops in small beads [2–5]. Convection through thin microporous membranes eliminates diffusion and pressure drop concerns, yielding a purification that ideally is limited by intrinsic sorption kinetics between the protein and ligand, thus minimizing processing time and maximizing purification efficiency.

Affinity membranes have been designed and evaluated in the form of flat sheet systems and stacks of membranes, hollow fibers, radial flow cartridges and tubes [6–12]. Ions and hydrophobic groups may also be attached to microporous membranes, and solutes are transported to binding sites primarily by convection. Despite promising results and the availability of several membrane adsorbers on the market, membrane chromatography technology has lagged

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behind column chromatography both in analytical and preparative biochromatography.

In an affinity system, breakthrough is defined as the point at which the solute being purified appears in the effluent solution [1]. The breakthrough curve is the effluent concentration profile as a function of the mass of solute loaded or the effluent volume. Ideally breakthrough would occur in affinity chromatography only after the protein and ligand reach saturation, at which point the effluent concentration would instantaneously increase from zero to that of the feed solution [6]. Actual breakthrough curves are broadened by the nonidealities of real flow systems, e.g., dead volume mixing and flow maldistribution, and by slow intrinsic sorption kinetics.

In industrial purification of high-value proteins, it is desirable to minimize the loss of product into the filtrate stream during the loading step [7,13]. Consequently, adsorption is allowed to proceed until breakthrough occurs, at which point the loading step is terminated. In contrast, in medical therapeutic applications the objective is to remove a pathogenic agent from blood or plasma, and adsorption is allowed to continue until the matrix is nearly saturated.

This work explores the performance of recombinant protein A/G affinity membranes for human and mouse IgG purification. The effects of a variety of variables on human and mouse IgG breakthrough curves, including flow-rate and feed solution concentration, are studied.

2. Experimental

2.1. Membrane

The affinity membranes were prepared from poly (methyl methacrylate, acrylonitrile and sodium methallyl sulfonate) membranes (Tech-sep, Miribel, France). The covalent linkage of protein A/G or protein A onto the polymer is made after chemical modification of the membrane [14]. First, a diamine, the 4,7,10 trioxatridecane-1,13-diamine (Sigma, Saint Quentin Fallavier, France), reacts with the methacrylate ester. Secondly, the bis-[*N*-sulfisuccinimidyl] substrate (Pierce, Madison, IL, USA) reacts with superficial amines of the preactivated membrane at one side and on the protein A/G or A on the other side. The membrane thickness was 50 μm and the mean pore diameter was 0.6–1 μm [15]. The void porosity, ϵ, was 0.70.

2.2. Cartridge

Protein A/G and protein A affinity membrane cartridges consisting of a stack of eight membranes were used in this work. The stack of eight membranes was sealed in a cylindrical, inox housing. A system of radial channels in the top and the bottom lid of the housing distributed the flow at the entrance and exit of the cartridge, respectively. Based on the 5.3-cm inner diameter of the gaskets and the 0.4-mm stack of eight-membrane thickness, the total flow-accessible membrane volume was calculated to be 0.88 ml.

2.3. Equipment

The cartridge was placed into a box of expansed polystyrene which was thermostated at 25°C to minimize temperature fluctuations during experiments. A spectrophotometer (Spectrometer UVmc^2, Safas, Monaco) connected to a computer (HP vectra, P133, Hewlett-Packard, Lyon, France) was used to record the effluent IgG concentration throughout the experiments. Fractions of the effluent were collected using a fraction collector (Ultrarac 7000, LKB, France). The pumping system consisted of a peristaltic pump (Minipulse, Gilson, Villiers-le-Bel, France).

Gel casting and migration for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis were done on a Blue vertical 100/C system, (Biowhittaker, Gagny, France) with a high-voltage generator (Biowhittaker).

3. Chemicals

All buffers and solutions were filtered through 0.22-μm filters (Millipore, Molsheim, France) before use. Buffers were stored at 4°C. The loading buffer was 0.1 mol l^{-1} glycine, 0.15 mol l^{-1} NaCl, pH 9, for human IgG separation and 0.1 mol l^{-1} phosphate, 0.15 mol l^{-1} NaCl, pH 6.5, for mouse IgG sepa-

ration. The elution buffer was 0.1 mol l^{-1} carbonate, 0.15 mol l^{-1} NaCl, pH 11.

Lyophilized human IgG was obtained from Laboratoire Français du Fractionnement et des Biotechnologies (Paris, France). Protein A was purchased from Interchim (Montluçon, France) and protein A/G from Pierce. Pure mouse IgG (93%) was obtained from Sigma.

Cell culture supernatants from murine hybridomas containing a monoclonal antibody (mouse $IgG1_K$) were kind gifts from Dr. Kessler (Laboratoire de Virologie, Faculté de Médecine Grange-Blanche, Lyon, France). A murine hybridoma (6C10E4E5H7F10) was used for monoclonal antibody production in a hollow fibre bioreactor [16].

4. Breakthrough curve (BTC) procedure

The flow-rate (0.1–10 ml min^{-1} or interstitial flow velocity $v = 1 \times 10^{-4}$ to 1×10^{-2} cm s^{-1}) through the system was adjusted using the loading buffer. After obtaining a baseline on the screen of the computer, IgG in loading buffer was pumped into the cartridge to produce the breakthrough curve. Unbound IgG was then rinsed from the cartridge using loading buffer (≈50 ml). Bound IgG was eluted using the elution buffer. After elution was complete, the cartridge was washed with the loading buffer (≈50 ml) to restore it to its initial conditions for carrying out the next experiment. Readings from the UV detector were checked to ensure that it returned to baseline at the end of the washing step, indicating that all unbound IgG had been removed and, at the end of the elution step, indicating that all adsorbed protein had been removed from the membrane binding sites [7].

The adsorbance of the IgG solution flowing through the spectrophotometer was measured at 280 nm. The IgG concentration was determined using an extinction coefficient of 1.2 mg^{-1} cm^{-1} for human IgG and 1.6 mg^{-1} cm^{-1} for mouse IgG (loading and elution buffers). For one breakthrough curve, 2.5-ml fractions were collected and protein concentration was measured by Coomassie Blue. These values were identical to the protein concentrations calculated from the absorbance detector. One set of eluant fractions was collected by the fraction collector for further analysis with SDS–PAGE.

5. Batch sorption measurements

The membranes (total membrane volume V_m = 0.88 ml) that had been freed from one cartridge were placed into a flask containing a 0.082 mg ml^{-1} IgG solution in a final volume of 100 ml (V_{sol} = 100 ml). The suspension was agitated at low speed for approximately 48 h at 25°C. Samples of 100 μl were taken periodically and placed into haemolysis tubes containing 0.4 ml of loading buffer. The samples were immediately frozen. Coomassie Blue were used to determine IgG concentrations. The optical density was measured at 595 nm. A calibration showed that optical density was linear with concentration over the range of examination (0.001–0.025 mg ml^{-1} IgG after dilution).

6. Electrophoresis

Analysis of the purity of the proteins eluted from the protein A/G affinity cartridge was performed by SDS–PAGE under denaturing conditions on 11% gels [17] using Blue Coomassie R250 to stain the separated bands and protein markers from 97.4 to 14.4 kg mol^{-1} (Amresco, Solon, OH, USA).

7. Results

Breakthrough curves up to ligand saturation were measured and used to study the effects of flow velocities, feed solution concentrations and protein A/G versus protein A membranes.

Fig. 1a and b shows the human IgG breakthrough curves and elution profiles from a protein A/G cartridge at flow-rates of 0.1, 1.0 and 10 ml min^{-1}. The breakthrough curves contain the following main features: low IgG binding prior to breakthrough, lack of membrane saturation and broadness. Little IgG bound to the cartridge prior to breakthrough at all three flow-rates. Decreasing the flow-rate do not increase the amount bound. Defining breakthrough as c/c_0 = 0.1, 1.2, 1.2 and 1.1 mg of IgG were loaded

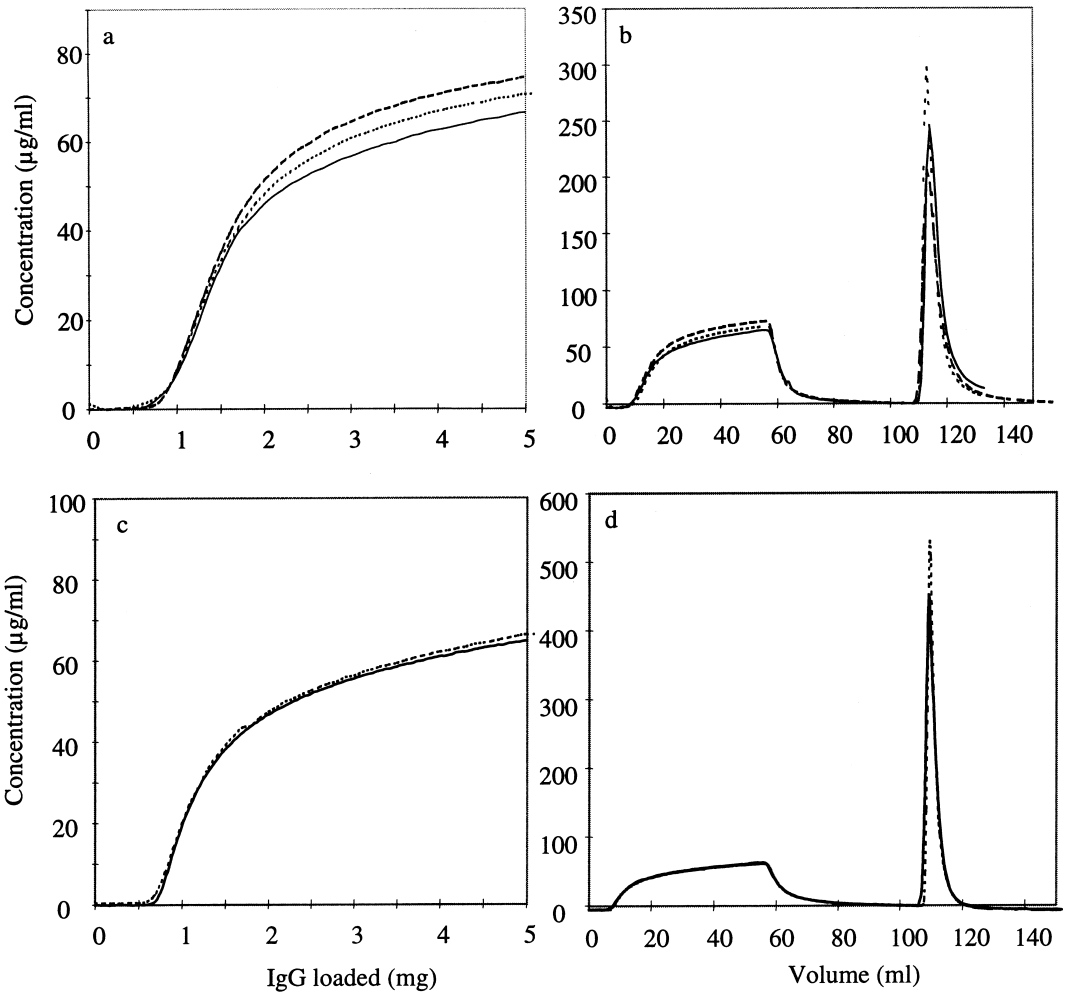

Fig. 1. (a) Effect of flow-rate on the breakthrough of human immunoglobulin G (100 μg ml^{-1}) from an affinity membrane cartridge containing immobilized recombinant protein A/G. Experiments were performed at each of three flow-rates, (—) 0.1, (···) 1.0 and (- - -) 10 ml min^{-1}. (b) Effect of flow-rate on the breakthrough curves and elution profiles. (c) Effect of flow-rate on the breakthrough of mouse immunoglobulin G (100 μg ml^{-1}) from an affinity membrane cartridge containing immobilized recombinant protein A/G. Experiments were performed at each of two flow-rates, (—) 1.0 and (···) 10 ml min^{-1}. (d) Effect of flow-rate on the breakthrough curves and elution profiles.

prior to breakthrough at flow-rates 0.1, 1.0 and 10 ml min^{-1}, respectively. Based on the area under the elution peaks, the mass of IgG eluted was 1.5±0.1 mg for each of the flow-rates.

Fig. 1c and d shows the mouse IgG breakthrough curves and elution profiles from a protein A/G cartridge at flow-rates of 1.0 and 10 ml min^{-1}. Very little difference was observed between the two breakthrough curves. Defining breakthrough as $c/c_0 = 0.1$, 1.0 and 0.9 mg of IgG were loaded prior to breakthrough in the 1.0 and 10 ml min^{-1} experiments respectively. The mass of IgG eluted was 1.6±0.1 mg for each of the flow-rates, based on the area under the elution peaks. Continued removal of IgG from the feed solution was observed until the end of all experiments. The slopes of all the breakthrough curves at the end of the experiments were very small, indicating a slow approach to membrane

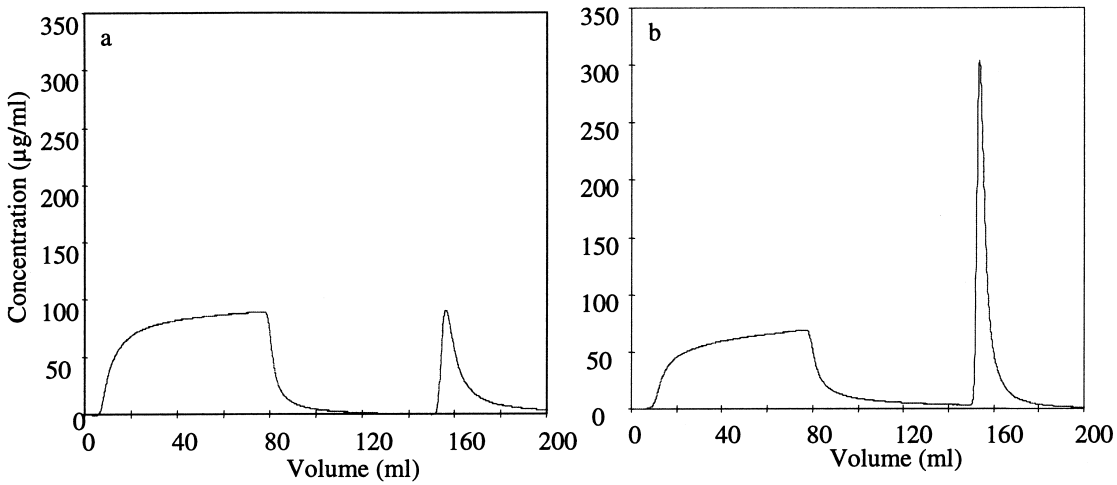

Fig. 2. Breakthrough curves and elution profiles of human immunoglobulin G (100 mg ml^{-1}) from a protein A affinity membrane cartridge (a) and a protein A/G affinity membrane cartridge (b). The flow-rate was 3.6 ml min^{-1}.

saturation. After 21.6 mg of a 0.1-mg ml^{-1} human IgG solution had been loaded at 3.6 ml min^{-1}, c/c_0 was approximately 0.99 (data not shown).

Fig. 2a and b shows the comparison between adsorption breakthrough curves and elution profiles using protein A and protein A/G affinity membranes cartridge, respectively. The concentration of human IgG was 0.1 mg ml^{-1} and the flow-rate was 10 ml min^{-1} for both experiments. The amount of IgG that had been loaded was 5 mg. The mass of IgG eluted based on the area under the elution peaks was 0.94 and 1.7 mg, for protein A and protein A/G experiments, respectively. This result showed the advantage of protein A/G affinity membranes over protein A affinity membranes for IgG purification.

Fig. 3a and b contain breakthrough curves and

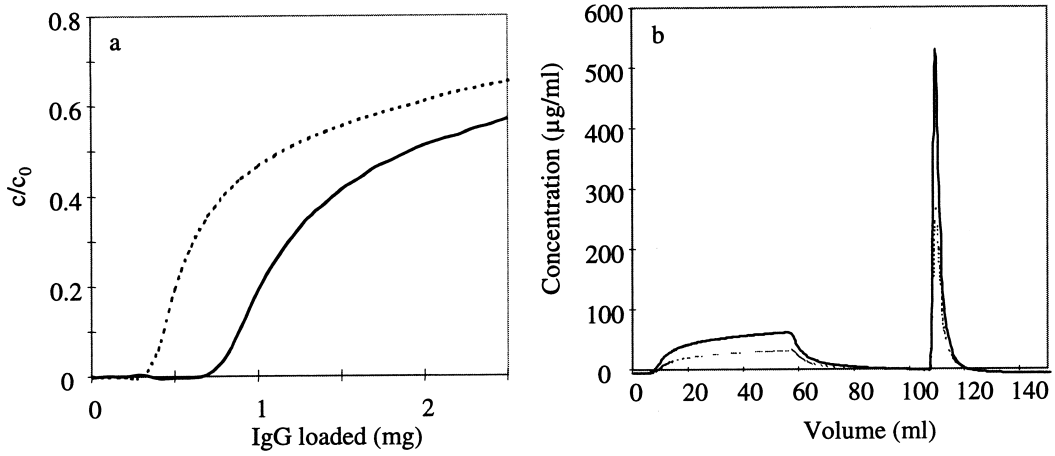

Fig. 3. (a) Effect of feed solution concentration on the breakthrough curve of mouse immunoglobulin G from an affinity membrane cartridge containing immobilized recombinant protein A/G. (···) 50 μg ml^{-1} and (—) 100 μg ml^{-1}. (b) Effect of feed solution concentration on breakthrough curves and elution profiles.

elution profiles for mouse IgG solution concentrations of 0.05 and 0.1 mg ml^{-1} at 10 ml min^{-1}. More IgG binds from the higher concentration feed solution (0.9 mg) prior to breakthrough defined as $c/c_0 = 0.1$ than the lower concentration (0.4 mg). Based on the area under the elution peaks, the mass of IgG eluted was 1.7 mg for 0.1 mg ml^{-1} solution and 0.9 mg for 0.05 mg ml^{-1} solution. The breakthrough curve shape is similar at both feed solution concentrations, but the curves are shifted horizontally from each other.

Small loss in capacity (18%) was detected over 4 weeks of storage. Possible leakage of immobilized protein A/G was examined by Coomassie Blue and gave negative results.

Adsorption measurements of IgG to immobilized protein A/G affinity membranes were conducted in batch experiments and are shown in Fig. 4. The equilibrium reached is 0.15 μmol l^{-1}. The integrated form of the rate equation of the Langmuir type given by Suen and Etzel [9], was fit to the experimental data to determine the association rate constant k_a for IgG binding to protein A/G affinity membranes:

$$\frac{c}{c_0} = 1 - \frac{2c_1 \sinh\left(\frac{VG}{2} k_a t\right)}{G \cosh\left(\frac{VG}{2} k_a t\right) + B \sinh\left(\frac{VG}{2} k_a t\right)} \quad (1)$$

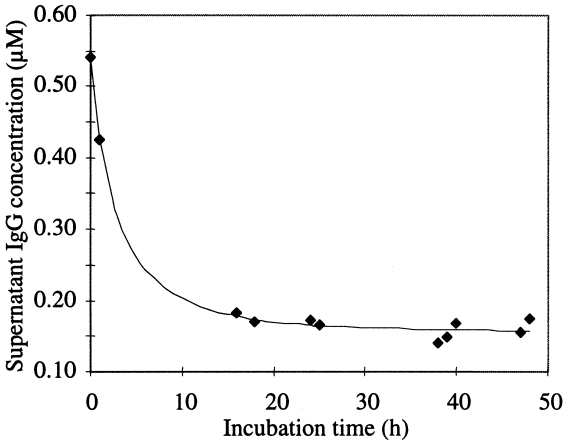

Fig. 4. Batch adsorption of mouse immunoglobulin G ($c_0 = 5.4 \times 10^{-7}$ mol l^{-1}) onto recombinant protein A/G immobilized onto affinity membranes. As indicated using a solid line, the data were fit to Eq. (1) resulting in 203 mol^{-1} l s^{-1} for k_a.

where

$$B = \frac{c_0}{V} + c_1 + \frac{K_d}{V} \quad (2)$$

$$G = \left(B^2 - \frac{4c_0c_1}{V}\right)^{\frac{1}{2}} \quad (3)$$

$$K_d = \frac{k_d}{k_a} \quad (4)$$

The initial IgG concentration, c_0, was 0.081 mg ml^{-1}. The ratio of membrane solid volume to solution volume, *V*, was calculated from

$$V = \frac{(1-\epsilon)V_m}{V_{sol}} \quad (5)$$

V was determined to be 0.0026. The total ligand capacity based on the solid volume of the membrane, c_1, was calculated from:

$$c_1 = \frac{(c_0 - c_{eq})V_{sol}}{(1-\epsilon)V_m} \quad (6)$$

Where c_{eq} was the IgG concentration in the solution at equilibrium. c_1 was determined to be 22 mg ml^{-1}. The total ligand capacity based on total membrane volume was 6.6 mg ml^{-1}. The value obtained from least-squares fitting Eq. (1) to the data was 203±45 mol^{-1} l s^{-1} for k_a. Unarska et al. [4] studied the reaction rate between human γ-globulin and immobilized protein A onto a microporous nylon membrane. The reaction rate coefficient was found equal to 0.086 mg^{-1} ml min^{-1} (215±35 mol^{-1} l s^{-1}) with 0.45-μm membranes and immersion flow conditions. We find k_a equal to 203 mol^{-1} l s^{-1}, which is close to the data of Unarska et al., although the assumptions of the two theoretical models are different.

Fig. 5 shows the purification of monoclonal antibody (mouse IgG_1) from cell culture supernatant. A 50-ml volume of the concentrated supernatant, containing approximately 70 mg/ml of protein (mainly bovine serum albumin) and 70 μg ml^{-1} of antibody, was applied to a protein A/G affinity membrane cartridge previously equilibrated with phosphate-buffered saline (PBS). Washing was done with 50 ml PBS and desorption was carried out using carbonate-buffered saline. During the whole process the flow-rate was maintained constant at 10 ml min^{-1}. The first peak represents the unbound fraction and the

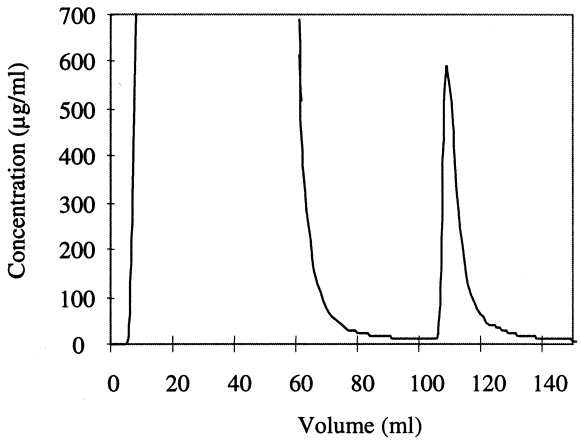

Fig.5. Purification of monoclonal antibody from cell culture supernatant. Concentrated supernatant containing 70 µg ml^{-1} of IgG1 and 70 mg ml^{-1} of protein was applied at a flow-rate of 10 ml min^{-1} to a protein A/G affinity membrane cartridge. Washing was performed using PBS and elution using carbonate buffer saline.

second the eluted antibody. The amount of purified IgG, based on the area under the elution peak was 5 mg ml^{-1} membrane volume. As judged by SDS–PAGE, the antibody was pure (Fig. 6) showing two bands corresponding to the heavy and light chains of IgG.

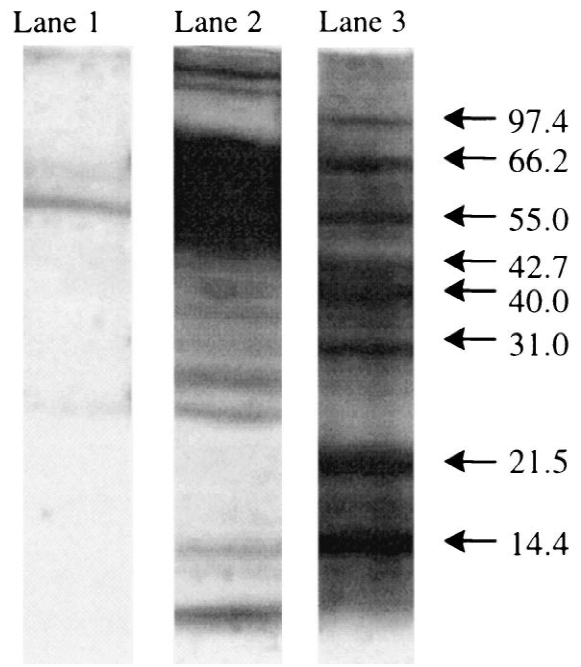

Fig. 6. SDS–PAGE. Lanes: 3, molecular weight markers (97–14 kg mol^{-1}); 2, cell culture supernatant; 1, monoclonal antibody eluted with carbonate-buffered saline.

8. Discussion

In order for protein to freely diffuse to the wall of a pore and bind before it passes through the membrane, the time scale for axial convection must greatly exceed the time scale for boundary layer mass transfer to the pore wall, i.e., $t_R >> t_{BLMT}$. As an order-of-magnitude approximation, $t_{BLMT} \approx d_p^2/4D$ [18]. In this work, t_R was 4–400 s, and t_{BLMT} was 4×10^{-3} s. Consequently, mass transport limitations due to film diffusion were negligible [19].

The time scales for protein diffusion to and association with the ligand were calculated and compared. The time scale for protein diffusion was l^2/D. The diffusion path length (l) was 25 µm, half the thickness of an individual membrane. The diffusion coefficient for IgG was 3.8×10^{-7} cm^2 s^{-1} [9]. Thus, the time scale for protein diffusion was 16.4 s. The average time scale for protein association with the ligand ($t_A = 1/k_a c_0$) was 9.1×10^3 s. The time scale for protein diffusion was negligible in comparison to the time scale for protein association with the ligand.

9. Conclusion

Breakthrough curves of immunoglobulins G solutions are measured for various flow velocities. We found that the flow velocity does not influence the BTC. However, the approach to saturation was very slow, as a small fraction of IgG continued to bind for a long time. The IgG residence times in the membrane during the breakthrough curve experiments were insufficient for complete binding of IgG to the membrane.

10. Notation

B constant defined by Eq. (2)

c	supernatant IgG concentration in solution (mg ml^{-1} or mol l^{-1})
c_{eq}	concentration at equilibrium (mg ml^{-1} or mol l^{-1})
c_1	ligand capacity based on the solid volume of the membrane (mg ml^{-1} or mol l^{-1})
c_0	initial IgG concentration (mg ml^{-1} or mol l^{-1})
d_P	pore size (cm)
D	axial diffusion coefficient (cm^2 s^{-1})
G	constant defined by Eq. (3)
k_a	association rate constant (mol^{-1} l s^{-1})
k_d	dissociation rate constant (s^{-1})
K_d	equilibrium dissociation constant (mol l^{-1})
l	diffusion path (cm)
L	membrane stack thickness (cm)
t	time (s)
t_A	time scale for protein–ligand association (s)
t_R	mean residence time of the fluid in the membrane (s)
t_{BLMT}	time scale for boundary layer mass transfer (s)
v	interstitial flow velocity (cm s^{-1})
V	ratio of membrane solid volume to solution volume
ϵ	void porosity

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