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2-Mercapto-5-benzimidazolesulfonic acid: an effective multimodal ligand for the separation of antibodies

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Abstract

The report describes the use of 2-mercapto-5-benzimidazolesulfonic acid (MBISA) as a ligand for the separation of antibodies by chromatography. The ligand shows a relatively specific adsorption property for antibodies from very crude biologicals at pH 5.0–5.5. At this pH range most of other proteins do not interact with the resin especially when the ionic strength is similar to physiological conditions. Several characterization studies are described such as antibody adsorption in different conditions of ionic strength, pH and temperature. These properties are advantageously used to selectively capture antibodies from very crude feed stocks without dilution or addition of lyotropic salts. Demonstration was made that the adsorption mechanism is neither based on ion exchange nor on hydrophobic associations, but rather as an assembly of a variety of properties of the ligand itself. Binding capacity in the described conditions ranges between 25 and 30 mg/mL of resin. The sorbent does not co-adsorb albumin (Alb) and seems compatible with a large variety of feedstocks. Quantitative antibody desorption occurs when the pH is raised above 8.5. The final purity of the antibody depends on the nature of the feedstock, and can reach levels of purity as high as 98%. Even with very crude biological liquids such as ascites fluids, cell culture supernatants and Chon fraction II + III from human plasma fractionation where the number of protein impurities is particularly large, immunoglobumins G (IgG) were separated at high purity level in a single step.

Keywords: Heterocyclic ligands; 2-Mercapto-5-benzimidazolesulfonic acid; Antibodies

1. Introduction

Antibody production from recombinant technologies and from natural sources is an area of growing interest especially with the promised large therapeutic applications. At present, whatever the source, antibodies represent the largest class of proteins currently produced in number and in mass.

With the maturation of therapeutic and diagnostic applications, demands for pure antibodies will increase rapidly and therefore it is anticipated that more and more effective production strategies will be explored. One important step in the production process is the purification from the very crude biological material where antibodies are expressed. They contain a large number of impurities that are to be completely removed since the final formulation of the injectable antibody must meet very stringent rules in terms of biological activity and safety. To recover costs associated with meeting such stringent requirements, it is expected that there will be pressure to reduce production costs and increase the efficiency of both antibody recovery and contaminant elimination of impurities.

Issues related to the production and purification of antibodies are extensively discussed in a number of published reports where it appears that separation technologies are analyzed under three different aspects: technical, biochemical and economical. The most currently used techniques for antibody separation from very crude feedstocks is affinity chromatography on protein A resins [1]. *Staphylococcus aureus* protein A represents an almost perfect docking ligand directed against a specific domain of Fc antibody that was naturally optimized as a result of evolutionary biology. This

Abbreviations: Alb, albumin; ELISA, enzymatic linked immuno system assay; HIC, hydrophobic interaction chromatography; HPLC, high performance liquid chromatography; IgG (or Ig), immunoglobumins G; MBISA, 2-mercapto-5-benzimidazolesulfonic acid; MEP, 4-mercaptoethyl pyridine; MS, mass spectyrometry; SELDI, surface enhanced laser desorption ionization; SDS, sodium dodecyl sulfate; Tr, transferrin; Tris, Tris(hydroxymethyl)aminomethane

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represents a high level of selectivity for antibody purification with strong advantage over all described non-natural ligands. This is the reason why protein A is largely used today at lab and preparative scale. Nevertheless, protein A, when used as ligand in a rather artificial situation like chromatography, is associated with a number of important practical problems [2,3]. Major issues are related to the difficulty to properly regenerate columns of protein A with current approved methods such as caustic washings. Protein A does not necessarily recognize all antibodies [4]; in some cases it must be added with specific salts for the adsorption of antibodies to occur. From a biochemical point of view it is to be recalled that the pH of antibody recovery after protein A adsorption is acidic and may create aggregates with subsequent partial denaturation and loss of biological activity. Moreover, the presence of proteases in the feedstock contributes to the ligand breakdown. Protein A can therefore leach from the solid phase by either a dissociation from the solid surface or because of its partial hydrolysis [5]. This is the reason why specific enzymatic linked immuno system assay (ELISA) tests are used to assay the presence of traces of protein A in the antibody preparations [6]. Possible contamination is one of the most important risks associated with antibody purification as it may induce either unwanted toxic effects since protein A is known as a toxic biological for human [7], or it may bring agonistic properties inducing false results [8]. Finally, on an economic standpoint, protein A resins represent a large investment due to the combination of a particularly elevated cost of the columns and the limited number of reuse. This initial investment necessarily impacts the production cost of antibodies.

In the recent past years several synthetic small sized ligands have been designed for the capture of antibodies. The major reason was to compete and possibly replace protein A in the process of antibody separation.

Thiophilic chromatography [9–11] and biomimetic affinity chromatography [12,13] have both been developed under various aspects. In spite of their effectiveness to adsorb immunoglobulins G both techniques suffer from specific limitations that depend on the type of chemical group involved. Pure thiophilic chromatography requires a relatively large amount of lyotropic salts, which is detrimental to the cost, on one hand, and induces disposal issues for salted solutions when the technology is used at large scale, on the other hand.

Among biomimetic ligands described in the domain of antibody separation it deserve to mention thiopyridine [14], mercaptothiazoline [15], 3-(2-mercaptoethyl)quinazoline-2,4 (1*H*, 3*H*)dione [16], 2, mercaptonicotinic acid [17], histidine [18] and 4-mercaptoethyl pyridine (MEP) [19]. This latter has been extensively studied because it can be used under similar conditions as protein A resins. Adsorption occurs in physiological conditions of pH and ionic strength, while elution is accomplished by decreasing both the ionic strength and the pH of the buffer.

Rational design of ligands by computer aided molecular modeling to mimic the binding of natural interactors was also extensively investigated. This approach involved the use of a triazine scaffold where different substituents where attached [20,21]. Adsorption of immunoglobumins G (IgG) with these synthetic ligands occurs at pH near neutrality, but binding is enhanced at relatively high salt concentrations. Desorption is achieved by lowering the pH to 3–3.5.

In all cited cases the attempt was to reach specificity as close as possible to that of protein A, however, co-adsorbed protein impurities were not the same. In particular some albumin (Alb) is co-adsorbed in most of described synthetic ligands. In most of the cases elution of IgG is frequently obtained by lowering the pH; this specific condition may be a limitation for either antibodies that tend to form aggregates or that are sensitive to acidic conditions.

In this paper a novel chemical ligand is described. It allows adsorbing antibodies very easily in physiological conditions of ionic strength and pH between 5.2 and 5.5; desorption occurs when raising the pH to 8.5–9.5.

Properties and use of this material are described with respect to the separation of poly and monoclonal antibodies.

2. Experimental

2.1. Chemicals and biologicals

Chromatography sorbents, such as immobilized 2-mercapto-5-benzimidazolesulfonic acid (MBISA) on cellulose beads (also called MBI HyperCel in this paper), MEP HyperCel, Protein A Ceramic HyperD and ProteinChip® Arrays for mass spectrometry analysis of proteins (surface enhanced laser desorption ionization (SELDI)–mass spectyrometry (MS)) were supplied by Ciphergen Biosystems Inc. (Fremont, CA, USA).

Feedstocks used for the capture and separation of antibodies were either mouse ascites fluids or supernatants of cells cultured in media containing fetal bovine serum or Cohn fraction II + III of human plasma. The mouse ascites fluid contained IgG1 monoclonal antibody at a concentration of 3.5 mg IgG/mL. The cell culture supernatant of rat hybridomas cultured in the presence of 10% fetal bovine serum contained IgG1 monoclonal antibodies at a concentration of about 0.3 mg/mL. Cohn fraction II + III, an ethanol precipitate of human plasma containing a large number of proteins, was a gift of Octapharma (Vienna, Austria). Protein concentration after solubilization either in 50 mM acetate buffer pH 5.5 containing 0.14 M sodium chloride or in phosphate buffered saline, was about 18 mg/mL. Estimated IgG concentration within all soluble proteins was of about 7.5 mg/mL.

Before use, all feedstocks were filtered to remove suspended particulate matter and loaded on the columns directly at pH 5.2 or 5.5 without further treatment.

Electrophoresis plates composed of 12% polyacrylamide were purchased from BioRad (Ivry-sur-Seine, France). Protein molecular weight standard, fine chemicals, plasticware

and buffers were from Sigma Chemicals (St. Louis, MO, USA).

2.2. Sorbent characterization

Binding capacity for human polyclonal IgG was measured in a column of 6.4 mm diameter and 100 mm long. Titration solution was a 5% hIgG solution in buffers of different pH from 3 to 10 with a conductivity close to 17 mS/cm, or in a 50 mM acetate buffer, pH 5.5 containing different amount of sodium chloride up to 1 M. Linear flow rate was of 50 cm/h; calculations of dynamic binding capacity were made at 10% breakthrough.

In order to understand the interaction between IgG and the solid phase, binding capacity studies were conducted in the presence of additives such as ethylene glycol, urea, polyvinyl alcohol and sodium chloride. The effect of temperature on IgG binding capacity was also measured.

2.3. Optimization of and desorption conditions

Conditions of antibody adsorption during the sorbent characterization, and optimization of antibody elution were studied in a matrix experiment crossing over different pHs and different concentrations of sodium chloride (see Table 1). The sample was a crude bovine serum conditioned in the adsorption buffer. pH range evaluated was between 7.5 to 9.5 using Tris–HCl buffers; sodium chloride concentrations ranged from 0 to 1 M.

The experiment was performed in a 96-well filtration plate (only half plate was used 48 wells under a configuration of 6×8 wells). Immobilized MBISA was loaded in each well (100 μL of settled resin). The vertical rows of wells (from "A" to "F") were devoted to different adsorption conditions and the horizontal columns of wells (from 1 to 8) were devoted to elution conditions.

After having loaded the bovine serum at pH 5.5, wells "A"–"F" of each column were washed with buffers according to Table 1 to eliminate non-adsorbed proteins. Elution of IgG was evaluated using different buffers in columns 1–8 according to Table 1.

Five microliter aliquots of each of the 48 eluted fractions were loaded on NP20 ProteinChip Arrays and analyzed by SELDI–MS. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of relevant fractions was also performed as described below.

2.4. Chromatographic separations

Capture and separations of antibodies were performed on small preparative columns packed with MBI HyperCel. The sorbent was packed classically into glass columns of either 6 or 11 mm diameter and 60–70 mm in length (see figure legends for details). Each column was washed extensively with all buffers used for the chromatographic separation and finally equilibrated in a 50 mM acetate buffer pH 5.2 (pH 5.5 for human plasma Cohn fraction) containing 0.14 M sodium chloride. Protein elution was monitored by UV absorbance at 280 nm.

Columns were loaded with the crude samples previously filtered and adjusted at pH 5.2 or 5.5 by addition of 0.5 M acetic acid.

The volume of ascites fluid was 6.6 mL; the volume of cell culture supernatants was 19 mL; the volume of redissolved Cohn fraction II + III in the buffer was 10 mL.

In all cases once the injection of the sample was done, the column was washed with the equilibration buffer to remove unbound impurities until the UV absorbance returned to baseline.

Bound antibodies were desorbed using 50 mM carbonate buffer pH 9.0–9.5, containing 0.14 M sodium chloride.

When elution was complete, columns were cleaned with 1 M sodium hydroxide and then re-equilibrated with the acetate buffer to start another separation run.

Separations on protein A columns were performed in classical conditions using a phosphate buffered saline for the adsorption and 0.1 M acetic acid for elution. All other conditions were similar to those described above including column dimensions.

2.5. Electrophoresis analysis of chromatographic fractions

Collected fractions from chromatography columns were analyzed by SDS–polyacrylamide gel electrophoresis under both reducing and non-reducing conditions. Concentration of polyacrylamide gel slabs was 12%. Protein solu-

Table 1
List of buffers used for the optimization of separation conditions of IgG in a matrix-like approach using a 96-well filtration plate

Adsorption buffers (horizontal rows from "A" to "F")	Desorption buffers (vertical rows from 1 to 8)
A: 50 mM acetate, 0.15 M NaCl, pH 5.0	1: 100 mM Tris–HCl, pH 7.5
B: 50 mM acetate, 0.15 M NaCl, pH 5.5	2: 100 mM Tris–HCl, pH 8.5
C: 50 mM acetate, 0.15 M NaCl, pH 6.0	3: 100 mM Tris–HCl, pH 9.5
D: 50 mM acetate, pH 5.25 (no NaCl)	4: 100 mM Tris–HCl, 0.15 M NaCl, pH 7.5
E: 50 Mm acetate, Ph 5.25, 0.15 M NaCl	5: 100 mM Tris–HCl, 0.15 M NaCl, pH 8.5
F: 50 mM acetate, pH 5.25, 0.5 M NaCl	6: 100 mM Tris–HCl, 0.15 M NaCl, pH 9.5
	7: 100 mM Tris–HCl, 1 M NaCl, pH 8.5
	8: 100 mM Tris–HCl, 1 M NaCl, pH 8.5

Protein detection was performed using ProteinChip Arrays in association with SELDI MS.

tion at a concentration of 0.5 mg/mL was made in standard Tris-glycine buffer. Protein migration was performed under 110 V for 35 min. Staining was performed using standard condition using Coomassie Blue. The purity of IgG was estimated by densitometry.

2.6. Determination of apparent dissociation constants

Drained MBI-HyperCel previously equilibrated with the adsorption buffer was mixed with an equal volume of 50 mM acetate buffer containing 140 mM sodium chloride, pH 5.2 (50%, v/v). The homogeneous suspension was transferred by 3 mL aliquots, corresponding to 1.5 ml of settled sorbent, to different tubes. To each tube another 1 mL of acetate buffer was added along with different amounts of pure IgG from 20 to 120 mg. Mixtures were then covered and shaken for 16 h at 23 °C. Experiments were performed in triplicate. Supernatants were then sampled and the concentration of IgG determined by high performance liquid chromatography (HPLC). From analytical data, a binding isotherm was established and the maximum binding capacity (q_{max}) as well as the apparent dissociation constants were calculated using the well-known Langmuir model.

3. Results and discussion

2-Mercapto-5-benzimidazolesulfonic acid used as ligand for adsorption of IgG was deducted from an extensive number of synthetic ligands described in the literature relating to the separation of IgG. From this review it appeared that heterocycles are among molecules that are repeatedly described for the capture of antibodies. The presence of sulfur atoms is also relatively frequently encountered among sites capable to interact with antibodies. First, heterocycles associated with thiophilic sorbents were described by Porath and coworkers [14,22] where pyridine rings were involved. Then, other heterocycles still associated with thiophilicity were described as good adsorbents for antibodies; they were based either on five-members heterocycles [15] or pyridinium groups [22] or even more complex heterocycles associated with aromatic rings [16,17]. Along these developments it appeared that heterocycles containing nitrogen were of interest for the specificity of immunoglobulins. The presence of a sulfur atom was also considered as an enhancer of the interaction; however, with such an approach antibodies were generally adsorbed in the presence of lyotropic salts.

Most of described methods for IgG capture resulted in co-adsorption of few other proteins among them significant amounts of albumin. To circumvent this difficulty it was envisioned to introduce a negatively charged group as part of the ligand construct that would repel certain proteins carrying the same charge at the pH of adsorption. MBISA (Fig. 1) corresponded quite ideally to what should be such a ligand: it contains a heterocycle (the imidazole ring) largely described as being able to interact quite selectively with antibodies [18]. It carries a mercapto group introducing thus a certain level of thiophilicity well known for its ability to interact with antibodies. To that structure a sulfonate group is attached on the aromatic ring where it is always negatively charged within a very large pH range contributing to the repulsion of proteins of the same charge when the environmental pH is above the isoelectric point of the protein in question. The environmental pH can be managed so that the sulfonic group could contribute to IgG adsorption under pH conditions where IgG are positively charged, namely pH below 6.

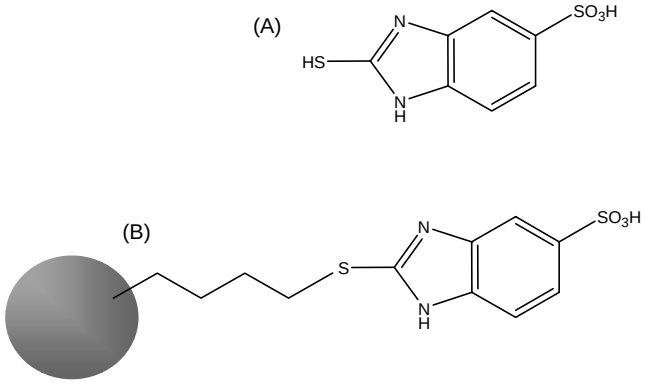

Fig. 1. Schematic representation of native 2-mercapto-5-benzimidazolesulfonic acid structure (MBISA; A) and the resin carrying MBISA chemically covalently attached on the bead (MEP HyperCel; B).

In a first time, studies have been conducted to characterize the solid phase in terms of binding capacity for serum IgG in various physicochemical conditions. IgG as well as albumin adsorption was determined at different pHs and different ionic strengths as reported in Fig. 2.

Adsorption appeared as clearly pH-dependent: when the pH is increased from 5 to 8 IgG adsorption decreases progressively down to zero. Adsorption of albumin took place essentially at a pH value below 5 with a maximum value at about pH 4. This is consistent with the mechanism of ion exchange where albumin at pH 5 is negatively charged.

At a constant pH of 5.5, adsorption did not appear as dependent on the ionic strength; however, optimal binding capacity was obtained with 0.14 M sodium chloride (Fig. 2B). Below this value, binding capacity decreased linearly from about 30 to about 15 (50 mM acetate buffer, pH 5.5). Above 0.14 M sodium chloride, binding capacity for IgG slightly decreased from about 30 mg IgG to 20–25 mg IgG/mL resin. These data gave good indication of what the conditions of adsorption and elution of IgG should look like. The pH of the adsorption buffer should be slightly acidic (pH between 5 and 5.5) to obtain a good selective IgG uptake performance while avoiding co-adsorption of albumin.

Under envisioned adsorption conditions, one could argue that the mechanism is similar to what happens on strong cation exchange chromatography. Experimental data (Fig. 3) clearly demonstrated that when used as an ion exchanger, MBI-HyperCel performed in a non-conventional manner. In

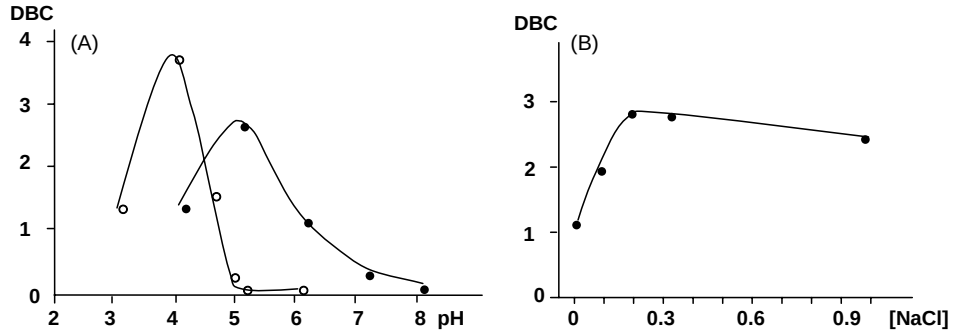

Fig. 2. Adsorption behavior of MBI HyperCel for human immunoglobulin G and albumin as a function of pH (A) and of sodium chloride concentration (B). Determinations were made using solutions of pure proteins at a concentration of 5 mg/mL in the appropriate buffer (see Section 2 for technical details). (○) human albumin; (●) human IgG.

acetate buffer pH 5.2 most of proteins were adsorbed and did not significantly elute under a sodium chloride gradient up to 1 M as it is the case with regular cation exchangers. This is consistent with previous data of adsorption of IgG in the presence of high concentrations of sodium chloride (Fig. 2). Antibodies were nevertheless desorbed when increasing the pH to 9.5. The sulfonate group anchored on the aromatic ring is in fact not equivalent to a regular sulfonate; the immediate presence of an imidazole ring has an influence on the apparent p*K*. Measured by means of a titration curve, the p*K* value of this acid was found close to 2.2 instead about 1.6–1.8 for a regular sulfonate group from strong cation exchange resins.

The ligand density of MBI-HyperCel resin on the solid matrix did not seem critical for IgG binding capacity. In fact a specific study showed a quite linear relationship between ligand density and binding capacity before reaching a plateau (data not shown). This contrasts significantly with the adsorption of IgG on a synthetic ligand called 4-mercaptoethyl pyridine (MEP HyperCel), for which a critical level of ligand density is necessary likewise in hydrophobic interaction chromatography (HIC). With these data one could conclude that hydrophobic forces are probably not the dominant factor for antibody adsorption on the MBI-HyperCel resin. The assumption was confirmed with the behavior of binding capacity as a function of temperature, a well known rule in HIC. When comparing data of protein binding capacity as a function of temperature (Fig. 4) it clearly appeared that the investigated resin showed a relative independence from temperature, contrary to hydrophobic-based resins where binding capacity increased linearly from about 10 to 45 °C. The initial increase of binding capacity of immobilized MBI HyperCel from 8 to 27 °C was attributed to the decrease in the viscosity of IgG solution with a consequent increase in diffusion rate within the pores. After 27 °C binding capacity decreased, a phenomenon that probably corresponds to the ion exchange effect in relation with the presence of a sulfonate group on ligand structure.

From further investigations it was found that binding capacity did not significantly change in the presence of ethylene glycol contrary to MEP HyperCel where the interaction with IgG is dominated by hydrophobic association

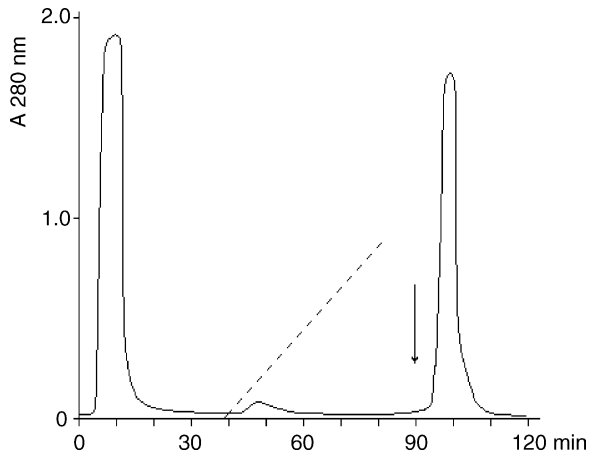

Fig. 3. Chromatography of bovine serum using MBI HyperCel under ion exchange conditions. Column: 11 mm i.d. × 70 mm; initial equilibration buffer: 50 mM acetate, pH 5.2; sample: 1 mL of bovine serum diluted in 1 mL of equilibration buffer; elution gradient: sodium chloride from 0.14 to 1 M in equilibration buffer (dotted line). Arrow indicates the injection of 50 mM carbonate buffer pH 9.5 containing 0.14 M sodium chloride (desorbed peak contains antibodies).

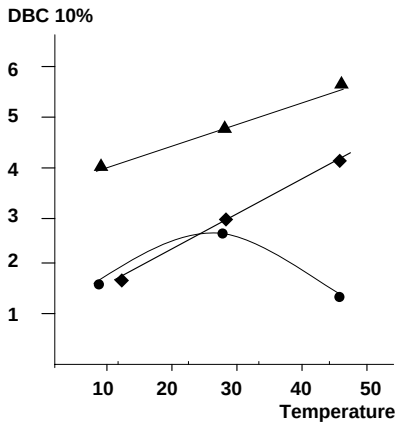

Fig. 4. Dynamic binding capacity (DBC) at 10% breakthrough of MBI HyperCel (●) as a function of temperature. Comparison was made with hydrophobic resins such as phenyl agarose (▲) and MEP HyperCel (◆).

[23]. Additionally the presence of polyvinyl alcohol (a potent inhibitor of hydrophobic associations) and urea (a strong chaotropic agent) did not significantly modify the adsorption capacity for antibodies (results not shown).

Based on these data, a determination of the apparent dissociation constant for IgG was made using conditions of adsorption defined as a 50 mM acetate buffer pH 5.5 containing 0.14 M sodium chloride. At 23 °C dissociation constant value was 1.26×10^{-6} M/L. This value is not very different from the one measured for protein A resins [24] and for MEP HyperCel [25] with respective values of 1.1×10^{-6} M/L and 2.9×10^{-7} M/L. The dissociation constant is of practical interest when dealing with expression systems where the antibodies are produced at very low concentrations.

Studies on sorbent characterization (behavior of IgG adsorption at different pH, see Fig. 2) suggested that desorption of IgG would occur when increasing the pH of the buffer. This was confirmed during the systematic study for the optimization of elution conditions using the combination of pH and sodium chloride concentration in a matrix-like manner. From SELDI MS analysis and SDS electrophoresis it appeared that the best conditions for IgG desorption was reached using a buffer of pH 9.0–9.5 containing at least 0.14 M sodium chloride.

Based on these preliminary characterization studies, real IgG separation trials were made starting from different crude feed stocks.

The first example deals with the separation of a monoclonal IgG1 from a crude rat ascites fluid. The sample was first adjusted with 1 M acetic acid to decrease the pH to 5.2 and then directly loaded onto a column of MBI HyperCel previously equilibrated with 50 mM acetate buffer containing 0.14 M sodium chloride pH 5.2.

As shown in Fig. 5, a large amount of proteins were found in the flowthrough and a significant peak was desorbed using the slightly alkaline carbonate buffer.

SDS polyacrylamide gel electrophoresis showed that no significant amount of IgG appeared in the flowthrough; purity of IgG1 obtained by desorption at pH 9.0 was estimated close to 85%. According to the electrophoresis pattern, no albumin was present in the eluate, but traces of several other proteins.

The second reported example was the separation of an antibody from a rat hybridoma cell culture supernatant containing 10% fetal bovine serum. The separation was performed in comparison to MEP HyperCel and Protein A Ceramic HyperD, both well known resins for the separation of antibodies. The concentration of expressed antibodies in the culture supernatant was about 300 μg/mL. Adsorption conditions were column-specific. For MEP HyperCel and protein A Ceramic HyperD the filtered sample was directly injected on the column without preliminary treatment; in the case of MBI HyperCel, pH of the sample was decreased to 5.2 by addition of 0.5 M acetic acid before loading. Elution conditions were also column-specific. For MEP HyperCel, desorption of antibodies was obtained with a 50 mM acetate buffer pH 4; for protein A column the desorption was reached with a 0.1 M acetic acid and for MBI HyperCel IgG1 were recovered using a 50 mM 50 mM carbonate buffer, pH 9 containing 0.14 M sodium chloride. From chromatography profiles represented in Fig. 6 it is interesting to see that protein A did not adsorb well antibodies while MBI and MEP resins showed a relatively large desorption peak. This result is actually consistent with known data on the low affinity of rat IgG for protein A [4]. The analysis of elution fraction by SDS electrophoresis evidenced that the separation of antibodies was in both cases very effective. Although the amount of antibody separated using protein A column was very low (most of them were present in the flowthrough of the column) the purity was as good as expected. With the MBI HyperCel column no antibodies were found in

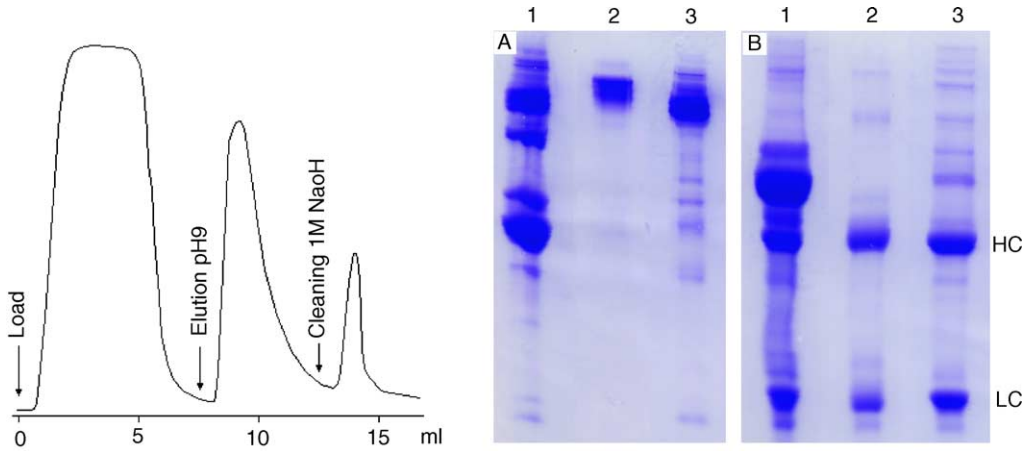

Fig. 5. Chromatographic separation profile of monoclonal IgG1 from a mouse ascites fluid using a MBI HyperCel column. Column: 6 mm i.d. × 50 mm (1.4 ml resin); equilibration buffer: 50 mM acetate, NaCl 0.14 M, pH 5.2; sample: mouse ascites fluid containing IgG1 at a concentration of 3.5 mg/mL; sample volume injected: 6.6 ml; linear flow rate: 26 cm/h; elution of antibodies: 50 mM carbonate buffer, 0.14 M NaCl, pH 9.0. Insert: SDS polyacrylamide gel electrophoresis in non-reducing (A) and reducing (B) conditions. From the left: crude sample (1); pure IgG standard (2); eluted fraction (3). LC: light chain; HC: heavy chain.

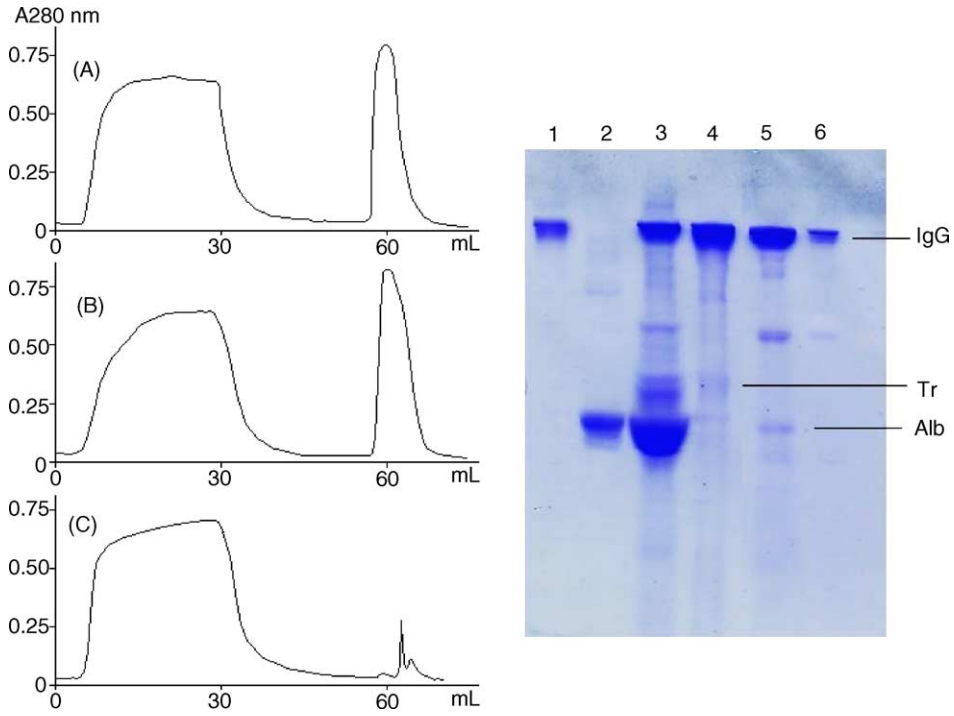

Fig. 6. Comparative separation rat IgG from hybridoma cell culture supernatant containing 10% fetal bovine serum using a MBI HyperCel column (A), MEP HyperCel (B) and protein A Ceramic HyperD (C). All column dimensions: 6 mm i.d. × 75 mm (2.1 ml resin). Equilibration buffer: 50 mM acetate, NaCl 0.14 M, pH 5.2 for MBI HyperCel; PBS for both MEP HyperCel and protein A Ceramic HyperD columns. Sample for all columns: 19 mL of hybridoma cell culture supernatant containing IgG1 at a concentration of 0.3 mg/mL equilibrated in the appropriate buffer. The supernatant was obtained from cells cultured in the presence of 10% fetal bovine serum. Linear flow rates: 48 cm/h. Elution of antibodies: 50 mM carbonate buffer, 0.14 M NaCl, pH 9.0 for column A; 50 mM acetate buffer pH 4.0 for column B; 0.1 M acetic acid for column (C). Insert: SDS polyacrylamide gel electrophoresis in non-reducing conditions. From the left: standard IgG, standard albumin, crude sample, eluate from column A, eluate from column B, eluate from column (C).

the flowthrough; purity of eluted antibodies was estimated higher than 90%.

An interesting capture of polyclonal antibodies was shown from the so-called paste II + III from Cohn fractionation of human plasma. This biological extract is an intermediate ethanol precipitate of human plasma fractionation from where a number of proteins of therapeutic interest are purified [26]. Fraction II + III obtained by precipitation with about 19% ethanol, contains several proteins among them the most known abundant are immunoglobulins; transferrin (Tr), albumin and some other alpha and beta globulins. Based on electrophoresis analysis (see insert in Fig. 7) IgG present in this fraction represent about 40% of total soluble proteins. The separation of IgG from Cohn fraction II + III was performed in parallel on MBI HyperCel and protein A Ceramic HyperD.

Under the above described conditions IgGs from Cohn fraction II + III were well adsorbed on both resins and totally desorbed by either increasing the pH to 9.0 for MBI HyperCel, or decreasing the pH to 3 for the protein A column. As shown in Fig. 7, elution peaks were both relatively large; a cleaning step using 1 M sodium hydroxide did not show a large amount of non-specifically adsorbed proteins. SDS-electrophoresis analysis showed that polyclonal human antibodies purity was of about 90% with the MBI HyperCel column and of about 95% with the protein A column.

Repeated separations did not show a decrease of separation performance as reported elsewhere [27].

Several other separations of antibodies from various antibody-containing biological liquids were performed using MBI HyperCel in conditions described in Section 2 (data not shown). They involved crude bovine serum, cell culture supernatants in protein free conditions and milk whey. In all cases the separation of antibodies (polyclonal and monoclonal) was equally possible with purities ranging from 85 to 99% depending on the composition and purity of the starting feedstock. Naturally the best purity was always obtained with protein-free cell culture supernatants.

Recently a study was conducted to separate polyclonal antibodies from rabbit serum (29) with purity of about 98%. In the same study polyclonal IgGs from other species such as pork and sheep were reported with similar results.

According to data described in the present study it is believed that this approach can be easily applied to the purification of antibodies at preparative scale. The antibody separated fraction from MBI HyperCel may be directly combined with other columns to polish out traces of protein impurities. pH and ionic strength of the collected frac-

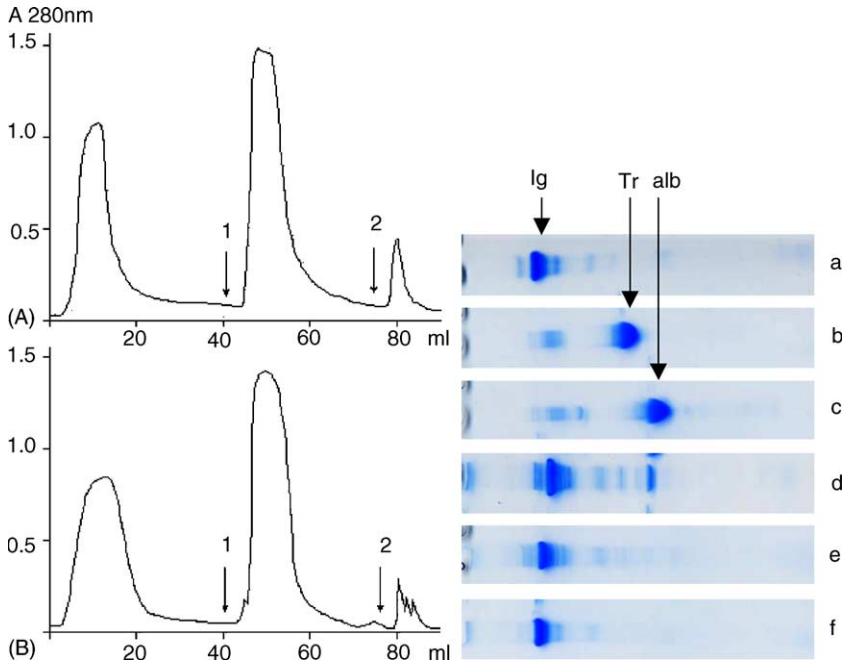

Fig. 7. Comparative separation of human polyclonal IgG from a Cohn human plasma fraction II + III using a MBI HyperCel column (A) or a Protein A Ceramic HyperD (B). Column: 11 m i.d. × 65 mm (6.5 ml resin); equilibration buffer for MBI HyperCel column: 50 mM acetate, NaCl 0.14 M, pH 5.5; equilibration buffer for protein A column: phosphate buffered saline; sample: extracted proteins from Cohn fraction II + III containing about 12 mg protein per mL; sample volume injected: 10 ml; linear flow rate: 50 cm/h; elution of antibodies from column A (arrow 1): 50 mM carbonate buffer, 0.14 M NaCl, pH 9.5; elution of antibodies from column B (arrow 1): 100 mM acetic acid. Column cleaning (arrows 2) was performed in both cases using 1 M sodium hydroxide. Insert: SDS polyacrylamide gel electrophoresis in non-reducing conditions. From the top to the bottom: pure human IgG "Ig" (a), pure human transferrin "Tr" (b), pure human albumin "alb" (c), redissolved Cohn fraction II + III (d), IgG recovered from MBISA column (e) and IgG recovered from protein A column (f).

tions are actually compatible with hydroxyapatite columns, IMAC columns and MEP HyperCel columns. Work is in progress with all these combinations to design complete processes.

4. Conclusion

Immobilized 2-Mercapto5--benzimidazolesulfonic acid (MEP HyperCel) clearly provides rapid and efficient purification of antibodies from a large variety of biological crude extracts. Antibody purity in a single pass ranges from 85% to more than 95%, according to the nature of the feedstock. In this respect the described immobilized ligand can be easily used for the production of pure antibodies for diagnostic and therapeutic applications in conditions compatible with large scale requirements. Albumin contamination is highly reduced or even in most of the cases eliminated as a result of the presence of a sulfonic acid group on the ligand structure. Albumin is in fact a protein contaminant generally difficult to remove whatever the purification scheme.

The whole interaction mechanism responsible for antibody adsorption is not yet fully understood; however, it is probably the result of multiple interactions and repulsion forces due to the presence of a sulfur atom and of an imidazole ring, both well described in the literature for antibody adsorption. The presence of a sulfonic acid group contributes to repulse protein species such as albumin that would adsorb concomitantly in the described conditions.

Interestingly enough the described solid phase is compatible with antibodies of different species and classes; this situation opens the way to the design of a number of separation processes starting from different feedstocks. Actually this resin could be used not only to separate antibodies from biological liquids described in this reported examples, but also from other non-conventional sources such as sweet whey, milk from hyperimmunized animals and from recombinant expression systems. An application of particular interest as reported is the purification of polyclonal antibodies from human plasma Cohn fractions where current yields based on alcohol precipitation are generally low.

Taken together, experimental data show that MBI HyperCel is an effective affinity-like sorbent for the separation of antibodies even compared to real affinity chromatography separation based on protein A ligand.

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