The Profinia™ Protein Purification System Simplifies Antibody Purification With Protein A

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Introduction

Immobilized Protein A from *Staphylococcus aureus* has been used for many years to purify antibodies from a variety of species (Hjelm et al. 1972). The high selectivity and stability of protein A has made it a popular choice for the purification of antibodies from a wide range of sample sources, including serum, ascitic fluid, and hybridoma cell culture supernatants. Mammalian antibodies are categorized into five major classes: IgA, IgD, IgE, IgG, and IgM. IgG is the predominant class of antibody in serum and is generated in large amounts during the secondary immune response. The IgG class of antibody is further divided into subclasses that vary depending upon the species and the properties of the heavy chain component. There are four subclasses of IgG in humans (IgG_1, IgG_2, IgG_3, IgG_4) and in mice (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3). The affinity of protein A for IgG varies considerably between species and IgG subtypes and has been extensively characterized (Duhamel et al. 1979, Schwartz 1990). In humans, protein A binds with high affinity to IgG_1, IgG_2, and IgG_4, but poorly to IgG_3. Among the four IgG subtypes in mice, protein A has the weakest affinity for IgG_1. The binding of antibodies to protein A is mediated, at neutral or alkaline pH values, through hydrophobic interactions involving a highly conserved histidyl residue located in the protein A binding site of IgG. The elution of IgG from immobilized protein A is commonly achieved by lowering the pH using an acidic buffer. Protein A-purified antibodies are then typically neutralized with a base, dialyzed against a neutral buffer, or desalted using a gel-filtration column to avoid acid-mediated hydrolysis and denaturation. The use of protein A affinity chromatography offers a number of advantages to alternative separation techniques based on ion exchange, immobilized metal affinity chromatography (IMAC), and hydrophobic interaction chromatography (HIC), due to its simplicity and high recoveries of the purified product.

The Profinia protein purification system is an automated chromatography instrument designed for the purification of affinity-tagged proteins and antibodies. An intuitive touch screen interface allows users to access preprogrammed chromatography methods or to customize any method for a particular application. With the Profinia system, antibodies can be purified and immediately desalted, precluding the need for neutralization, dialysis, or additional chromatography steps. The system is specifically designed for ease of use and for routine purification of 1–100 mg of IgG, using either 1 ml or 5 ml prepacked protein A cartridges. In this study, the purification, yield, and purity of IgG recovered from human, rabbit, mouse, rat, and goat sera using protein A-based separations are described. In addition, the effect of varying the elution buffer composition on the elution of IgG from protein A cartridges was investigated. Lastly, the purification of a monoclonal antibody from a hybridoma cell culture supernatant using the Profinia system and its subsequent functional analysis by an ELISA is presented.

Methods

Sample Preparation

Pooled human serum was obtained from Bioreclamation, Inc. Normal sera from rabbit, rat, goat, and mouse were obtained from Millipore Corporation. All serum types were stored as aliquots at –20°C. Prior to chromatographic separations, serum samples were thawed, diluted 1:5 (v/v) with phosphate-buffered saline (PBS), pH 7.4, and filtered through a 0.2 µm filter.

Antibody Purification From Serum

Antibody purifications were carried out on the Profinia protein purification system (Bio-Rad Laboratories, Inc.), using 1 ml Bio-Scale™ Mini Affi-Prep® protein A cartridges (Bio-Rad). For protein A desalting separations, a 10 ml Bio-Scale™ Mini Bio Gel® P-6 desalting cartridge (Bio-Rad) was used in tandem with the protein A cartridge. All separations were performed by selecting the Profinia Protein A and G method and carried out at room temperature. The protein A cartridge binding and wash buffer for all purifications was PBS, pH 7.4.

Sample loading, column washing, and elution steps were carried out at 1 ml/min. Five ml of sample was loaded onto the protein A cartridge, and unbound proteins were collected as a single flow-through fraction equal to the volume of the sample. The cartridges were then washed with 12 column volumes (CVs) of PBS and the wash fraction was collected. Elution buffer was pumped through the Bio-Scale Mini Affi-Prep protein A cartridge until the IgG peak was detected. The detection and collection of the elution peak as a single fraction is carried out using the Profinia automatic peak detection algorithm. (The pH was adjusted to 3.0 for sodium citrate and glycine elution buffers in all separations.) For the Profinia Protein A and G method, the purified antibody was collected in 4 ml of elution buffer, and a 75 µl aliquot was subsequently neutralized with 25 µl of 1 M Tris, pH 8.0 for SDS-PAGE. For the Profinia Protein A and G plus desalting method, 3.0 ml of the purified antibody in elution buffer was automatically diverted to the desalting cartridge, exchanged into PBS, and collected as a single 4 ml fraction. The yield of IgG was determined by measuring the absorbance of the elution fraction at 280 nm.

Monoclonal Antibody Purification

The monoclonal antibody (mAb) CCRC-M96 (IgG_3) was generated as described previously (Puhlmann et al. 1994) using tomato xyloglucan (XG) covalently coupled to BSA as the immunogen. CCRC-M96 was purified from 20 ml of hybridoma cell culture supernatant using the Profinia Protein A and G plus desalting method. A control experiment was conducted under the same experimental conditions with 5 ml of sterile, uninoculated RPMI1640 hybridoma cell culture medium. Samples stored at –20°C were thawed and filtered through a 0.2 µm filter prior to chromatography. Chromatography parameters were identical to those for serum sample purification using sodium citrate elution buffer as described above. The protein concentration of column fractions was measured using the Quick Start™ Bradford protein assay (Bio-Rad) with BSA (Fraction V, Fisher Scientific) in water as a standard.

ELISA

Protein A column fractions were analyzed for the presence of CCRC-M96 using an ELISA. CCRC-M96 has been demonstrated to bind with high avidity to tomato XG (unpublished results). Incubations were carried out in 96-well Costar 3598 microtiter plates (Corning Incorporated) at room temperature, unless otherwise specified. The suspension, dilution, and washing buffer was 0.1% (w/v) nonfat milk powder in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5 (TBS), unless otherwise specified. All volumes were 50 µl unless otherwise specified.

Plate wells were coated with tomato XG by evaporating 10 µg/ml of XG in ddH_2O to dryness at 37°C overnight. Nonspecific binding sites were blocked by incubating wells with 200 µl of 1% (w/v) nonfat milk powder in TBS for 1 hr. A dilution series (14 steps, 2-fold dilution factor) of each of the column fractions (load, flowthrough, wash and elution) was set up in coated and blocked wells. After a 1 hr incubation, the wells were washed (3 x 300 µl), and a 1:5000 (v/v) dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich, Inc.) was added to the wells. After a 1 hr incubation, the wells were washed (5 x 300 µl), and 3,3',5,5'-tetramethylbenzidine substrate in ddH_2O (TMB substrate kit, Vector Laboratories) was added according to the manufacturer's instructions. Color was allowed to develop in the dark until the desired intensity was achieved (~15 min). The reaction was then stopped by adding 50 µl of 0.5 M sulfuric acid in ddH_2O. The absorbance was immediately measured spectrophotometrically (absorbance at 450 nm, subtracting the absorbance at 595 nm), using a Bio-Rad Model 680 microplate reader. All measurements were done in triplicate. Control wells without XG coating, hybridoma supernatant, secondary antibody, or substrate were assayed in parallel with the column fractions.

SDS-PAGE

For serum samples, the protein A cartridge flow-through, wash, and elution fractions were diluted 1:10 with Laemmli gel loading buffer under reducing conditions and separated by SDS-PAGE using Criterion™ Tris-HCl gels 4–20% linear gradient (Bio-Rad). Gels were stained with Bio-Safe™ Coomassie stain (Bio-Rad). Approximately 1 µg of the purified antibody was loaded onto the gel for comparative analysis. For CCRC-M96 purification, column fractions were analyzed by SDS-PAGE (Laemmli 1970) using a Ready Gel® Tris-HCl gel 4–15% linear gradient (Bio-Rad). Loaded volumes of all fractions were proportional to the relative fraction volumes. The gel was silver stained (Shevchenko et al. 1996) and imaged with the Molecular Imager® Gel Doc™ XR imaging system (Bio-Rad). Precision Plus Protein™ standards were used for all molecular weight determinations.

Experion Analysis

The Experion™ automated electrophoresis system (Bio-Rad) was used to determine the purity of all IgG elution fractions recovered from serum. Purified IgGs were prepared for analysis under reducing conditions using the Experion Pro260 kit according to the protocol detailed in the Experion instruction manual.

Results and Discussion

Purification of IgG From Human Serum

A major advantage of antibody purification using protein A chromatography is its simplicity. Antibodies are bound to immobilized protein A, washed, and subsequently eluted with an acidic buffer. However, prolonged contact with acidic buffers can be detrimental to the integrity of antibodies, and an additional neutralization or chromatography step is often required. The Profinia purification system is particularly well suited to antibody purification since the purified antibody in acidic elution buffer can be immediately exchanged into a defined buffer of choice, eliminating the need for

neutralization, dialysis, or further chromatography steps. In addition, the Profinia system utilizes peak detection algorithms to automatically separate the starting sample into the column flow-through, wash, and purified fractions.

A representative purification of antibodies from 1 ml of human serum using the Profinia Protein A and G plus desalting method is shown in Figure 1A. The sample was applied to the protein A cartridge using PBS for both the binding and wash steps. Bound antibodies were then eluted with 0.1 M sodium citrate, pH 3.0 and exchanged into 4 ml of PBS by diverting the purified sample to the desalting cartridge. The separation resulted in the recovery of 8 mg of purified IgG in 48 min. A sample of each column fraction was analyzed under reducing conditions by SDS-PAGE. Under these conditions, the heavy and light chain components of IgG migrate at approximately 50 kD and 25 kD, respectively.

Comparison of Glycine and Citrate Elution Buffers

Citrate and glycine buffers are commonly used for the elution of antibodies bound to immobilized protein A. To compare the chromatographic properties of each elution buffer, two protein A affinity separations were carried out, differing only with respect to the buffer used to elute the bound antibodies. Figure 1B shows an overlay for the purification of IgG from human serum using either sodium citrate or glycine for the elution buffer. Each separation resulted in a recovery of approximately 8 mg of IgG with no significant difference in purity (Figure 1B, right panel). However, an increase in retention time for the elution peak was observed for the glycine elution buffer compared to that for citrate. In addition, the antibody eluted in approximately 4 CVs (4 ml) using the glycine elution buffer compared to 3 CVs with the citrate buffer. As a result, glycine at this concentration and pH would be unsuitable for the Profinia Protein A and G plus desalting method since the maximum volume that can be diverted to the 10 ml desalting column is 3.0 ml. Thus, a 0.1 M glycine elution buffer at pH 3.0 would result in a significant decrease in antibody recovery using integrated desalting. However, if antibody purification using glycine is desired, increasing the concentration of glycine in the elution buffer results in a narrower elution peak. Figure 1C shows the purification of IgG from the same serum sample using increasing concentrations of glycine (0.10 M, 0.25 M, and 0.50 M) at pH 3.0. No significant effect on the purity or yield of the recovered antibody was observed with an increased concentration of glycine (Figure 1C, right panel). A glycine concentration of 0.25 M resulted in an elution peak narrow enough (3 ml) to permit complete buffer exchange with no significant loss in antibody recovery. Figure 1D shows the result of increasing the concentration of the glycine elution buffer to 0.25 M for the Profinia Protein A and G plus desalting method for the purification of IgG from human serum. The yield of IgG recovered using the method was similar for both the citrate (8.1 mg) and glycine (8.5 mg) separations (Figure 1D, right panel).

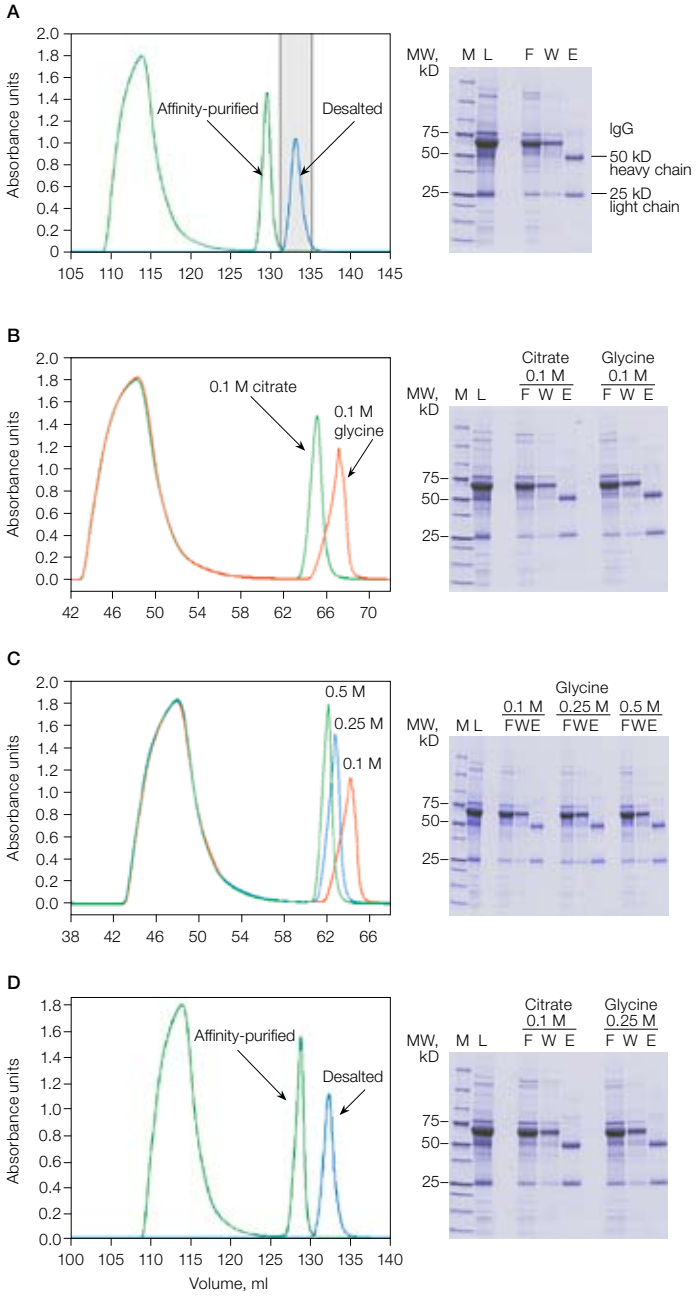

Fig. 1. Purification of IgG from human serum using the Profinia protein purification system. Left panels represent chromatograms from purifications of IgG using 1 ml of human serum. The Profinia system monitors the absorbance at 280 nm from the affinity and desalting cartridges. **A**, protein A affinity purification (–) and desalting (–) profiles using 0.1 M citrate elution buffer; **B**, comparison of protein A elution profiles using 0.1 M glycine, pH 3.0 (–) and 0.1 M citrate, pH 3.0 (–) elution buffers; **C**, protein A purification profiles using three concentrations of glycine, pH 3.0 (0.1 M (–); 0.25 M (–); 0.5 M (–)) for elution; **D**, protein A affinity purification (–) and desalting (–) profiles using 0.25 M glycine elution buffer. Panels on the right display images from SDS-PAGE analyses of protein fractions from each experiment. M, Precision Plus Protein standards; L, load; F, flowthrough; W, wash; E, elution. The gel in 1D includes IgG purified using 0.1 M citrate as the elution buffer for comparison.

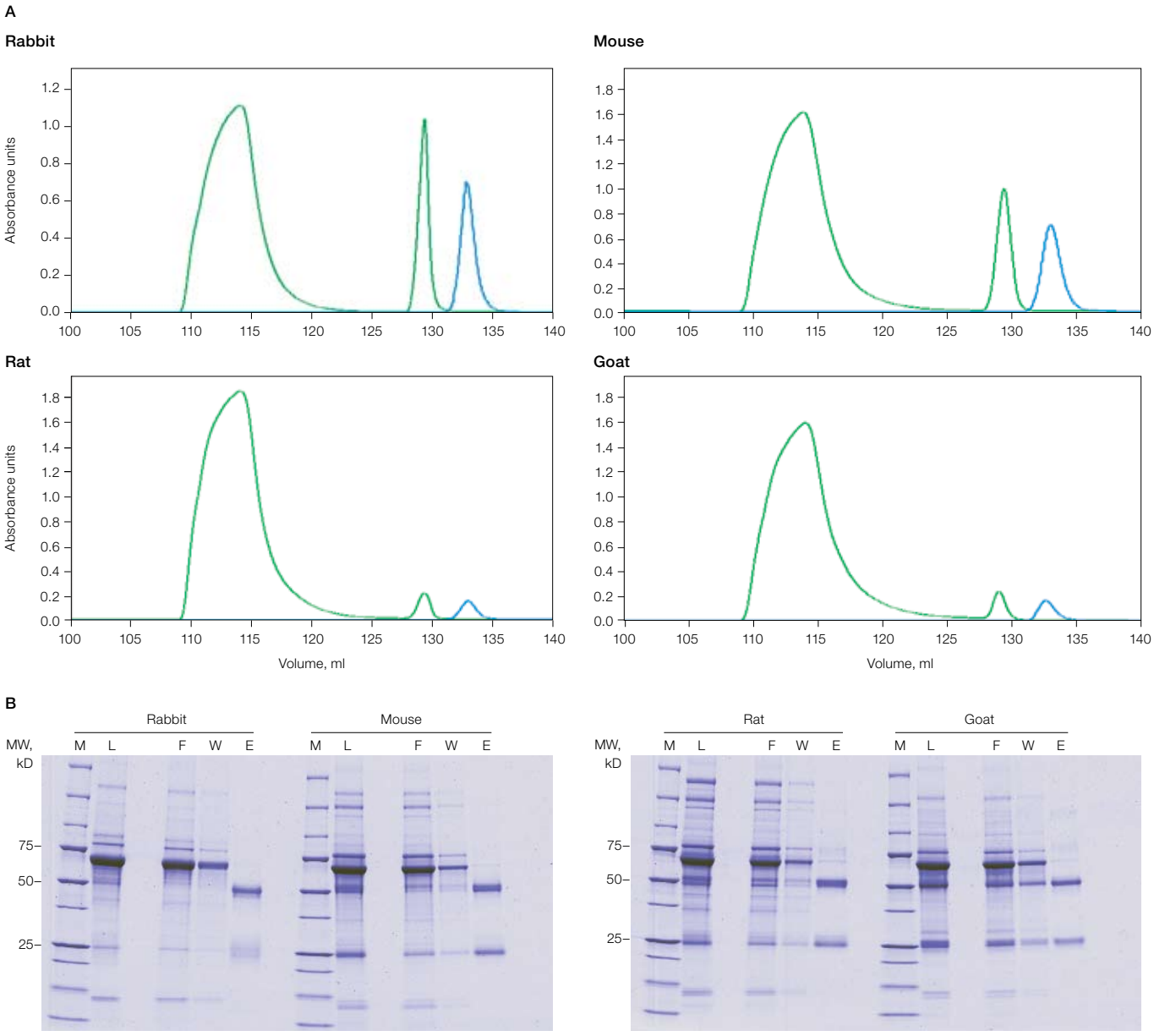

Fig. 2. Purification of IgGs from different species using the Profinia protein purification system. A, chromatograms of protein A affinity purification (–) and desalting (–); **B**, SDS-PAGE of protein A column fractions under reducing conditions. M, Precision Plus Protein standards; L, load; F, flowthrough; W, wash; E, elution.

Purification of IgG From Various Serum Types

Subclasses of IgG from various species are known to bind to immobilized protein A with a wide range of affinities (Hober et al. 2007). To illustrate the differential binding of IgG to protein A, equal volumes of serum (1 ml) from rabbit, goat, mouse, and rat were purified under identical chromatography conditions (Figure 2A). Separations for each serum type were carried out in triplicate, resulting in an average IgG yield of 4.5 mg for rabbit, 5.8 mg for mouse, and 1.1 mg for both rat and goat. These results are consistent with the relative binding properties of different IgG subclasses from various species to immobilized protein A. Column fractions from representative separations were analyzed by SDS-PAGE under reducing conditions and shown in Figure 2B. The Experion automated electrophoresis system was used to analyze the purity of the protein A-purified IgGs from each serum type. The Experion system combines electrophoresis, detection, analysis, and digital documentation of protein samples. The Experion software automatically generates an electropherogram and a table reporting the purity of the protein (Figure 3). The yield and purity of IgGs from all protein A desalting separations is summarized in Figure 4. The purity of recovered IgG was consistently 90% or greater, regardless of the source of serum or amount of IgG recovered.

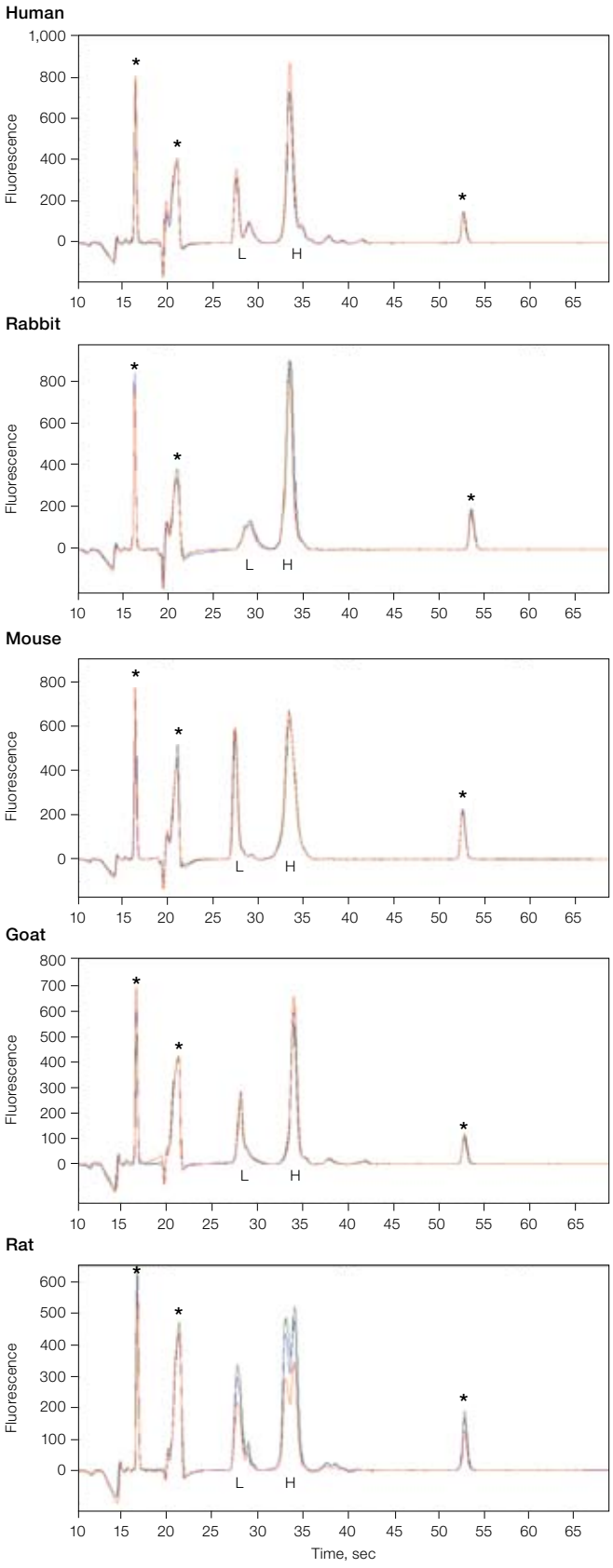

Fig. 3. Purity analysis of purified IgG samples. Experion electropherograms of protein A elution fractions for human, rabbit, mouse, goat, and rat. Each electropherogram is an overlay from three independent purifications. *, Experion alignment peaks; H, heavy chain; L, light chain.

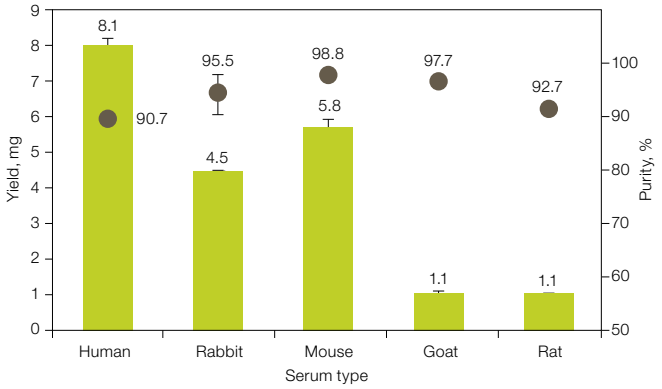

Fig. 4. Yield and purity of IgG samples. Average yield (▪) and purity (●) of IgGs from triplicate purification runs from 1 ml of serum.

Purification of CCRC-M96

The specificity of mAbs has made them an invaluable research and diagnostic tool. In addition, mAbs can be perpetuated indefinitely from hybridomas and are routinely isolated from cell culture supernatants using immobilized protein A. Figure 5A shows an example of the purification of 0.24 mg of mAb CCRC-M96 from 20 ml of hybridoma cell culture supernatant using the Profinia Protein A and G plus desalting method. A comparable chromatogram, except for the notable exclusion of the elution peak, was obtained in the control experiment carried out with uninoculated hybridoma culture medium (data not shown). The crude hybridoma culture supernatant and the various fractions eluting from the column were analyzed by SDS-PAGE with silver staining (Figure 5B). Quantitation of proteins in the various fractions demonstrated high and comparable total protein amounts for the flow-through fractions from both hybridoma supernatant and hybridoma culture medium, and residual protein amounts for both wash fractions (data not shown). A low but measurable protein amount was obtained from the hybridoma supernatant elution fraction, whereas no measurable protein amounts were obtained from the hybridoma culture medium supernatant (data not shown).

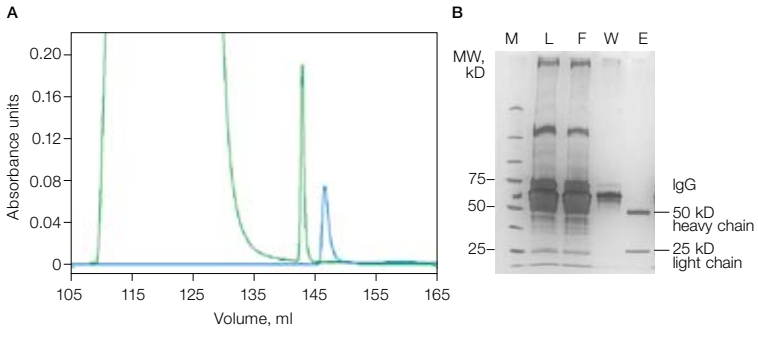

Fig. 5. Purification of mAb CCRC-M96. A, chromatogram showing protein A affinity purification (–) and desalting (–) profiles of mAb CCRC-M96 from 20 ml of hybridoma cell culture supernatant; **B**, SDS-PAGE analysis of each column fraction under reducing conditions. M, Precision Plus Protein standards; L, load; F, flowthrough; W, wash; E, elution.

The various column fractions were then evaluated for the presence of CCRC-M96 binding activity using an ELISA (Figure 6). Significant and identical binding activity of CCRC-M96 to tomato XG was observed for the load and elution fractions. In contrast, at least 1,000-fold lower binding was detected in the wash and flow-through fractions. The data in Figure 6 support the notion that the integrity and specificity of the CCRC-M96 antibody was not compromised by the chromatographic conditions used for the Profinia Protein A and G plus desalting method. No CCRC-M96 binding was observed in the uninoculated hybridoma culture medium fractions (data not shown), as expected. In summary, the Profinia Protein A and G plus desalting method resulted in the highly efficient purification of fully active and electrophoretically homogeneous CCRC-M96 from the complex protein mixture present in the initial hybridoma cell culture supernatant.

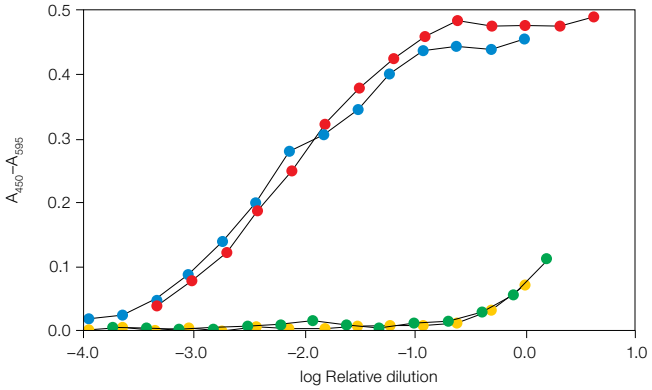

Fig. 6. Binding activity of purified CCRC-M96. ELISA of protein A column fractions assayed for tomato XG. Relative dilutions were calculated by normalizing fraction volumes to the volume loaded (20 ml). (●), load; (●) elution; (●), wash; (●), flowthrough.

Conclusions

Our results show that the Profinia protein purification system is ideal for the efficient purification of antibodies using protein A. A significant advantage of the Profinia system is the integrated tandem column configuration allowing for the purified antibody to be exchanged from an acidic elution buffer to a defined neutral buffer of choice. The preprogrammed methods are fully automated and require much less hands-on time than traditional gravity-flow or syringe-based methods.

In addition to purification of IgG from serum, the Profinia system is particularly well suited to the purification of monoclonal antibodies from the supernatants of hybridoma cell cultures using immobilized protein A. The large sample volumes of hybridoma cell culture supernatants can easily be accommodated by the Profinia system, and integrated peak detection automatically fractionates and collects the starting sample into the flow-through, wash, and purified samples. To accommodate antibodies from samples that bind poorly to protein A, the Profinia system is compatible with prepacked chromatography cartridges (such as protein G) from other suppliers using a simple adaptor (luer to 10-32 adaptor fittings kit).

The Profinia system also provides an optional software package to monitor real-time chromatography parameters and generates publication-quality chromatograms and reports. Separations carried out on the Profinia system require a minimum amount of chromatography experience and allow researchers to focus more on the downstream applications of the purified product than with the details of the purification process itself.

Acknowledgments

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