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Comparison of hydrophobic charge induction chromatography with affinity chromatography on protein A for harvest and purification of antibodies

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Abstract

Efficient harvest and recovery of high-purity monoclonal antibodies was achieved using hydrophobic charge induction chromatography (HCIC). Both simple and complex feedstocks were studied, including protein-free cell culture supernatant and the clarified/concentrated milk of transgenic goats. Viral clearance studies demonstrated a 4-log reduction of MVM virus (minute virus of mice), along with substantial reduction of DNA content. Sorbent characterization studies confirmed that HCIC is based on the pH-dependent behavior of a dual-mode, ionizable ligand. Binding, based on hydrophobic interaction, was achieved under near-physiological conditions, and in the absence of lyotropic salt. Desorption was accomplished under mild conditions — pH 4.0. At this pH, both ligand and antibody carry a net positive charge, and desorption occurs on the basis of electrostatic charge repulsion. pH-based control of chromatographic function was demonstrated. Chromatography on this antibody-selective HCIC sorbent was evaluated as a cost-effective, process-compatible alternative to affinity chromatography protein A sorbents.

Keywords: Hydrophobic charge induction chromatography; Affinity chromatography; Preparative chromatography; Monoclonal antibodies; Proteins; Protein A; Immunoglobulins; Mercaptoethylpyridine

1. Introduction

Affinity chromatography on protein A sorbents is widely used for harvest and initial purification of monoclonal antibodies. Nevertheless, there is growing interest in alternative sorbents to accomplish this process step, particularly in pilot and production-scale applications. The goal, broadly acknowledged among process developers and production engineers, is an antibody-selective sorbent that would provide greater chemical stability and longer service life at lower cost than protein A sorbents. This need is reflected in the focus of technical symposia and in literature describing the development of sorbents based on various biomimetic ligands [1–3].

An ideal alternative to protein A sorbents would permit desorption under milder conditions than those frequently used during affinity chromatography. Similarly, binding would be achieved without need for addition of high concentrations of glycine or salt to promote binding. Hydrophobic charge induction

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chromatography (HCIC) is a technique whose fundamental mechanism and practical operating characteristics appear well suited to evaluation against these goals. Named and described by Burton and Harding [4], HCIC is based on the pH-dependent behavior of dual-mode, ionizable ligands. The ligand contains structural features to support hydrophobic binding. When pH of the mobile phase is reduced, both the ionizable ligand and the target molecule take on net positive electrostatic charge. The resulting electrostatic charge repulsion overcomes hydrophobic binding interactions, and desorption of the biomolecule occurs. The HCIC sorbent described here carries a ligand that is antibody-selective. Effective use of this sorbent in harvest and purification of antibodies has been described and its stability to repeated cleaning with 1 *M* sodium hydroxide has been demonstrated [5]. Further study of the sorbent is reported here, with particular emphasis on performance characteristics of importance during process-scale purification of therapeutic antibodies.

Findings presented here demonstrate that binding of antibody is achieved under near-physiological conditions, without need for addition of glycine or salts to promote binding. As in earlier studies [5], feedstocks were applied to the column without adjustment of pH or ionic strength. Elution of antibody is achieved using low ionic strength buffer at moderate pH (e.g., 50 m*M* sodium acetate buffer, pH 4.0). (Buffer formulations are described fully in the Experimental section). By eliminating costs of supplementary salt or glycine, as well as costs of related waste disposal, process economy should be enhanced. Moreover, the use of low-ionic-strength buffer during elution eliminates the need for extensive diafiltration or dilution preliminary to the next chromatographic step — typically, ion-exchange chromatography. This, too, can be expected to reduce process costs.

In his comprehensive monograph on antibody purification [6], Gagnon points out that despite its high selectivity, harvest using protein A sorbents does not eliminate the need for subsequent chromatographic steps required to assure appropriate clearance/removal of virus, DNA, endotoxin, non-specific antibodies, and significantly, leached protein A. Such schemes, he observes, may include ''as many steps as non-affinity schemes'' and that ''advantages associated with higher initial purity may be offset by higher material and validation costs'' [6]. These issues are similarly addressed in another recent review [7].

In studies described here, HCIC is challenged with respect to the goals and characteristics identified above. The work includes isolation of antibody from both simple and complex feedstocks, including harvest from highly dilute feedstock. Studies to assess viral clearance and reduction of DNA content are presented.

2. Experimental

2.1. Chromatography: materials, equipment and general procedures

The HCIC sorbent, MEP HyperCel, was obtained from Life Technologies (Rockville, MD, USA). Buffer salts were of the highest available purity, and were obtained from Life Technologies or Sigma (St. Louis, MO, USA). Other chemicals were of reagent grade. Chromatography was performed using conventional glass columns equipped with adjustable flow adapters, and suitable for low/medium pressure applications, <3 bar (Amicon, Danvers, MA, USA; KronLab, Sinsheim, Germany; Pharmacia, Piscataway, NJ, USA).

Tris–HCl buffers were prepared using the indicated concentration of Tris base and were adjusted to the stated pH using hydrochloric acid. Sodium acetate buffers were prepared using the specified concentration of acetic acid and were adjusted to the indicated pH using sodium hydroxide. Sodium phosphate buffers were prepared by combining stock solutions of disodium hydrogenphosphate and sodium dihydrogenphosphate, along with pure water, to provide the specified pH and phosphate concentration. Phosphate-buffered saline (PBS) contained 150 m*M* sodium chloride unless otherwise indicated.

Columns were packed by suspending a quantity of gravity settled sorbent in a volume of equilibration buffer sufficient to provide a 67% slurry (e.g., 20 ml sorbent, 10 ml of buffer). After assuring that lines and frits were free of entrapped air, and that 0.5–1 cm of buffer remained above the lower frit, the

slurry was introduced into the column. Columns were packed at 600 cm/h using equilibration buffer, typically 50 m*M* Tris–HCl or 50 m*M* PBS. When the bed was fully consolidated, the flow adaptor was adjusted (typically, under flow) to bring the frit into contact with the packed sorbent. Preliminary to first use, the column was washed with 1 *M* sodium hydroxide followed by equilibration buffer.

Isolation and purification of antibody from cell culture supernatant, clarified/concentrated milk of transgenic goats, and test feedstocks for viral clearance studies was performed as described in figure captions accompanying the chromatograms.

2.2. Analysis of chromatographic fractions and feedstocks

Chromatographic fractions were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) and proteins were visualized using Coomassie blue dye. SDS–PAGE was conducted according to classical procedures with Laemmli buffers [8]. Unless otherwise noted, 12% acrylamide gels were employed. Gels shown in Figs. 4, 6 and 12 were cast in the laboratory [8], while those shown in Figs. 10 and 11 were purchased pre-cast (BioRad Labs., Hercules, CA, USA). Western blot analyses shown in Figs. 10 and 11 were performed using anti-goat IgG–alkaline phosphatase and anti-human IgG–alkaline phosphatase conjugates (Boehringer Mannheim, Roche Molecular Biochemicals). Detection/visualization was accomplished using combined BCIP (5-bromo-4-chloro-3-indolyphosphate *p*-toluidine) and NBT (nitro-blue tetrazolium chloride) reagent (Kirkegaard and Perry Labs.).

As described in Section 2.3, murine IgG_1 and IgG_{2b} produced in cell culture were proprietary products of Life Technologies. Concentration of these antibodies was determined by an enzyme-linked immunosorbent assay (ELISA) procedure that is, likewise, proprietary to Life Technologies. The assay is specific for murine IgG, but is not isotype-specific. Thus, this method was also used to determine murine polyclonal IgG during viral clearance studies described in Sections 2.5 and 3.3. During studies to isolate monoclonal antibody from the milk of transgenic goats, IgG concentration was determined by protein A assay using a 100-μl sorbent cartridge from PE Biosystems (Norwalk, CT, USA). Analytical procedures used to determine viral load and to assess DNA content are described in Section 2.5.

2.3. Cell culture

Cell culture was conducted using a Bellco (Bellco Glass, Vineland, NJ, USA) 30-l stirred tank bioreactor. Protein-free growth media were employed, either CD-Hybridoma or Hybridoma-SFM (Life Technologies). Unless otherwise specified, cell culture supernatants (CCSs) were based on use of CD-Hybridoma. The murine hybridoma clones employed and the monoclonal antibodies produced are proprietary to Life Technologies. In order to limit appearance of host-cell protein and DNA in the culture broth, cultures were terminated after day 4 or 5, depending on the clone. The bioreactor was then drained, and the supernatant was clarified using a Flexstand microfiltration apparatus (A/G Technologies, Needham, MA, USA) fitted with a 0.45-μm filter (A/G Technologies) and equipped with a Labtop 350 rotary lobe pump (Teknoflow/Flow-Tech, Atlanta, GA, USA). Further filtration, using a 0.22-μm filter (Durapore, Millipore, Bedford, MA, USA) was conducted preliminary to storage of the product in sterile containers.

2.4. Determination of dynamic binding capacity, and its dependence on pH, ionic strength and IgG concentration

Determinations of dynamic binding capacity (DBC) were conducted using human polyclonal IgG (Sigma) and were based on frontal analysis. Test feedstocks were applied to the column (10×0.66 cm) at 75 cm/h and DBC was determined at 10% breakthrough. During studies to assess the influence of pH on DBC, test feedstocks contained 5 mg IgG/ml and were formulated using 50 m*M* sodium phosphate–citrate buffers prepared to provide pH values shown in Fig. 2. During studies to assess the influence of ionic strength on DBC, test feedstocks

were prepared using 50 m*M* sodium phosphate buffer, pH 7.2, and contained 5 mg IgG/ml. During studies to assess the influence of IgG concentration on DBC, test feedstocks were prepared in 50 m*M* sodium phosphate buffer containing 150 m*M* sodium chloride. IgG concentrations ranged from 0.05 to 5 mg/ml.

2.5. Viral and DNA clearance studies

Test feedstock was formulated using a hybridoma CCS containing a low concentration of IgG_{2b} (<3 μg/ml). Clarified CCS, 40 ml, was combined with solutions (10 ml, total) containing 10 mg of commercially-purified murine polyclonal IgG and ~$1.6 \cdot 10^8$ plaque forming units (pfu) of MVM virus (minute virus of mice). The murine antibody had been purified by affinity chromatography on a protein A sorbent and was obtained from Jackson Immunoresearch (Westgrove, PA, USA) or Rockland Immunochemicals (Gilbertsville, PA, USA). MVM viral culture was obtained from Life Technologies. The feedstock was also augmented with tetrasodium EDTA sufficient to provide a final concentration of 10 m*M*.

Chromatography on MEP HyperCel was conducted as described in Fig. 5. Viral titers were determined by tissue culture infective dose sufficient to produce 50% monolayer infection ($TCID_{50}$) assay [9]. Immediately prior to assay, chromatographic fractions were sterilized by filtration using 0.22-μm Millipore GV syringe filters (Danvers, MA, USA). Fractions obtained during elution with 50 m*M* sodium acetate buffer, pH 4.0 (see Fig. 5), were neutralized prior to assay by adding an equal volume of 200 m*M* dibasic sodium phosphate, which brought the pH to 7.0 and the osmolarity to 300–315 mOsmols.

Samples of chromatographic fractions were taken and DNA was amplified using a polymerase chain reaction (PCR) procedure [8]. Primers employed were directed to the L1 consensus sequence of the murine genome; 40 PCR cycles were conducted using Platinum TAq (Life Technologies). Products were evaluated by electrophoresis on 1% agarose gel [8].

2.6. Isolation of monoclonal antibody expressed in the milk of transgenic goats

Milk expressed by transgenic goats was a proprietary product of Genzyme Transgenics (Framingham, MA, USA), as was the humanized monoclonal IgG_1 contained in the milk. Milk was collected following induced lactation. Raw milk was concentrated twofold using a Pall-Filtron (Northborough, MA, USA) Omega membrane having a nominal molecular mass cut-off (MWCO) of 300,000. This membrane passed transgenic monoclonal antibody and retained the cream, casein micelles and most of the bacterial and cellular load. After a twofold concentration, the collected filtrate was used to begin diafiltration through a Pall-Filtron Omega membrane having a MWCO of 30,000. This second membrane served to capture and concentrate the antibody. The two membrane-filtration systems were coupled, as shown in Fig. 8. The design and operation of the coupled system is discussed in Section 3.4.

The HCIC procedure employed to isolate and purify antibody from the clarified, concentrated milk is described in Fig. 9 and discussed in Section 3.4. The HCIC-purified antibody was compared with a reference product, isolated and purified using affinity chromatography on protein A sorbent. The reference material was a product of Genzyme Transgenics.

2.7. Isolation of antibody from dilute protein-free CCS

Clarified CCS, 2000 ml, containing ~2.7 μg IgG_{2b}/ml was augmented with EDTA sufficient to provide a concentration of 10 m*M* in the feedstock. This was accomplished using a 200 m*M* EDTA stock solution prepared using tetrasodium EDTA and adjusted to ~pH 8.2 using acetic acid. (The tetrasodium form of EDTA should be employed.) The feedstock was applied to a 5.0×1.0 cm column of MEP HyperCel. The load was applied at 76 cm/h until 1 l had been applied. Remaining feedstock was applied at 300 cm/h, and chromatography was continued at this linear velocity. The column was washed with 50 m*M* Tris–HCl buffer, pH 8.0 until absorbance (280 nm) returned to baseline. The IgG fraction was then eluted using 50 m*M* sodium acetate

buffer, pH 4.0. Chromatographic fractions were analyzed by SDS–PAGE, as shown in Fig. 12.

3. Results and discussion

3.1. The chromatographic mechanism, and the influence of pH, ionic strength and antibody concentration on sorbent function

The mechanism of HCIC and its application to antibody purification is illustrated in Fig. 1. The sorbent, MEP HyperCel, carries a ligand derived from 4-mercaptoethylpyridine (4-MEP). Such nitrogen heterocycles have been shown to have particular selectivity for immunoglobulins. Additional selectivity is conferred by the presence of a sulfur atom proximal to the heterocyclic ring [10–13].

The pK_a of 4-MEP is 4.8. Thus, under near-neutral or alkaline conditions, the ligand is uncharged and behaves much like a phenyl group to bind antibody by hydrophobic interaction. However, the distinct structure of the ligand provides the antibody-selective characteristics for which this sorbent was designed. The pK_a of the ligand also provides sorbent characteristics that support antibody recovery under mild conditions. Desorption is achieved by reducing the pH of the mobile phase to confer a net positive charge on both ligand and antibody. At pH values in the range of 4.0–4.5, the ligand — and most antibodies — carry a predominant positive charge. Thus, desorption is achieved under conditions significantly milder than those typically employed during affinity chromatography on protein A sorbents.

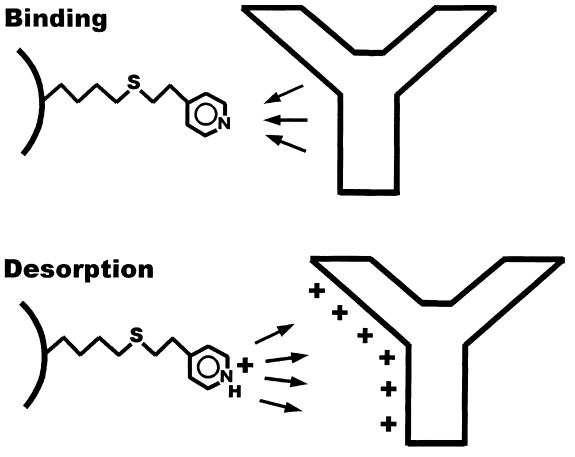

Fig. 1. A representation of the mechanism of hydrophobic charge induction chromatography, along with key structural features of the ligand. The antibody-selective ligand is derived from 4-mercaptoethylpyridine (4-MEP; pK_a 4.8). Hydrophobic binding under near-physiological conditions is illustrated. Desorption, typically conducted at pH 4, is based on electrostatic charge repulsion.

The fundamental chromatographic mechanism and specific characteristics of the sorbent were confirmed by studies to determine the influence of pH on the DBC for human polyclonal IgG. DBC was determined at 10% breakthrough; data are shown in Fig. 2. Over a pH range that encompasses values representative of typical feedstocks (pH~6.5–8.5), DBC ranged from approximately 21–33 mg IgG/ml sorbent. As anticipated, when pH of the test feedstock

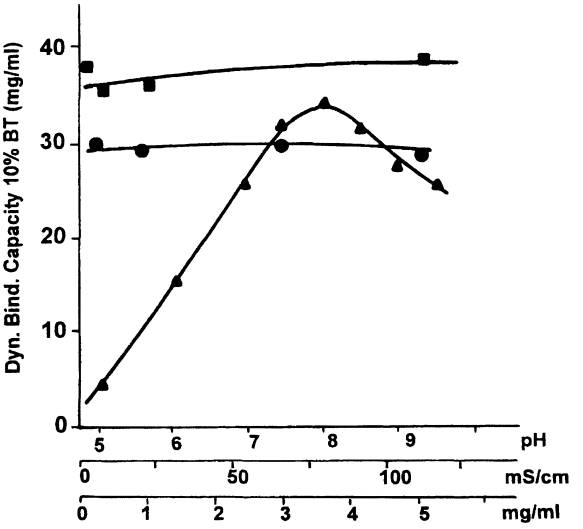

Fig. 2. Dynamic binding capacity of MEP HyperCel for human polyclonal IgG. The influence of pH (▲), ionic strength (●) and IgG concentration (■) are illustrated. Values were determined at 10% breakthrough (BT) as described in Section 2.4. Determinations at varying pH were conducted in various buffers (5 mS/cm conductivity) containing 5 mg IgG/ml. Determinations at varying ionic strength were conducted in 50 m*M* sodium phosphate buffer, pH 7.2, containing 5 mg IgG/ml and concentrations of sodium chloride up to 1 *M*. Determinations at varying IgG concentrations were conducted in 50 m*M* sodium phosphate buffer containing 150 m*M* sodium chloride, pH 7.2.

was reduced toward the pK_a of 4-MEP, there was a distinct decline in DBC. Based on these findings, desorption of antibody is typically conducted at pH 4.0. Somewhat higher values (≤4.5) may be appropriate in some applications.

Also illustrated in Fig. 2, DBC was found to be independent of ionic strength over values ranging from approximately 50 m*M* to 1 *M* total ionic strength. Such behavior is consistent with that observed in related studies [4,5,10,11]. As a practical matter, data shown in Fig. 2 indicates that typical feedstocks can be applied to MEP HyperCel without preliminary adjustment of ionic strength or pH.

The dependence of DBC on IgG concentration was also evaluated, and is shown in the same figure. Only a modest reduction in DBC was observed as IgG concentration in the test feedstock was reduced from 5 mg/ml to 50 μg/ml. Thus, capture of antibody from dilute feedstock may be accomplished without need for preliminary concentration. This principle was further demonstrated during studies discussed in Section 3.5.

Continuing studies with human polyclonal IgG, bovine polyclonal IgG, humanized IgG_1, murine IgG_1 and murine IgG_2 suggest that dynamic binding capacity for IgG is independent of subclass or species. Determined at 10% breakthrough, values range from approximately 30–40 mg IgG/ml sorbent. Moreover, binding of IgA, IgE and antibody fragments has also been demonstrated (data not shown). Dynamic binding capacity for IgM is ≤20% of that typically observed for IgG. Reduced binding capacity for IgM is attributed to steric/exclusion effects.

3.2. Isolation of monoclonal antibody from protein-free cell culture supernatant

A typical chromatogram illustrating harvest of a monoclonal antibody (murine IgG_1) from protein-free CCS is shown in Fig. 3. The CCS, containing 200 μg IgG/ml, was clarified by microfiltration before loading but was unadjusted with respect to pH or ionic strength. The chromatographic sequence is straightforward. After loading, the column was washed with equilibration buffer (50 m*M* Tris–HCl buffer, pH 8.0) until absorbance returned to baseline. The IgG fraction was then eluted under the influence of 50 m*M* sodium acetate buffer, pH 4.0. Based on SDS–PAGE analysis (see Fig. 4, lanes 2, 6, 7, 8), product purity was ≥95% and recovery was ~92%. A duplicate separation (see Fig. 4, lanes 2–5), gave equivalent purity, but with recovery of ~98%. Higher recovery in the duplicate separation is consistent with the observation that a quantity of IgG was visible in the post-load wash during the *initial* separation (Fig. 4, lane 7), while no IgG was visible in the wash during the *duplicate* run (lane 4). No IgG was visible in the load-flowthrough fractions during *either* separation (lanes 3 and 6). In overview, *both* separations provided high product recovery. Recovery values of 92–98% are entirely compatible with process-scale application of HCIC. Indeed, during earlier studies [5], murine IgG_1 was isolated from a protein-free cell culture supernatant at ~99% purity, with recovery of 98%. Additional examples

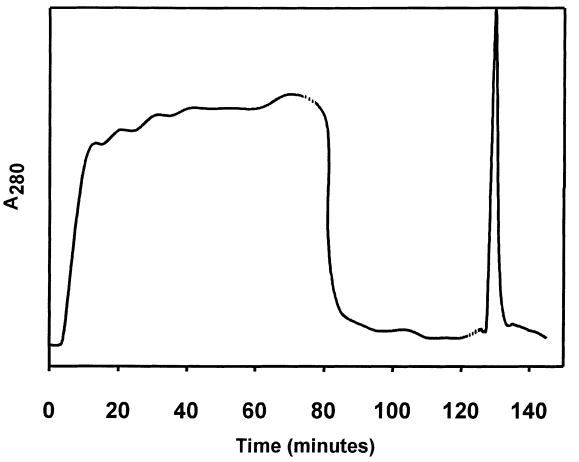

Fig. 3. Harvest and purification of murine IgG_1 from protein-free cell culture supernatant. Chromatography was conducted using a 9.0×2.5 cm column of MEP HyperCel, operated at 150 cm/h throughout. Detector sensitivity was 0.5 AUFS at 280 nm. The column was equilibrated in 50 m*M* Tris–HCl, pH 8.0. Clarified cell culture supernatant, 1000 ml, containing 200 μg IgG/ml, was applied after which the column was washed with equilibration buffer until absorbance returned to baseline. The IgG fraction was then eluted under the influence of 50 m*M* sodium acetate, pH 4.0. (Short segments of broken line were inserted at two locations where flow disturbance produced erratic detector response).

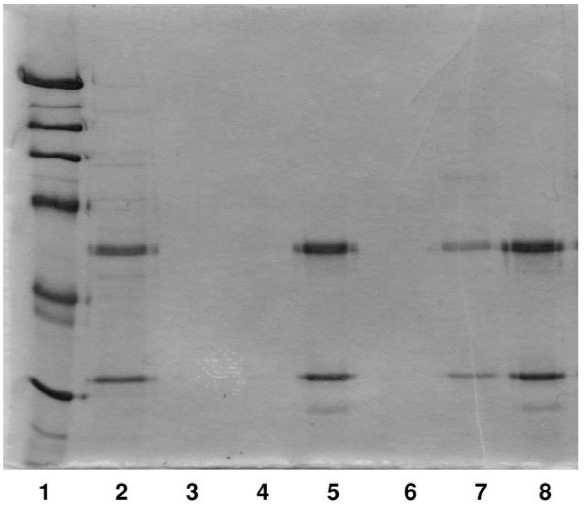

Fig. 4. SDS–PAGE analysis of chromatographic fractions collected during purification of IgG_1 from protein-free cell culture supernatant. (Refer to the chromatogram in Fig. 3 and to Section 3.2). SDS–PAGE was conducted under reducing conditions and gels were stained using Coomassie Blue dye. Lanes: 1=markers; 2=feedstock; 3=load flowthrough (duplicate separation, see text); 4=wash, pH 8 (duplicate separation, see text); 5=eluate, pH 4.0 (duplicate separation, see text); 6=load flowthrough (see Fig. 3); 7=wash, pH 8.0 (see Fig. 3); 8=eluate, pH 4.0 (see Fig. 3).

of representative chromatography are presented below.

3.3. Viral clearance and reduction of DNA content

Schemes for purification of therapeutic antibodies are designed with particular focus on achieving and demonstrating required levels of viral and DNA clearance. Accordingly, studies were conducted to assess viral and DNA clearance achieved during HCIC. Experiments were designed to provide an appropriate challenge with regard to both viral load and protein load applied to the column. At the same time, the load was formulated to reflect the background composition and DNA content of a representative protein-free CCS. Toward this end, 40 ml of clarified protein-free CCS was combined with solutions (10 ml, total) containing 10 mg of affinity-purified murine polyclonal IgG and $1.6 \cdot 10^8$ pfu of MVM virus. Murine polyclonal IgG comprised ~99% of the antibody present in the load. (The concentration of monoclonal IgG_{2b} in the CCS was <3 μg/ml). In connection with findings discussed in Section 3.5, the feedstock also contained 10 m*M* EDTA.

As shown in Fig. 5, test feedstock equivalent to 64 column volumes (CVs), was applied to the column. Collected fractions were analyzed by SDS–PAGE as shown in Fig. 6. The IgG fraction was judged to be >95% pure, while recovery was ~83%. Albumin was a component of the viral stock solution, and was identified during SDS–PAGE. It is present in the feedstock (lane 2) at a concentration of approximately 70 μg/ml, compared with 200 μg IgG/ml. Albumin is visible in the load-flowthrough and wash fractions (lanes 3 and 4), but not in the target IgG fraction (lane 5). Concentration of IgG in the target fraction is ~800 μg/ml. Thus, both concentration and purification were achieved. Studies demonstrating a substantially greater degree of product concentration are discussed in Section 3.5.

Significant viral clearance was achieved during

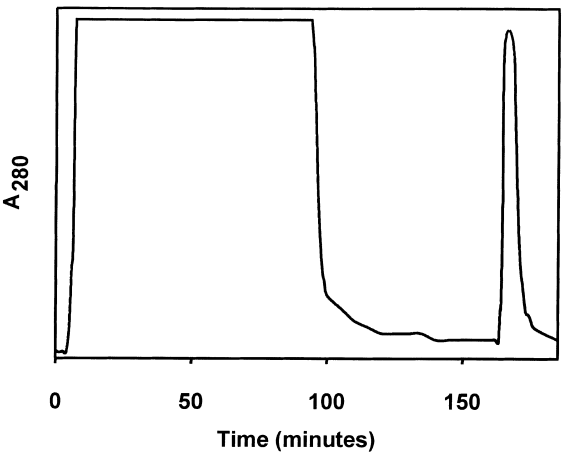

Fig. 5. Chromatographic study to assess viral clearance and DNA removal. Chromatography was conducted using a 4.0×0.5 cm column of MEP HyperCel, operated at 150 cm/h throughout. Detector sensitivity was 0.5 AUFS at 280 nm. The column was washed with 1 *M* sodium hydroxide and then equilibrated with 50 m*M* Tris–HCl, pH 8.0 prior to loading. The test feedstock is described in Section 2.5. The chromatographic sequence was analogous to that described in Fig. 3.

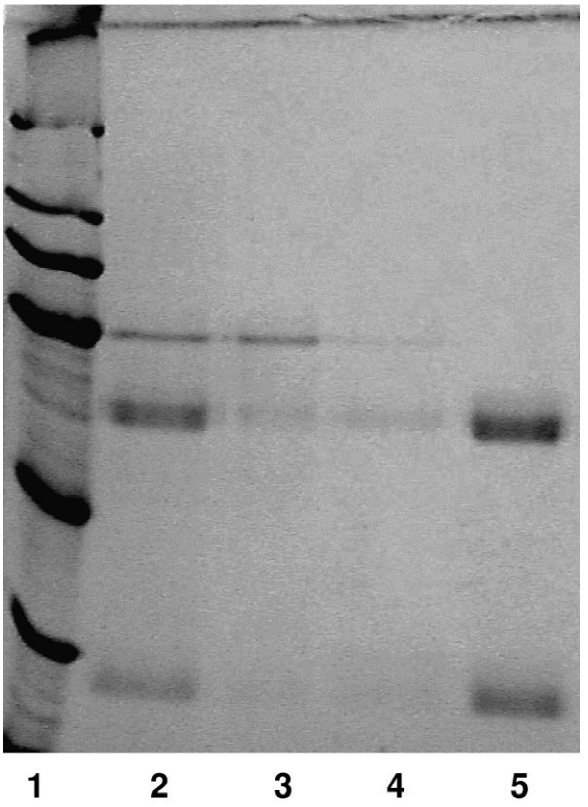

Fig. 6. SDS–PAGE analysis of fractions collected during chromatography to assess viral clearance and DNA removal. (Refer to the chromatogram in Fig. 5). Electrophoresis was performed as described in Fig. 4. Lanes: 1=markers; 2=test feedstock (see Section 2.5); 3=load flowthrough; 4=wash, pH 8.0; 5=eluate, pH 4.0.

HCIC. The IgG fraction contained $1.2 \cdot 10^4$ pfu, compared with $1.6 \cdot 10^8$ pfu in the feedstock — a 4-log reduction. The MVM virus is sufficiently acid-stable, that no reduction in concentration need be attributed to exposure to elution buffer (pH 4.0). By way of comparison, it is reported that viral clearance achieved during affinity chromatography on protein A Sepharose ranges from 3 to 5 logs [14]. Overall, HCIC can be expected to provide a significant contribution to the total viral clearance required in a purification scheme.

In order to evaluate reduction in DNA content, chromatographic fractions were collected and DNA content was assessed using PCR procedures followed by electrophoretic analysis of the DNA-amplified samples. During PCR, primers directed against the murine L-1 consensus sequence were used to amplify a 250 kilobase pair (kb) fragment. The L-1 consensus sequence accounts for approximately 5% of the murine genome. Analysis of DNA-amplified samples is shown in Fig. 7. Comparing lane 3 (the IgG eluate) and lane 4 (the feedstock), it is clear that a substantial reduction in DNA content was achieved during chromatography. Although the findings have not been quantified, electrophoretic analysis suggests that a large fraction of DNA passed unretained in the load-flowthrough fraction (lane 1) and in the post-load wash, pH 8 (lane 2). The effectiveness of this sorbent in reducing DNA burden may be related, in part, to the fundamental mechanism of the ligand. During elution at pH 4, the ligand carries a predominant positive charge. Any DNA still present would be expected to bind to the column, while IgG passes unretained. The post-elution wash with 1 *M* sodium hydroxide would be expected to rid the column of residual DNA.

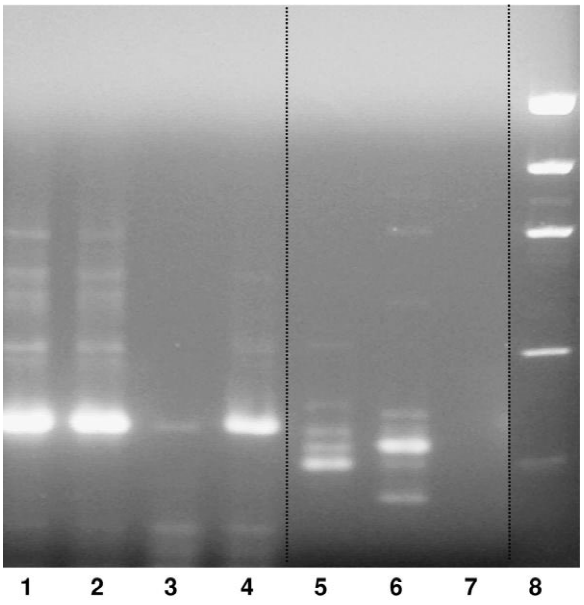

Fig. 7. Electrophoretic analysis of DNA-amplified chromatographic fractions. Fractions were collected during a study to assess viral clearance and DNA removal. (Refer to chromatogram in Fig. 5). Procedures for DNA amplification and electrophoresis are described in Section 2.5. Lanes: 1=load flowthrough; 2=wash, pH 8.0; 3=eluate, pH 4.0; 4=test feedstock (see Section 2.5); 5=control; no forward primer; 6=control, no reverse primer; 7=control; no enzyme; 8=mass ladder.

Overall, these data indicate that HCIC can be used to isolate high-purity antibody from CCS with good recovery, while at the same time providing significant contributions to viral clearance and reduction of DNA content.

3.4. Isolation of monoclonal antibody expressed in the milk of transgenic goats

Antibodies are routinely isolated from sources more complex than protein-free CCS. Accordingly, earlier studies [5] addressed isolation of monoclonal antibodies from samples such as CCS containing 5% fetal bovine serum and ascites fluid. In these studies, it was determined that supplementary wash steps could be used to enhance desorption of impurity components — particularly albumin — preliminary to elution of the IgG fraction. The elution sequence developed during the cited study was employed during current work to isolate monoclonal antibody from the milk of transgenic goats. (This phase of the current study was conducted as an active collaboration in the laboratory of Dr. Eszter Birck-Wilson, Genzyme Transgenics).

Transgenic milk employed in this work contained a humanized monoclonal IgG_1. Preliminary to chromatography, the milk was clarified and concentrated using the TFF apparatus shown in Fig. 8. First, a membrane having a MWCO of 300,000 was used to retain lipid globules, casein micelles, most of the bacterial and cellular load, and other large impurities. IgG and other proteins pass in the diafiltrate. The IgG-containing filtrate was then directed to a membrane having a MWCO of 30,000 where IgG and other proteins were retained and concentrated. As shown in the figure, the diafiltrate from the 30,000 MWCO membrane was returned to the process tank supplying feed stream to the 300,000 MWCO membrane. Using this approach, clarification and concentration were accomplished without use of process buffer, and dilution of the process stream is avoided. The process was continued until a sufficient fraction of IgG is passed through the 300,000 MWCO membrane and collected by the 30,000 MWCO membrane.

The clarified/concentrated feedstock applied to the column (15.2 ml, 15.4 mg IgG/ml) provided 31 mg IgG per ml of sorbent. As shown in Fig. 9, the chromatographic sequence included two supplementary wash steps, as mentioned above. Following the post-load wash with equilibration buffer, the column was washed with pure water, and then with 25 m*M* sodium caprylate in equilibration buffer. These wash steps may be used to promote desorption of impurity components that are *less hydrophobic* than the target antibody. As demonstrated above, IgG binding is salt-independent over a broad range of conditions. However, reducing the ionic strength of

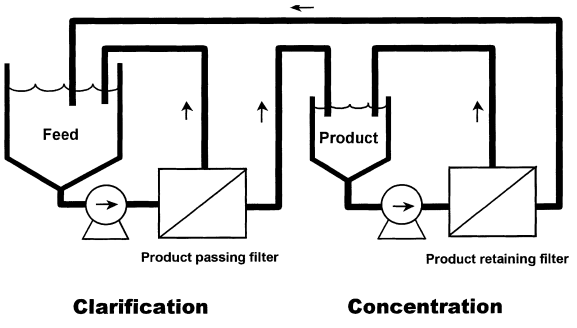

Fig. 8. Apparatus for clarification and concentration of transgenic milk (Genzyme Transgenics).

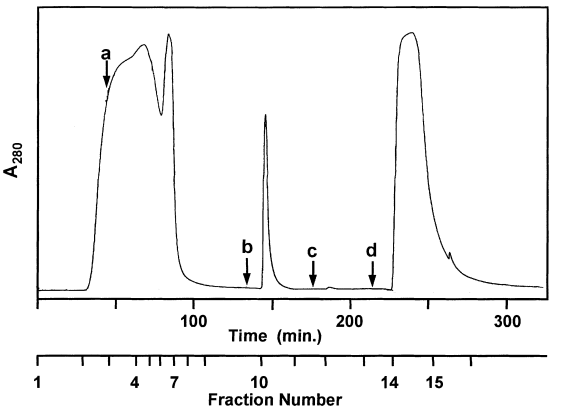

Fig. 9. Harvest and purification of IgG_1 from clarified, concentrated transgenic milk. Chromatography was conducted using a 8.0×1.1 cm column of MEP HyperCel. The column was operated at 47 cm/h during the first 30 min, and at 147 cm/h thereafter. Detector sensitivity was 1 AUFS at 280 nm. The column was equilibrated with 50 m*M* Tris–HCl, pH 7.4, after which the feedstock (15.2 ml containing 15 4 mg IgG/ml) was applied. The column was then washed with: (a) equilibration buffer; (b) pure water; (c) 25 m*M* sodium caprylate in equilibration buffer, pH 7.4. The IgG fraction was eluted under the influence of 50 m*M* sodium citrate, pH 4.0 (d).

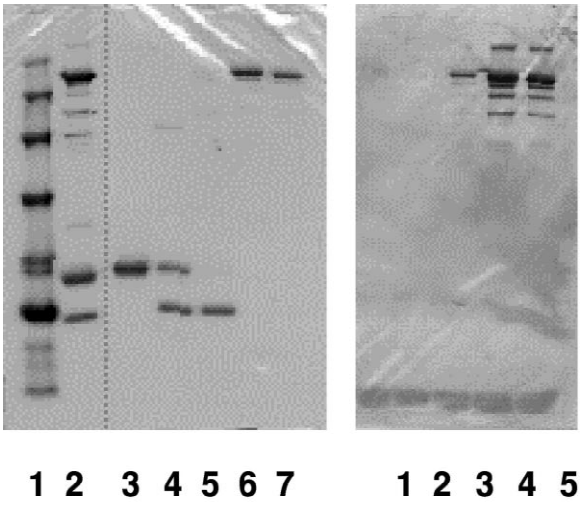

Fig. 10. SDS–PAGE and Western blot analysis of chromatographic fractions collected during purification of IgG_1 from clarified, concentrated transgenic milk. (Refer to the chromatogram in Fig. 9). SDS–PAGE was performed under non-reducing conditions and gels were stained using Coomassie Blue dye. Western blot analysis was performed using antibody–alkaline phosphatase conjugates, as described in Section 2.2. SDS–PAGE (left gel). Lanes: 1=markers; 2=feedstock; 3=fraction 4; 4=fraction 7; 5=fraction 10; 6=fraction 14 (1.2 μg IgG loaded); 7=fraction 15 (0.8 μg IgG loaded). Western blot analysis (right gel). Lanes: 1=fraction 4; 2=fraction 7; 3=fraction 10; 4=fraction 14; 5=fraction 15.

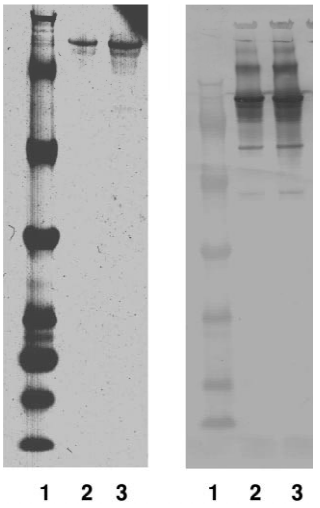

Fig. 11. Analysis of IgG_1 isolated from clarified, concentrated transgenic milk by affinity chromatography on protein A sorbent. SDS–PAGE and Western blot analysis were performed as described in Fig. 10. SDS–PAGE (left gel). Lanes: 1=markers; 2=purified IgG (1 μg IgG loaded); 3=purified IgG (2 μg IgG loaded). Western blot analysis (right gel). Lanes: 1=markers; 2=purified IgG; 3=purified IgG.

the mobile phase from 50 m*M* buffer to pure water can be employed as a useful technique to prompt desorption of less hydrophobic proteins. The mild detergent-like properties of sodium caprylate can also promote desorption of such proteins. In some applications, a small quantity of antibody may be desorbed along with impurity components during either of these steps.

As shown in Fig. 10, selected fractions were analyzed by SDS–PAGE and Western blot procedures. In the feedstock, bands corresponding to α-lactalbumin and β-lactoglobulin (14 000 and 18 000 molecular mass, respectively) are prominent (see PAGE, lane 2). These impurities are also prominent in load/wash flowthrough fractions (see PAGE, lanes 3 and 4). Based on both SDS–PAGE and Western blot studies, it appears that IgG is fully retained well into the descending region (Fraction 7) of the wash with equilibration buffer (see PAGE, lane 4 and Western, lane 2). These findings are consistent with a dynamic binding capacity of ~31 mg IgG/ml sorbent. Further studies may indicate that higher binding capacity values can be attained for this antibody.

During the wash with pure water (fraction 10), a significant quantity of α-lactalbumin was desorbed, along with a minor quantity of IgG (see PAGE, lane 5 and Western, lane 3). Based on SDS–PAGE, the principal IgG fractions (fractions 14 and 15) were judged to be >95% pure. These fractions were compared with product isolated by affinity chromatography on protein A. The affinity-purified product was analyzed by SDS–PAGE and Western blot procedures, as shown in Fig. 11. PAGE, lane 2 seen in Fig. 11 (affinity-purified antibody) and PAGE, lanes 6 and 7 seen in Fig. 10 (HCIC-purified antibody) represent comparable quantities of IgG applied to the gel. Based on comparison of these SDS–PAGE gels, product purified by HCIC was judged to be at least as pure as that obtained using affinity chromatography. Based on Western blot analysis, product purified by HCIC was judged to be more homogeneous than that isolated by affinity chromatography. Thus, HCIC is seen to be effective in harvest and purification of antibodies from both simple and complex feedstocks.

In studies with other complex feedstocks [5], it was demonstrated that HCIC can be combined with a simple anion-exchange procedure to yield very high purity IgG. This approach was used to isolate and purify a murine monoclonal IgG_1 from CCS supplemented with 5% fetal bovine serum. Purity of the final product was >97%. The anion-exchange procedure served to bind residual albumin (and other acidic impurities) while IgG passed unretained. The procedure was particularly effective in removing residual albumin. In the example cited, the IgG fraction isolated following HCIC was ~70% pure — albumin was the principal impurity. Given the routine use of anion-exchange chromatography to support viral clearance and reduction of DNA content, this two-step procedure represents a practical and efficient means of isolating high purity antibody from feedstocks containing abundant albumin.

3.5. Isolation of monoclonal antibody from high-volume loads of dilute protein-free CCS

Studies were conducted to assess and enhance chromatographic performance in applications where antibody is to be harvested from large volumes of dilute, protein-free CCS. A parallel objective was to assess and mitigate the influence of synthetic iron carrier present in the feedstock. (Protein-free growth media contain synthetic iron carrier in place of transferrin). Typically, such iron carriers are formulated by combining an organic chelating agent with an inorganic iron salt. The influence of such components can be particularly significant when large volumes of feedstock are applied to the column.

These studies were prompted, in part, by an observation that in some applications, the sorbent took on a light brown coloration when protein-free CCS was applied to the column. A similar effect could be produced by applying of solutions of ferrous lactate or gluconate to the column. These observations suggested that components of the iron carrier might be associating with the sorbent, presumably via interaction with the pyridine group. As reported in the literature, various pyridine-containing structures are known to complex with iron ions [15,16]. In order to test the hypothesis, clarified protein-free CCS containing a murine IgG_{2b} was augmented with 10 m*M* tetrasodium EDTA (see Section 2.7) and applied to the column. It was anticipated that the presence of this chelating agent in the feedstock would limit association of iron carrier with the sorbent. Indeed, during initial trials, addition of EDTA dramatically reduced appearance of brown color during loading.

Studies were continued with an experiment in which ~500 CVs of dilute feedstock (2.7 μg IgG_1/ml) were applied to a 3.9 ml column of MEP HyperCel, as described in Section 2.7. The chromatographic sequence was analogous to those shown in Figs. 3 and 5. Based on SDS–PAGE analysis (see Fig. 12), the IgG fraction, was >95% pure, while recovery was approximately 87%. Equally important, IgG in the target fraction had been concentrated >35-fold relative to its concentration in the feedstock. Indeed, the feedstock was sufficiently dilute that IgG was not visible following SDS–PAGE analysis of the feedstock (lane 1), but was clearly visible in the target fraction (lane 6). IgG recovery was substantially greater than that observed in experiments where EDTA was omitted from this dilute feedstock. In such cases, recovery values typically did not exceed 50%. Based on these findings, it is recommended that addition of tetrasodium EDTA, 10 m*M*, be considered for evaluation in any application where loading of protein-free CCS leads to uptake of brown color by the sorbent, or in cases where recovery values <80% are obtained. In the event that recovery declines over a series of separations, it is recommended that the column be washed with 100 m*M* EDTA, pH 7.2 (tetrasodium EDTA adjusted to pH 7.2 with acetic acid) following elution of the IgG fraction. The EDTA wash should be conducted *before* the column is washed with 1 *M* sodium hydroxide. This approach is designed to avoid formation of poorly soluble iron hydroxide in the event that traces of iron remain on the column. Work-in-progress indicates that this wash procedure is a useful technique to preserve and enhance sorbent function.

Beyond specific issues related to chromatographic optimization, this phase of the study demonstrates a valuable and novel use for a chromatographic sorbent. Namely, the simultaneous harvest and concentration of antibody from highly dilute feedstock.

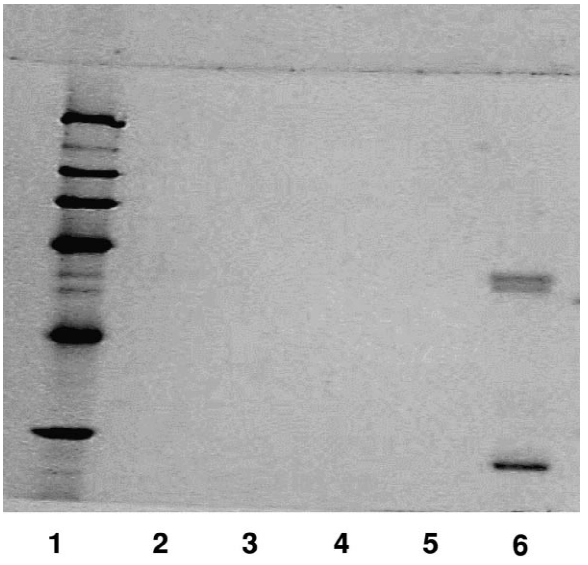

Fig. 12. SDS–PAGE analysis of chromatographic fractions collected during harvest and purification of IgG_{2b} from dilute, protein-free cell culture supernatant. Chromatography was performed as described in Section 2.7. Electrophoresis was performed as described in Fig. 4. Lanes: 1=markers; 2=feedstock; 3=load flowthrough (fraction 1); 4=load flowthrough (fraction 2); 5=wash, pH 8.0; 6=eluate, pH 4.0.

A column of MEP HyperCel may be evaluated to replace traditional ultrafiltration equipment as a device for antibody concentration.

4. Conclusions

Hydrophobic charge induction chromatography provided efficient isolation and purification of antibodies from protein-free cell culture supernatants and from the clarified/concentrated milk of transgenic goats. Product purities were ≥95% in a single chromatographic step, while recovery values ranged from ~83 to ~98%. A humanized IgG_1 isolated from transgenic milk was >95% pure, and was at least as pure as product isolated using affinity chromatography on protein A sorbent. Homogeneity of the HCIC-purified antibody exceeded that of affinity-purified product.

Viral clearance studies demonstrated a 4-log reduction of MVM virus, along with substantial reduction of DNA content. The sorbent was shown to be effective in capture of antibody from a large volume (~500 CVs) of highly dilute feedstock, supporting the concept that a column of MEP HyperCel may serve as an effective *device* for concentration of antibody from dilute feedstocks. This approach may be a cost-effective alternative to ultrafiltration in schemes where feedstock is concentrated by ultrafiltration preliminary to chromatography.

Studies were conducted to characterize and mitigate the influence of synthetic iron carrier present in protein-free CCS. Augmenting such feedstocks with 10 m*M* tetrasodium EDTA appeared to limit association of the sorbent with iron carrier, providing enhanced chromatographic performance in appropriate applications. Evaluation of this technique is recommended in cases where interaction with synthetic iron carrier is suspected.

In both current work and earlier studies [5], it was seen that typical feedstocks may be applied to the column without preliminary adjustment of pH or ionic strength and without addition of salts or glycine to promote binding. Elution was achieved under conditions significantly milder (e.g., pH 4) than those typically employed during affinity chromatography. Equally important, the sorbent has been shown to be chemically stable to repeated cleaning with 1 *M* sodium hydroxide [5]. Moreover, the cost of the sorbent is approximately 25% that of typical protein A media. Additional economies may be anticipated in connection with the use of dilute buffer (e.g., 50 m*M*) during elution of antibody. This practice eliminates the need for extensive diafiltration or dilution in advance of ion-exchange chromatography — often employed as the second chromatographic step in schemes for antibody purification. Finally, ongoing studies suggest that binding of IgG is independent of subclass or species. Binding of IgA, IgE, IgM and antibody fragments has also been demonstrated. Thus, the sorbent displays broad, practical specificity.

Taken together with the results of earlier studies [5], findings presented here demonstrate that HCIC on this antibody-selective sorbent represents a cost-effective alternative to affinity chromatography on protein A sorbents.

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