Protein A Removal From IgG on CHT™ Ceramic Hydroxyapatite Support

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Introduction

Affinity chromatography with immobilized protein A from *Staphylococcus aureus* or from recombinant sources has been used for the purification of immunoglobulins since the 1970s (Gagnon 1996). Much of its popularity is due to the simplicity of the method itself, the high product purity and recovery, and protein A's selectivity to IgG class and species. Good clearance of DNA, virus, and endotoxin is often obtained, plus the immobilized protein A is relatively resistant to chemical regeneration and sanitization procedures.

Despite these advantages, conditioned media and cellular lysates frequently contain varying amounts of proteases, which can release fragments of protein A from immobilizing supports (Bloom et al. 1989). This is of great concern to the diagnostics and biopharmaceutical industries due to the wide range of immunomodulatory effects associated with protein A and its fragments (Gagnon 1996).

A number of chromatographic methods have been used to separate protein A from IgG. Cation exchange chromatography is attractive because conditions can be devised in which protein A does not bind to the support while IgG is retained. However, process development is often required and chaotropic agents may be needed to disrupt the protein A-IgG complex. Anion exchange chromatography has been used, but suffers from the disadvantage that the strength of the protein A-IgG association is enhanced by elevated pH. Since both IgG and protein A are retained, coelution of protein A fragments with IgG can occur (Bloom et al. 1989). Use of hydrophobic interaction chromatography for protein A-IgG separation has also been reported. Process development is again required since high salt concentrations increase the affinity of protein A for IgG. Coelution of protein A and IgG is common, reducing contaminant clearance and product yield.

Mariani et al. (1989) developed a simple method for the purification of IgG_1 in the presence of protein A on hydroxyapatite (Bio-Rad HPHT). They started with an ascites fluid ammonium sulfate fraction. In recent years, HPHT has been replaced by the more robust CHT ceramic hydroxyapatite, which was designed for process-scale separations. In this report, we show that CHT can resolve IgG_1 from an IgG_1-protein A complex in unfractionated conditioned media. We have also optimized conditions for the separation of protein A and an IgG_4 by cation exchange chromatography on UNOsphere™ S support.

Results and Discussion

Initial studies in our laboratory indicated that recombinant protein A and IgGs from various sources exhibited virtually identical retention times on CHT under several elution conditions. We noted, however, that when small amounts of recombinant protein A were mixed with mouse ascites fluid containing IgG_1, an additional peak of higher retention time than any other component in the system was generated. This finding inspired more detailed studies.

We obtained a conditioned medium containing about 0.24 mg/ml of mouse IgG_1. When this medium or recombinant protein A was applied directly to a CHT column at pH 7.0 and eluted with a sodium phosphate gradient, peaks with essentially identical retention times were obtained (Figure 1, pink and green traces). However, upon mixing increasing amounts of recombinant protein A with the IgG_1-containing medium, an additional peak was seen with a higher retention time than any other in the chromatogram. In concert, the IgG_1 peak appeared to diminish in intensity until, at the highest recombinant protein A concentration, most of the IgG_1 peak was depleted. This suggested that a stable complex between IgG_1 and protein A was being formed in these experiments and that this complex had a higher retention time than the other species present.

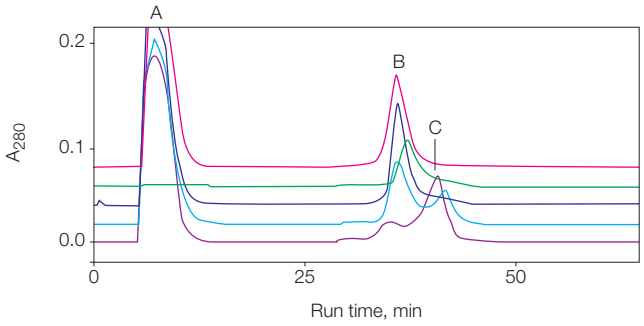

Fig. 1. CHT chromatography of conditioned medium containing murine IgG_1 and spiked with increasing amounts of recombinant protein A. Pink line, murine IgG_1, 6.0 mg; green, recombinant protein A, 4.2 mg; dark blue, 0.48 mg IgG_1 + 0.05 mg protein A; light blue, 0.48 mg IgG_1 + 0.1 mg protein A; purple, 0.48 mg IgG_1 + 0.2 mg protein A. Proteins were mixed in a total volume of 6.0 ml buffer A (5 mM sodium phosphate buffer, pH 7.0). The column was a 7 x 50 mm Bio-Scale™ column packed with CHT Type I, 20 µm (catalog #158-2000) equilibrated with buffer A. Elution was with a 20 column volume gradient to 200 mM sodium phosphate buffer, pH 7.0. at a flow rate of 1.5 ml/min. Detection was by absorption at 280 nm on a BioLogic™ HR system. The fractions at the apex of peaks A, B, and C were analyzed by SDS-PAGE (Figure 2). Scale offset for comparison.

Mariani et al. (1989) did not attempt to demonstrate that the additional peak they had observed on HPHT upon mixing partially purified IgG_1 and protein A was actually a complex of the two proteins. To show this, we picked a ratio of IgG_1 to recombinant protein A of about 0.48 to 0.15 mg, which we had calculated would produce IgG_1 and IgG_1-protein A complex peaks of approximately equal intensity on elution from the CHT column. The peaks were well resolved and of nearly equal height.

Fractions were collected and fractions from each of the three main peaks (A, B, and C) were examined with SDS-PAGE under reducing and nonreducing conditions. The gel was stained with an experimental fluorescent stain that is about 50 times more sensitive than Coomassie Blue (Figure 2). Lanes 2 and 3 of this gel show a commercially purified recombinant protein A standard, which appears to contain numerous trace impurities or degradation products. Lanes 4 and 5 contain the conditioned medium and show that IgG_1 is the main component. The unbound peak A (lanes 6 and 7) appears to contain little or no protein and is probably indicator dye and other medium additives. Lanes 8 and 9 are from peak B and show this fraction to be the purified IgG_1 as expected. However, lanes 10 and 11 are from peak C, the putative IgG_1-protein A complex, which show two bands in addition to the IgG_1 components. One of these bands comigrates with the main protein A band. The other band has a slightly higher mobility and is probably a proteolytic fragment of the recombinant protein A.

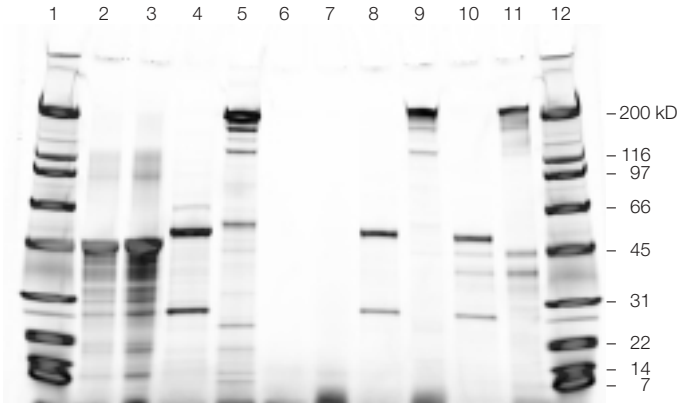

Fig. 2. SDS-PAGE analysis of CHT column fractions. Lanes 1 and 12, broad range molecular weight standards (catalog # 161-0317); lanes 2 and 3, protein A standard, reduced and unreduced; lanes 4 and 5, IgG_1 in conditioned medium, reduced and unreduced; lanes 6 and 7, peak A, reduced and unreduced; lanes 8 and 9, peak B, reduced and unreduced; lanes 10 and 11, peak C, reduced and unreduced. The gel was a 4–20% Ready Gel® precast gel.

In biopharmaceutical production processes, additional polishing steps are often included to ensure a pure product. We evaluated cation exchange chromatography on UNOsphere S as a potential additional polishing step.

Protein A has an isoelectric point of 5.1, and an examination of its calculated titration curve revealed that below pH 5.1, the negative charge on protein A increases rapidly, while above this pH, the change is much more gradual. Thus, protein A and a human IgG_4 both bind to UNOsphere S below pH 5.1. At pH 6.0, which does not favor interaction between protein A and IgG_4, the two proteins could be separated (Figure 3). The proteins were run individually and mixed at various ratios, but retention times and peak shape did not vary significantly. This suggests that little or no protein A-IgG_4 complex was formed under these conditions (unpublished data).

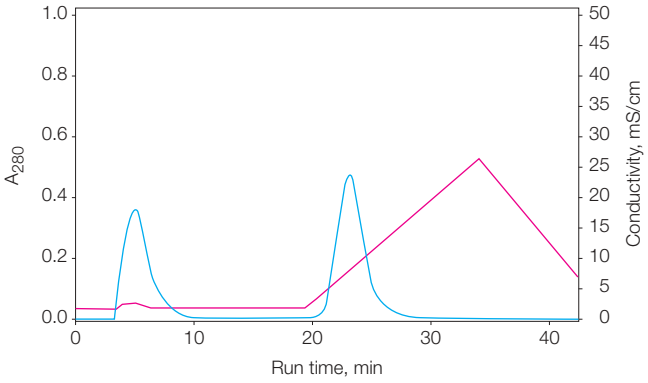

Fig. 3. Separation of recombinant protein A and human IgG_4 on UNOsphere S support. The protein A and IgG_4 (30 and 4.8 mg, respectively) were mixed in a total volume of 6.0 ml of buffer containing 50 mM MES, 1.0 mM NaH_2PO_4, pH 6.0. Proteins were applied to a 7 x 50 mm Bio-Scale column containing UNOsphere S (catalog # 156-0111). Elution was by a 20 column volume gradient from 0 to 0.5 M NaCl. Flow rate was 2.0 ml/min. Light blue line, A_{280}; pink line, conductivity. A BioLogic HR chromatography system was used.

Recovery of IgG from the protein A spiking experiment on CHT (see table) was about 64%. Almost 30% of the applied IgG was recovered in the protein A-IgG complex. About 85% of the IgG and 98% of the protein A applied to the UNOsphere S column was recovered.

We have shown that both supports offer high recovery and are effective in removing protein A from IgG from conditioned medium. These and other studies (Bio-Rad bulletins 2774 and 2780) also show high purity of the IgG recovered from a complex mixture using CHT and UNOsphere S. CHT and UNOsphere S chromatography are effective for protein A removal and as polishing steps in biopharmaceutical purification processes.

Table. Protein recovery from CHT and UNOsphere S columns.

	Protein Recovery (%)*		
Support	**IgG**	**IgG-Protein A Complex**	**Protein A**
CHT**	63.8	28.9‡	ND
UNOsphere S†	84.8	ND	98.6

* Recovery calculated by A_{280} of all pooled fractions.

** The CHT column load was conditioned culture medium containing 0.48 mg IgG_1 and 0.1 mg recombinant protein A. Conditions were as in Figure 1.

† The UNOsphere S column load was 5.0 mg purified human IgG_4 and 30 mg recombinant protein A. Conditions were as in Figure 3.

‡ Expressed as %IgG_1 recovered. This value was reduced by 10% to account for the protein A contribution to absorbance (the extinction coefficient of protein A is 10% that of IgG).

References

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