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A dual-mode approach to the selective separation of antibodies and their fragments☆

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Abstract

A novel chromatography method for the separation of antibodies is described. The adsorption of antibodies on the solid phase involves interaction with a ligand that combines mild hydrophobic characteristics and some degree of molecular recognition with a derivative of pyridine. This combined effect results in the adsorption of antibodies in the absence of lyotropic salts. When environmental pH is changed, the ligand becomes ionically charged, allowing the desorption of antibodies. The mechanism of adsorption, involving hydrophobic associations and ionic related interaction, is here qualified as dual-mode. Studies on the determination of the apparent dissociation constant for immunoglobulins G are presented. Adsorption of antibodies from crude feedstocks typically occurs without adjustment of pH or ionic strength. The sorbent is then washed with a buffer to eliminate protein impurities and, when lowering the environmental pH, antibodies are desorbed. The solid-phase material is used for the separation of antibodies from an ascites fluid and from a cell culture supernatant, followed by a polishing step on an hydroxyapatite column. Preliminary studies, related to the ability of the solid phase to separate antibody fragments, are also reported. In these studies, it has been demonstrated that both Fab and Fc fragments from polyclonal IgG are adsorbed to the solid phase under typical binding conditions. Under other defined physico-chemical conditions (ionic strength and pH), separation of both fragments in a single step has been achieved.

Keywords: Antibodies

1. Introduction

In the last few years, preparations of monoclonal and recombinant antibodies have grown to become the largest class of proteins in clinical-phase development intended for therapeutic and diagnostic applications.

Important progress has been made in this domain, resulting in the development of genetically engineered ‘humanized’ antibodies [1], single-chain antibodies [2], phage display [3,4] and fusion antibodies [5]. In addition, alternative expression systems to recombinant cells were perfected, such as expression in eggs (yolk and white) as well as in transgenic animals [6] and transgenic plants [7].

Complementary to expression systems are purifi-

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cation protocols designed to obtain very pure antibodies.

During the last two decades, a wide variety of strategies for the purification of antibodies have been developed and numerous sorbents have been designed for their separation, employing different adsorption–desorption principles (see refs. [8–10] for review).

At present, the most widely used technique to capture antibodies is affinity chromatography on immobilized Protein A sorbents. The high specificity of Protein A for the Fc antibody domain provides excellent chromatographic selectivity and facilitates the isolation of products in a single step [11]. Due to its biospecific interaction with antibodies, Protein A resins allow for the direct capture of antibodies from unadjusted feedstock at near-neutral pH and physiological ionic strength [12,13]. However, various practical complications associated with Protein-A chromatography have come under increasing attention, such as the labile nature of ligand, which cannot be subjected to clean-in-place procedures using sodium-hydroxide solutions. Moreover, Protein A resins carry with them specific issues, such as the possibility of contaminating eluted antibodies as a result of whole ligand leakage from the solid phase [14] or ligand fragments leakage from a partial breakdown by proteases present in the feedstock. This situation requires special consideration as part of the overall antibody-purification scheme [15]. In order to avoid complications related to Protein A sorbents, several alternative chromatographic separation techniques are currently available. Ion-exchange chromatography [16], hydrophobic-interaction chromatography [17] and thiophilic chromatography [18] are among the most popular. However, while these technologies overcome a number of the major weaknesses associated with the use of Protein-A sorbents, they are imperfect solutions. The feedstream often has to be diluted, added with lyotropic salts, or its pH has to be adjusted; these operations are expensive and impractical and add to the cost of waste handling.

More recently, a new antibody separation technique called ‘hydrophobic charge induction chromatography’ has been introduced [19]. A sorbent that employs this technology does not require feedstock preconditioning, such as concentration, dilution, pH adjustment or addition of specific salts, to bind antibodies optimally. Proteins are adsorbed directly from a variety of sources under physiological conditions and elution is achieved by simply lowering the pH. The ligand is non-proteinaceous and cannot be degraded by proteases present in the feedstock and withstand repeated cleaning cycles with sodium hydroxide without loss of function.

In this study, the isolation and purification of antibodies using hydrophobic charge induction chromatography is described. Antibodies selectively captured first from crude feedstocks are then purified on other columns such as hydroxyapatite as a second step. The separation of antibody fragments prepared through enzymatic breakdown is also described.

2. Experimental

2.1. Chemicals and biologicals

Preparative chromatography sorbents such as MEP HyperCel (sorbent for hydrophobic charge induction chromatography), HA Ultrogel (hydroxyapatite composite column) and Protein A Ceramic HyperD were supplied by Life Technologies (Rockville, MD, USA). A TSK-GSW-3000 column was purchased from Tosoh (Japan). Electrophoresis plates composed of 12% polyacrylamide were purchased from BioRad (Ivry-sur-Seine, France). Protein molecular-mass standard, fine chemicals, plasticware and buffers were from Sigma (St. Louis, MO, USA).

Feedstocks used for the capture and separation of antibodies were an ascites fluid and a cell culture supernatant containing 5% fetal bovine serum.

The mouse ascites fluid contained IgG_1 antibody at a concentration of 6.4 mg/ml. It was used after filtration and dilution (1:1, v/v) with 50 m*M* Tris–HCl buffer, pH 8.

The cell culture supernatant containing 5% fetal bovine serum was prepared from a hybridoma culture. The expressed antibody was an IgG_1 with a concentration of 100 μg/ml. This feedstock was filtered to remove suspended particulate matter and was loaded on the column without further treatment.

2.2. Sorbent characterization

The binding capacity for polyclonal human immunoglobulins G (hIgG) on MEP HyperCel under

different physico-chemical conditions was measured by frontal analysis in a column of 6.6 mm diameter that was 50 mm long. Titrated samples of hIgG at concentrations ranging from 0.05 to 5 mg/ml were made in phosphate–citrate buffers with pH values ranging from 5 to 10 and a conductivity close to 5 mS/cm. Titrated samples of hIgG were also prepared in a phosphate buffer, pH 7, containing different concentrations of sodium chloride up to 1 *M*. The linear flow-rate for chromatography was of 70 cm/h; all calculations of dynamic binding capacity were made at 50% breakthrough.

2.3. Determination of the apparent IgG–MEP dissociation constant

A 50-μl volume of a 50% (v/v) MEP HyperCel suspension in 25 m*M* phosphate containing 150 m*M* sodium chloride, pH 7.2 (PBS), was transferred to a 96-well filtration plate. To each well, 200 μl of a solution of hIgG of differing concentrations were added. The concentration of hIgG varied from 0.02 to 5 mg/ml. The experiment was performed in triplicate. Mixtures were then covered to avoid water evaporation and were shaken for 60 min at 23°C. The filtration plate was then centrifuged and filtrates were collected in the receiving plate.

The receiving 96-well plate containing all filtrates was installed inside a HPLC autosampler; 50 μl of solution from each well was then used for the quantification of the hIgG concentration by HPLC. Each hIgG peak was used for the determination of the amount of hIgG in the solution.

The amount of hIgG in each well represented the non-adsorbed hIgG on the MEP HyperCel resin. From these data, a binding isotherm was built and the maximum binding capacity (q_{max}) as well as the apparent dissociation constant were calculated using the well-known Langmuir model.

2.4. Chromatographic separations of whole antibodies

Capture and separation of antibodies was performed in columns. MEP HyperCel was packed into a glass column with a diameter of 6.6 mm and a length of 100 mm. The packed bed was washed extensively with the buffer sequence used for the chromatographic separation and was finally equilibrated in PBS.

In one experiment, the column was loaded with 5 ml of ascites fluid, diluted (1:1, v/v) with 50 m*M* Tris–HCl buffer, pH 8 (see Section 2.1). The load corresponded to 8.8 mg of antibody.

The column was then washed extensively with PBS to eliminate the non-adsorbed proteins. Non-IgG adsorbed proteins (mostly albumin) were desorbed using the same buffer containing 25 m*M* sodium caprylate. Antibodies were then desorbed using 50 m*M* acetate buffer, pH 4.0.

In a second experiment, 300 ml of cell culture supernatant containing 5% fetal bovine serum were loaded. The load corresponded to 30 mg of antibody. In this experiment, the same wash and elution conditions were used as detailed above.

Minor impurities in the IgG_1 elution pool from serum-containing cell culture supernatant were subsequently removed using a column of hydroxyapatite.

The eluate from the MEP HyperCel purification step was first adjusted to pH 8 and then loaded onto a column of HA Ultrogel (100 mm×6.6 mm I.D.) that was previously equilibrated with a 10-m*M* phosphate buffer, pH 8. After washing out the non-adsorbed proteins, IgG_1 was desorbed by raising the ionic strength using a 500-m*M* KCl solution. Protein impurities still adsorbed on the column were desorbed by increasing the phosphate buffer concentration to 100 m*M*, pH 6.8.

In both experiments, columns were cleaned using two column volumes of 1 *M* sodium hydroxide solution.

2.5. Chromatographic separation of antibody fragments

A 100-mg amount of hIgG was dissolved in 100 m*M* phosphate buffer, pH 7.5, containing 20 m*M* cysteine and 2 m*M* EDTA. To this solution, 2 mg of pure papain were added and the mixture was incubated for 2 h at 37°C. Proteolysis was stopped by the addition of 1 ml of 20 m*M* iodoacetamide in the same buffer, to inhibit the enzymatic reaction. The resulting product was analyzed by polyacrylamide gel electrophoresis to verify the presence of both Fc and Fab fragments. To find conditions to separate hIgG fragments, a Protein A Ceramic HyperD

column was first used. Fab was separated from Fc using classical conditions. Briefly, a column (165 mm long×6.6 mm I.D.) packed with Protein A HyperD was equilibrated with PBS. Papain-treated hIgG was dialyzed against the equilibration buffer. This solution was then loaded onto the pre-equilibrated sorbent bed and PBS was used to wash the column. Most Fab fragments were found in the flow-through, whereas Fc was bound to the column. The Fc fragment was desorbed by lowering the pH to about 3 using a 100-m*M* acetic acid solution. The Fab and Fc fractions collected were used separately to study their behavior on a MEP HyperCel column.

To determine the optimal separation conditions, the ionic strength and pH of the load, wash and elution steps were varied from 5 to 15 mS/cm and from 7 to 4, respectively. This approach resulted in the following final separation protocol.

Enzymatically hydrolyzed hIgG solution was dialyzed against 100 m*M* Tris–acetate, pH 5.5, containing sodium chloride (5 mS/cm) and loaded onto the MEP HyperCel column that had been equilibrated in the same buffer. Fab fragments were mostly found in the flow-through fraction. Fc fragments were then eluted by decreasing the pH to 4.5 using the same Tris–acetate buffer. At the end of the separation cycle, the column was cleaned using 1 *M* sodium hydroxide solution.

2.6. Analytical determination of chromatographic fractions

The protein fractions collected were analyzed by polyacrylamide gel electrophoresis. Analysis by GPC–HPLC was performed on a 4-μm TSK-GSW-3000 column, 30 cm long and with a diameter of 4.6 mm using a 25-m*M* phosphate buffer, pH 7.4, containing 150 m*M* sodium chloride as the liquid phase. The flow-rate was 0.2 ml/min.

3. Results and discussion

While a number of chromatographic methods are currently employed for antibody purification, they frequently suffer from practical or economic problems. For instance, adjustments to feedstock composition must be made with most chromatographic separation techniques, as detailed in comprehensive reviews [9,10] and related publications [20–22]. Such adjustments, whether by dilution, adjustment of pH or addition of salt, lead to a significant increase in process labor and cost. Moreover, adjustment of feedstock pH can sometimes lead to precipitation of antibody.

The use of ion-exchange chromatography requires that the feedstock is diluted, diafiltered or dialyzed to lower the ionic strength and to modify the pH so that it is compatible with the adsorption phase. The use of hydrophobic interaction chromatography as well as thiophilic chromatography necessitates an increase in the ionic strength by adding lyotropic agents up to a concentration of 1 *M* or more [23,24].

With biospecific affinity sorbents [25–28], antibody adsorption can be achieved under physiological conditions. However, very frequently, the binding capacity is low and/or the resin is not compatible with the harsh feedstream and caustic washings necessary for long-term use of the column.

As an alternative, Necina et al. [20] described a non-conventional cation-exchange sorbent that provided efficient antibody binding at physiological ionic strength with no prior feedstock concentration. The pH of the feedstock, however, must be adjusted upon loading.

As published by Burton and Harding [29], hydrophobic charge induction chromatography provides the basis for an ideal adsorbent for proteins. Over a pH range of 6 to 8, the ligand is uncharged. Under these conditions, the ligand and the spacer arm behave much like a hydrophobic site and bind the protein by hydrophobic association. Ligand density and the spacer-arm structure are designed so that binding occurs under physiological conditions without the need for the addition of lyotropic salt. When the pH of the mobile phase is lowered, the ligand becomes ionically charged. Under these conditions, the protein also carries a similar net positive charge, therefore, desorption occurs on the basis of electrostatic repulsion between the solid-phase sorbent and the protein. In the case of MEP HyperCel, the ligand is 4-mercaptoethyl-pyridine, a well known basic structure that enhances the specificity for immunoglobulins [30]. The selectivity of various nitrogen-heterocyclics for immunoglobulins was demonstrated by Schwarz et al. [31]. It has been shown that

selectivity is further enhanced by the presence of a sulfur atom proximal to the pyridine ring. Analogous heterocyclic ligands with more traditional thiophilic structure have also recently been described as improved compounds for the adsorption of antibodies in a salt-independent manner [32,33].

As evidenced in Table 1, adsorption of IgG on a MEP HyperCel column is a function of pH. The optimal pH for adsorption is 7–9 at physiological ionic strength. When the pH is lowered to 6, the binding capacity decreased by a factor of two, and at a pH value below 5, no IgG was adsorbed on the column. This behavior indicates that desorption of IgG can be easily achieved by changing the pH. When ionic strength is varied by the presence of different amounts of sodium chloride, it appeared that the binding capacity did not change significantly. These data indicate that no adjustment of feedstock ionic strength is required in order to obtain high IgG binding capacities on the MEP HyperCel column. Table 1 also shows that the adsorption capacity of the column for IgG does not depend on the concentration in the feedstock, at least in the range of explored concentrations, i.e., between 0.05 and 5 mg of antibodies per ml of solution.

In order to determine the adsorption strength of immunoglobulins G to the sorbent, the binding isotherm was determined and the apparent dissociation constant calculated. Under the described conditions and using polyclonal IgG (see Experimental section), calculations resulted in an apparent dissociation constant of 2.9×10^{-7} mol/l and a maximal binding capacity of 48.2 mg/ml (Fig. 1). This high-affinity value compared to classical resins is of particular relevance when considering the dissociation constants of IgG–Protein A complexes [34].

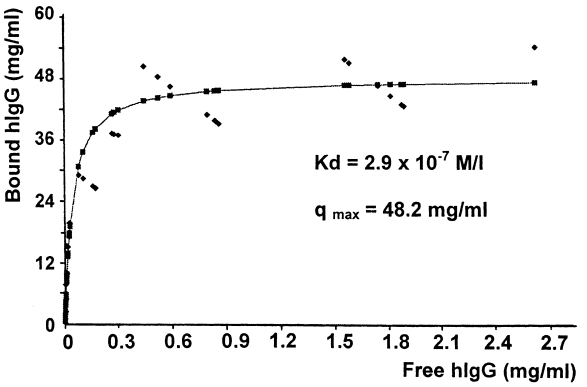

Fig. 1. Equilibrium adsorption isotherm for human polyclonal immunoglobulins G in phosphate-buffered saline.

It is of note that the density of pyridine-based ligand on the sorbent was 102 μmol/ml of packed resin. In this situation, it is likely that several 4-MEP ligands interact with a single molecule of antibody.

As a consequence of the dissociation constant value, of the specificity of pyridine-based ligand for the antibodies and of the high binding capacity, the described sorbent is very attractive for the separation of monoclonal IgG antibodies from crude extracts. Fig. 2 illustrates the isolation of IgG_1 from an ascites fluid. Sample loading was performed after dilution with an equal volume of loading buffer (50 m*M* Tris–HCl buffer, pH 8). Under these conditions, the IgG_1 was fully retained. No traces of antibody were detected in the flow-through fraction, or during further washing with loading buffer. Elution was achieved by lowering the pH to 4.0 with an acetate buffer. From the chromatogram and accompanying sodium-dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) results, it is demonstrated

Table 1
IgG binding capacity of MEP HyperCel at 50% breakthrough as a function of pH, sodium chloride molarity and antibody concentration

Binding capacity as a function of pH		Binding capacity as a function of ionic strength		Binding capacity as a function of IgG concentration	
pH in 150 m*M* NaCl; IgG, 5 mg/ml	IgG binding capacity (mg/ml)	Ionic strength (mS/cm) at pH 7.0; IgG, 5 mg/ml	IgG binding capacity (mg/ml)	IgG concentration (mg/ml) at pH 7.0 and 150 m*M* NaCl	IgG binding capacity (mg/ml)
3.5	0.0	5	63.4	0.050	44.2
4.5	18.1	10	70.4	0.250	50.8
5.5	44.5	15	63.3	1.000	47.2
6.5	59.4	20	59.7	5.000	73.4
7.5	67.0	30	54.1	–	–
8.5	61.1	–	–	–	–

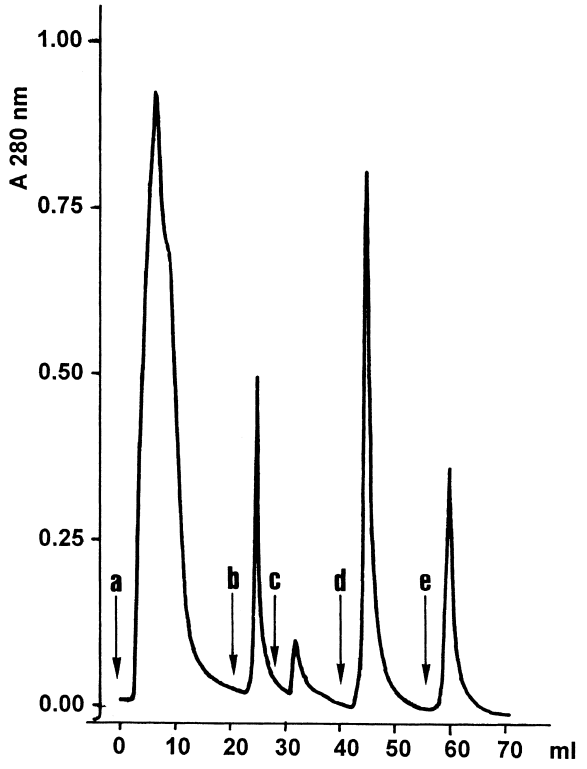

Fig. 2. Monoclonal IgG_1 separation from a crude ascites fluid on a MEP HyperCel column. Column: 100 mm×6.6 mm I.D. Equilibration buffer: 50 m*M* Tris–HCl, pH 8. Loaded sample (a) 2.5 ml of ascites fluid diluted with an equal volume of 50 m*M* Tris–HCl buffer, pH 8. Washing solutions: 25 m*M* sodium caprylate in 50 m*M* Tris–HCl buffer, pH 8 (b), followed by demineralized water (c). Eluting solution (d): 50 m*M* acetate buffer, pH 4.0. Cleaning solution (e): 1 *M* sodium hydroxide. Linear flow-rate: 70 cm/h.

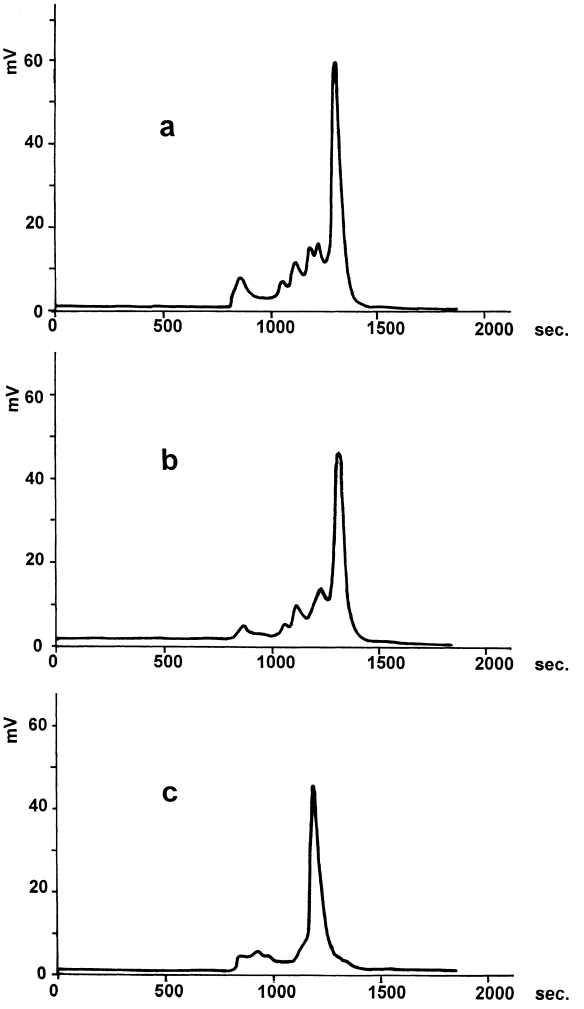

Fig. 3. GPC–HPLC analysis of fractions collected using MEP HyperCel chromatography of ascites fluid (see Fig. 2). Column: TSK-GSW-3000 pre-packed (300 mm×4.6 mm I.D., with a 4-μm particle diameter. Sample volume, 20 μl. Running buffer: 25 m*M* phosphate, 150 m*M* sodium chloride, pH 7.4. Flow-rate: 2 ml/min. (a) crude ascites fluid; (b) flow-through; (c) elution fraction.

that most of the protein impurities are not retained during the load and initial wash steps. Further washes with 25 m*M* sodium caprylate in loading buffer desorbed a significant amount of protein impurities, including albumin (data not shown). The mild detergent-like properties of sodium caprylate promoted the desorption of hydrophobic species such as the weakly bound albumin. With the described wash sequence, immunoglobulin purity of 83% was obtained in a single step. These findings were confirmed by high performance gel filtration chromatography, as shown in Fig. 3. Recovery of immunoglobulin was 79% and the purification factor was 5.2.

Similar conditions were successfully used for the separation of monoclonal antibodies from a cell culture supernatant supplemented with 5% fetal bovine serum. Although a wash with sodium caprylate was performed to eliminate hydrophobic adsorbed proteins, the purity of the antibodies was about 69%. To complete the purification process, a second, hydroxyapatite, column was used.

Fig. 4A shows the separation of antibodies from the first column. The serum-containing cell culture

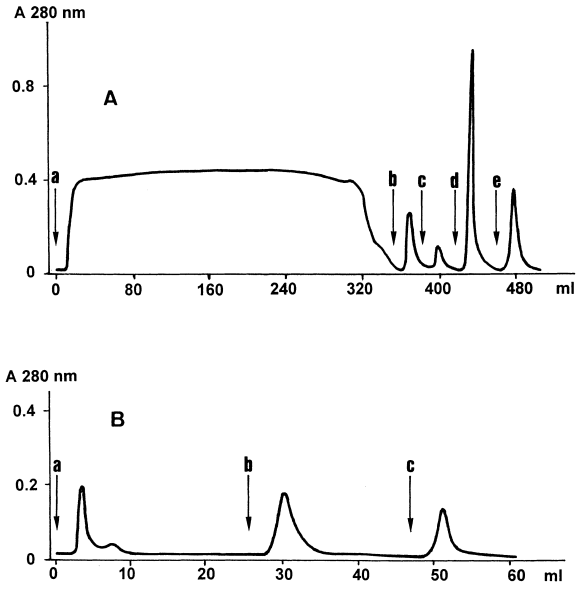

Fig. 4. Purification of monoclonal IgG_1 from a cell culture supernatant on a MEP HyperCel column (A) followed by a hydroxyapatite column (B). Column dimensions: 100 mm×11 mm I.D. (A) Sample injection: 300 ml of cell culture supernatant containing 5% fetal bovine serum (a); column washing with 25 m*M* sodium caprylate in equilibration buffer (b); water wash (c); elution of antibodies with 50 m*M* acetate buffer, pH 4.0 (d); column cleaning with 1 *M* sodium hydroxide (e). (B) Sample injection (a): eluted antibody fraction from MEP HyperCel column (see fraction 'd' above); elution of monoclonal antibodies with 500 m*M* potassium chloride (b); column cleaning: 100 m*M* sodium phosphate buffer, pH 6.8 (c). Linear flow-rates for both columns: 70 cm/h. Collected fractions were analyzed by SDS–PAGE under reducing conditions and by GPC–HPLC (see Fig. 5).

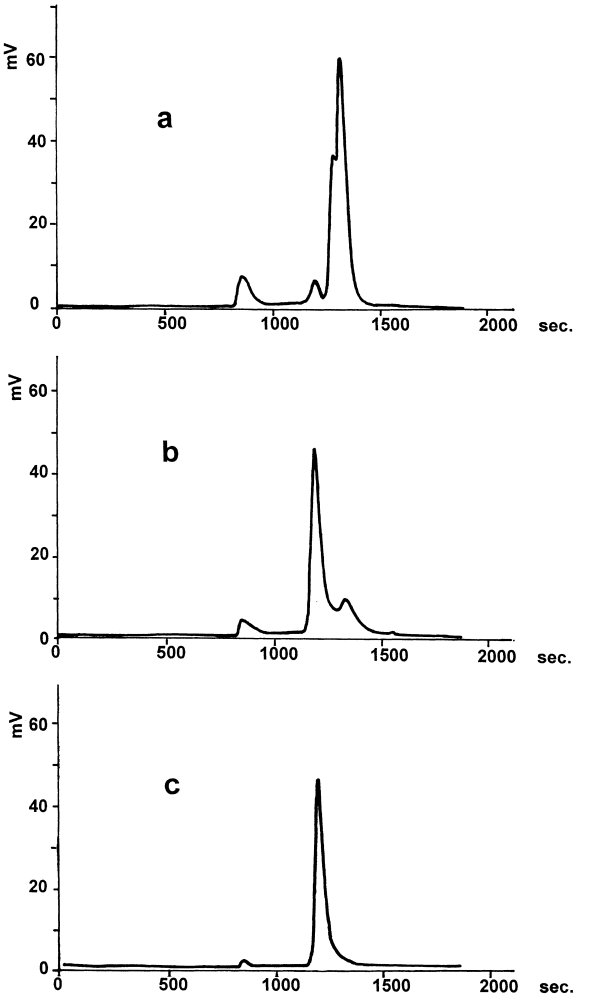

Fig. 5. GPC–HPLC analysis of fractions collected by MEP HyperCel and HA Ultrogel chromatography (see Fig. 4). Column: TSK-GSW-3000 pre-packed (300 mm×4.6 mm I.D.), with a 4-μm particle diameter. Sample volume: 20 μl. Running buffer: 25 m*M* phosphate, 150 m*M* sodium chloride, pH 7.4. Flow-rate: 2 ml/min. (a) crude cell culture supernatant; (b) IgG fraction from the MEP HyperCel column; (c) IgG from the hydroxyapatite column.

supernatant was loaded and, after two washes, the IgG_1 fraction was recovered by eluting at pH 4. The purity of the collected IgG_1 was estimated by HPLC and SDS–PAGE. The purification factor and percentage recovery were 40 and 76%, respectively. This antibody-enriched fraction was purified to 98–99% purity using a HA Ultrogel column, as shown on Fig. 4B.

Elution of antibodies was performed by means of a phosphate buffer, pH 8, containing 500 m*M* potassium chloride. Protein impurities were found in the flow-through and in the regeneration step performed using 100 m*M* phosphate buffer, pH 6.8.

As shown by gel filtration HPLC analysis (Fig. 5), the final purity of the IgG_1 was estimated to be 99%, compared to the purity in crude feedstock and in the MEP HyperCel pool, which were 1.5 and 69%, respectively.

Hydroxyapatite sorbents have been described in a number of protocols for the separation of polyclonal and monoclonal antibodies [35–37]. Phosphate buffers are generally used for this type of chromatography; their initial concentration ranges between 1 and 20 m*M* and the pH ranges between 6.5 and 8. Elution is generally obtained by increasing the phosphate-buffer concentration. However, with anti-

bodies, phosphate buffers may not be sufficient to separate albumin traces from the antibody [38]. Interestingly, the interaction between acidic proteins and hydroxyapatite is not sensitive to a sodium-chloride gradient. Cationic proteins, such as most of the antibodies, can therefore be desorbed by a potassium chloride gradient. This is an advantage in their purification because all acidic contaminants, such as DNA, endotoxins and other acidic proteins, will remain adsorbed on the resin. This is why hydroxyapatite was used as a second column after antibody capture. MEP HyperCel was also evaluated for its ability to separate Fc and Fab fragments from enzymatically hydrolyzed immunoglobulins G. It clearly appeared that both fragments can be adsorbed on the column at neutral pH and their desorption can be achieved sequentially by decreasing the pH. Fc fragments are adsorbed more tightly on MEP HyperCel than Fab as a result of their higher hydrophobicity. On a pH gradient, they are desorbed after Fab fragments (data not shown).

Under well-defined conditions of ionic strength and pH (see Fig. 6a), Fab fragments can be found in the flow-through while adsorbed Fc fragments are recovered by dropping the pH to 4.5. Analytical results of collected fractions showed that Fab contained very low amount of Fc fragments and the Fc fraction contained some non-hydrolyzed antibodies (Fig. 6b). To date, the best way to separate these antibody fragments is using Protein A chromatography, as Protein A is a well known Fc fragment binder [39]. Fig. 6b, lanes 3 and 4, show, however, that, in spite of the use of a bioaffinity adsorption step on Protein A, there is still some cross-contamination of the separated fragments. Comparatively, MEP HyperCel column allows these fragments to be separated quite satisfactorily.

Although with recombinant production of antibody fragments there is no special need to discriminate between Fab and Fc, the separation properties of MEP HyperCel may be of practical interest. Production of Fab fragments from whole immunoglobulins (polyclonal and monoclonal) is increasingly popular and is already performed on a large scale for the preparation of pharmaceuticals such as antisera against snake venom, tetanus toxin, etc. In these cases, pure polyclonal antibodies (generally from horses) are hydrolyzed with papain to cut the Fc

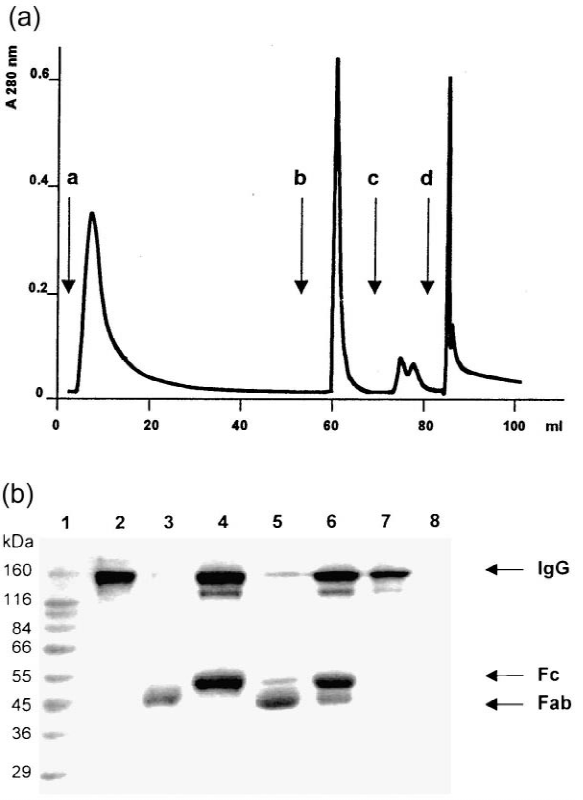

Fig. 6. (a) Separation of Fc–Fab fragments on a MEP-HyperCel column from human IgG hydrolyzed with papain. Column dimensions: 30 mm×6.6 mm I.D. Equilibration buffer: 100 m*M* Tris–acetate, sodium chloride 5 mS/cm conductivity, pH 5.5. Loaded sample (a): 2 ml of papain hydrolyzate (see 'Experimental' section) dialyzed against equilibration buffer. First elution solution (b): 100 m*M* Tris–acetate containing sodium chloride up to 5 mS/cm, pH 4.5. Second elution solution (c): 100 m*M* Tris–acetate buffer containing sodium chloride up to 5 mS/cm conductivity, pH 3.0. Cleaning solution (d): 1 *M* sodium hydroxide. Linear flow-rate: 100 cm/h. (b) 10% SDS–PAGE of fractions collected by MEP-HyperCel chromatography (see Fig. 6a). Lane 1: markers (human IgG, 160 000 M_r; β-galactosidase, 116 000 M_r; fructose-6-phosphate kinase, 84 000 M_r; albumin, 66 000 M_r; glutamic dehydrogenase, 55 000 M_r; ovalbumin, 45 000 M_r; glyceraldehyde-3-phosphate dehydrogenase, 36 000 M_r; carbonic anhydrase, 29 000 M_r). Lane 2: standard human IgG. Lane 3: flow-through from Protein A column (Fab). Lane 4: eluate from Protein A column (Fc). Lane 5: flow-through from MEP HyperCel column. Lane 6: first eluted peak at pH 4.5 (Fc fragment). Lane 7: second eluate at pH 3.0 (excess of IgG). Lane 8: 1 *M* sodium hydroxide cleaning fraction.

fragment from the antibody prior to chromatographic separation. These processes are generally large-scale, low cost applications where the use of Protein A sorbents is considered too expensive to be of practical value.

The behavior of MEP HyperCel for antibody fragments therefore opens the way to the capture/purification of related fusion proteins, especially when they contain Fc fragments. Separation methods for such structures are in progress.

4. Conclusion

Hydrophobic charge induction chromatography, conducted using 4-MEP as ligand, provides effective adsorption of antibodies under physiological conditions. Antibodies are adsorbed at nearly neutral pH and at a binding capacity of 30–35 mg/ml of sorbent (10% breakthrough), which is independent of antibody concentration. This phenomenon is attributed to the relative specificity of the ligand for antibodies, to the mild hydrophobic association and to the high ligand density on the solid phase. This combination yields an apparent dissociation constant of 2.9×10^{-7} mol/l and a maximal binding capacity close to 50 mg/ml.

Effective capture of antibodies from ascites fluid and from a cell culture supernatant containing 5% fetal bovine serum was demonstrated with purities that reached 83 and 69%, respectively, in a single step.

Elution of IgG is achieved under relatively milder conditions (pH 4.0) compared to the ones employed during affinity chromatography on Protein A sorbents. The likelihood of aggregate formation or inactivation is thus reduced.

Monoclonal antibodies from crude cell culture supernatants containing high concentrations of albumin can be brought to high levels of purity (>98%) by the use of a simple hydroxyapatite chromatography following hydrophobic charge induction chromatography.

Although the investigated adsorbent does not have a true bioaffinity interaction on the Fc fragment of IgG, it can also be effectively used to separate Fab from Fc antibody fragments under predetermined conditions of pH and ionic strength.

The unique properties of this material make it an effective tool for the purification of antibodies from different feedstocks and for the separation of recombinant or enzymatically produced IgG fragments. This material should also be used for the purification of fusion proteins comprising Fc domains.

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