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Journal of Chromatography A, 1163 (2007) 105–111
JOURNAL OF CHROMATOGRAPHY A
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Fragments of protein A eluted during protein A affinity chromatography

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Received 28 March 2007; received in revised form 6 June 2007; accepted 7 June 2007
Available online 12 June 2007

Abstract

Protein A affinity chromatography is a common method for process scale purification of monoclonal antibodies. During protein A affinity chromatography, protein A ligand co-elutes with the antibody (commonly called leaching), which is a potential disadvantage since the leached protein A may need to be cleared for pharmaceutical antibodies. To determine the mechanism of protein A leaching and characterize the leached protein A, we fluorescently labeled the protein A ligand in situ on protein A affinity chromatography media. We found that intact protein A leaches when loading either purified antibody or unpurified antibody in harvested cell culture fluid (HCCF), and that additionally fragments of protein A leach when loading HCCF. The leaching of protein A fragments can be reduced by EDTA, suggesting that proteinases contribute to the generation of protein A fragments. We found that protein A fragments larger than about 6000 Da can be measured by enzyme linked immunosorbent assay, and that they can be more difficult to clear than whole protein A by cation-exchange chromatography.

Keywords: Antibody; Affinity chromatography; Leached protein A; Fluorescence detection

1. Introduction

Recombinant monoclonal antibodies are an important class of biological pharmaceuticals that are used to treat a wide variety of indications including infectious disease, cardiovascular disease, cancer, and autoimmune disease [1–5]. Protein A affinity chromatography is commonly used to purify recombinant monoclonal antibodies [6–8]. Protein A is a protein of 42 000 molecular weight with five homologous antibody binding regions and a C-terminal region for cell wall attachment [6,9–11]. The sequence and structure of protein A has been well established [12–14]. When immobilized on a column, protein A provides a method for purifying antibodies.

Protein A affinity chromatography has many benefits for process scale purification of therapeutic antibodies, including its ability to specifically bind antibodies at high capacity and achieve high purity [15–18]. However, a drawback to protein A affinity chromatography is that protein A ligand from the column often co-elutes with the antibody in the column eluate [19,20], an effect which is often referred to as protein A leaching. There is some evidence that protein A may cause adverse physiological events [21], and in many cases there may be a regulatory expectation that leached protein A should be cleared during purification of antibodies for human use [22]. We have found that protein A is sometimes difficult to clear by downstream processes and a significant part of the development of antibody purification processes is designed around clearance of protein A [23]. Characterizing the mechanism of protein A leaching could lead to more efficient antibody purification processes.

The mechanism of protein A leaching is not well understood. To investigate the mechanisms of protein A leaching, we used fluorescently labeled Prosep vA and MabSelect affinity resins to visualize the leached protein A in column eluates when the labeled resin is exposed to purified IgG1 and harvested cell culture fluid (HCCF) solutions. The direct detection of leached protein A by fluorescent imaging demonstrates that one leaching mechanism is fragments of protein A co-eluting with antibody. We show that these fragments have significance to process chromatography practitioners by demonstrating that some protein A fragments are active in the protein A enzyme-linked immunosorbent assay (ELISA) while others are not, and that some fragments may be more difficult to clear than intact protein A.

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doi:10.1016/j.chroma.2007.06.012

2. Experimental

Wild type protein A and Prosep vA affinity chromatography media were obtained from Millipore (Billerica, MA, USA). The MabSelect protein A ligand, MabSelect affinity chromatography media, SP-Sepharose FF media, and PD-10 columns were from GE Healthcare (Uppsala, Sweden). The Alexa Fluor-488 TFP ester was obtained from Molecular Probes, Invitrogen. Thermolysin was obtained from Sigma–Aldrich.

Labeling of wild-type protein A and the MabSelect protein A ligand with amine reactive Alexa Fluor-488 label was performed according to manufacturer's protocols. Separation of protein A Alexa Fluor-488 conjugate from un-reacted label was performed using PD-10 gel filtration media. The label:protein molar ratio was determined using UV–vis spectroscopy at 280 nm for protein A and 495 nm for Alexa Fluor-488. A correction factor of 0.11 was used for the absorbance of Alexa Fluor-488 at 280 nm according to manufacturer's protocols. Labeling of the immobilized protein A on Prosep vA and MabSelect resins was performed using a 50% resin slurry in 0.2 M carbonate buffer pH 9.0. Following the labeling reaction, the excess un-reacted label was removed by sequential washing with buffer.

Chinese hamster ovary (CHO) cell culture fluid containing recombinant humanized monoclonal IgG1 was obtained from Genentech (South San Francisco, CA, USA). Cell culture fluid containing antibody at titers ranging from 0.7 to 1.2 g/L was processed by continuous centrifugation and depth filtration to remove cells and cellular debris, followed by sterile filtration before use in protein A affinity chromatography. We used HCCF from three different antibodies with high homology, denoted HCCF-1, HCCF-2, and HCCF-3. Purified monoclonal IgG1 was also obtained from Genentech; this antibody was diluted with phosphate-buffered saline (PBS) to 1 g/L, and is denoted PBS/IgG1. The purified antibody was from an intermediate pool, just before the final purification step.

The level of protein A in our samples was determined by a sandwich ELISA [24]. Chicken anti-protein A antibody was immobilized on microtiter wells; protein A binds to the coat antibody. The amount of bound protein A was detected with chicken anti-protein A labeled with biotin, followed by streptavidin-horseradish peroxidase and then the substrate *o*-phenylenediamine dihydrochloride and hydrogen peroxide. The reaction was stopped by adding sulfuric acid. The product was quantified by reading an absorbance at 490 nm. All samples were initially diluted to 0.2 mg/mL antibody product in assay diluent. Samples were then serially diluted two-fold with standard diluent, which contained 0.2 μg/mL antibody product. Samples were assayed as a dilution series to ensure that antibody excess was reached. Values were calculated as the average of all results within the reporting range (0.78–25 ng/mL).

For size-exclusion chromatography (SEC) of model protein A fragments, 800 μg of wild type protein A was incubated with 20 μg of thermolysin for 5 min. This mixture was injected onto a 16 mm × 60 mm Sephacryl-200 SEC column. The column was run at a flow rate of 1 mL/min in phosphate buffered saline. Peaks that eluted during size exclusion chromatography were collected and analyzed by spectrophotometry, protein A ELISA mass spectrometry, and sequencing. The concentration was determined by absorbance at 280 nm, assuming an extinction coefficient of $E^{1\%} = 1.65$.

For N-terminal sequencing of protein A peptides from thermolysin digestion, samples were exchanged into 0.2% acetic acid by dialysis. Aliquots containing approximately 600 pmol of sample were applied to Micro TFA filters (Applied Biosystems, Foster City, CA, USA) and subjected to 10 cycles of Edman degradation in a Procise 494 protein sequencer (Applied Biosystems). The PTH (phenylthiohydantoin) amino acid derivatives were analyzed on-line using the Applied Biosystems Model 140C gradient system and Model 785A detection system. Molar values for the PTH amino acids were calculated by peak height comparison to an external standard mixture containing 10 pmol of each PTH amino acid derivative.

For mass spectrometry analysis of protein A peptides, equal amounts (0.5 μL) of the peptide mixture and THAP matrix (prepared by dissolving 2 mg 2,4,6-trihydroxyacetophenone in 1 mL of CH3CN/10 mM dibasic ammonium citrate 1:3) were combined on a matrix-assisted laser desorption/ionization (MALDI) stainless-steel plate and dried under vacuum. Mass spectra were obtained using a Voyager Elite-DE MALDI time-of-flight (TOF) mass spectrometry (MS) instrument operated in the reflector and positive mode using delayed extraction. Other operating conditions included an accelerating voltage of 25 kV, grid 78%, guide wire 0.08%, and a delay of 250 ns. The Voyager Elite instrument was manually calibrated using a known standard mixture. This mixture contains peptides above and below the mass range of interest and provides up to 0.02% accuracy.

3. Results

The immobilized protein A ligand on Prosep vA and Mabselect resins was fluorescently labeled with Alexa Fluor-488 probe through conjugation with primary amines of lysine in protein A. We confirmed labeling of the immobilized protein A on Prosep vA and MabSelect by proteolytic digest of the labeled resins followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel fluorescence detection. Peptides of labeled protein A ranging in molecular weights from 40 to 6 kDa were released from the resins by proteolysis with thermolysin and were detected in the solution supernatants (Fig. 1). The free labeled protein A (lanes 2 and 6) both have two distinct bands, one of which is intact protein A monomer (42 kDa for Prosep and 34 kDa for Mabselect). Prosep protein A has a lower molecular weight band which may be a fragment, while MabSelect protein A has a higher molecular weight band which may be a dimer formed by the free cystine used for immobilization. The disulfide linked dimer is probably reduced during immobilization. The labeled peptides of protein A released from Prosep vA and MabSelect resins (lanes 4 and 8) have a molecular weight distribution similar to one another as well as to the digestion products from proteolysis of free labeled protein A (lanes 3 and 7). However, the digestion pattern for free labeled protein A (lane 3) contains a dominant band between ∼25 and 27 kDa that is not visible in the digests of the labeled Prosep vA and MabSelect resins or the free labeled MabSelect protein A

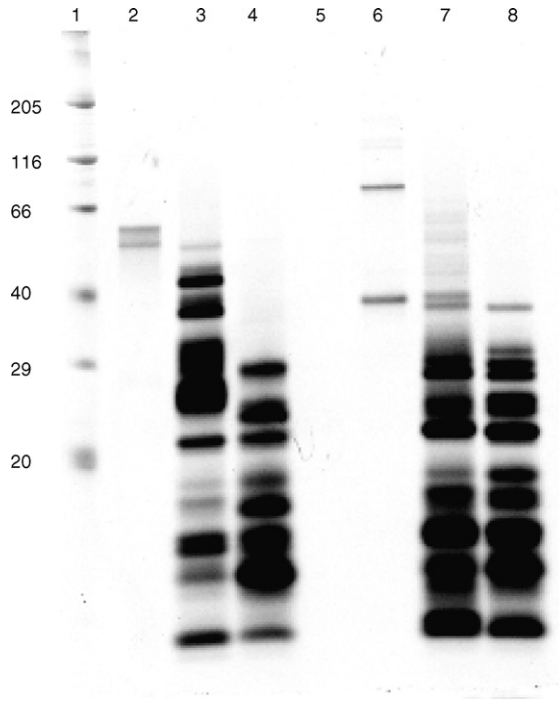

Fig. 1. Proteolytic digests of Alexa-Fluor-488 labeled Prosep vA and MabSelect resin. Labeled resins (0.5 mL) were incubated with 1 μg of Thermolysin protease for 3 h with mixing on a rotary wheel. The resin supernatant was isolated by centrifugation and analyzed by non-reduced SDS-PAGE coupled to in-gel fluorescence detection of Alexa Fluor-488 labeled protein A. Samples were prepared in Laemmli sample buffer followed by heating at 80 °C for 4 min. Samples were run on a 10% Bis/Tris gel in 1 × 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. Fixed gels were scanned on a Typhoon Trio Imager using 532 nm excitation with a 526 SP emission filter. Lanes: (1) FITC-labeled molecular weight marker; (2) wild type protein A-488; (3) wild type protein A-488 thermolysin digest; (4) Prosep vA-488 thermolysin digest; (6) MabSelect protein A-488; (7) MabSelect protein A-488 thermolysin digest; (8) MabSelect-488 resin thermolysin digest.

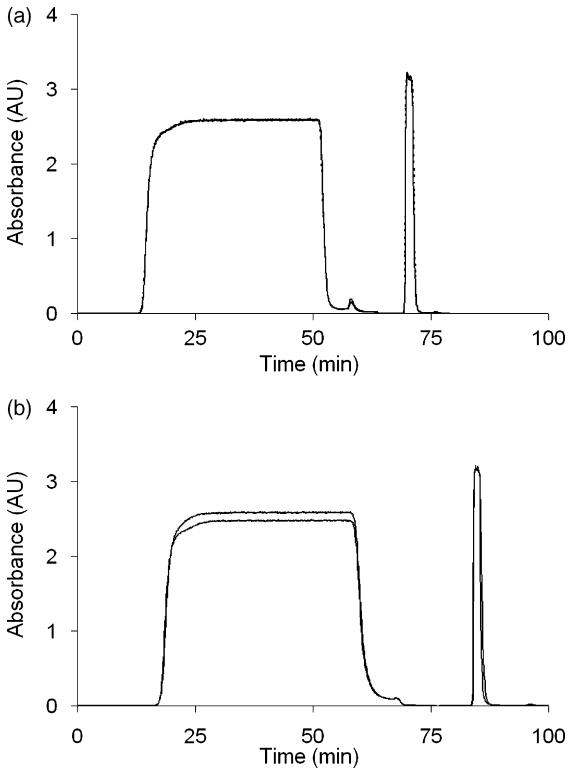

Fig. 2. Overlay of protein A chromatogram for the purification of HCCF on labeled (a) Prosep vA and (b) MabSelect resins with unlabeled resin. A 0.66 cm inner diameter × 14 cm length column was packed with either labeled or unlabeled Prosep vA chromatography media. Four buffers were used. Buffer A was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1; buffer B was 0.4 M potassium phosphate pH 7.0; buffer C was 0.1 M acetic acid, pH 2.9; and buffer D was 0.1 M phosphoric acid, pH 1.7. The columns were initially precycled using 3 column volumes each of buffer B and buffer C. Following column precycle, the columns were equilibrated with 5 column volumes of buffer A, loaded to 14 g/L, washed with 3 column volumes of buffer A, washed with three column volumes of buffer B, washed with 3 column volumes of buffer A, eluted with 3.5 column volumes of buffer C, and regenerated with 3 column volumes of buffer D. All chromatography phases were performed at 420 cm/h.

ligand. These results suggest that there are common sites of fluorophore conjugation on the immobilized protein A on Prosep vA and MabSelect as well as on the free protein A ligand. The digests of free and immobilized protein A from MabSelect (lanes 7 and 8) show greater similarity to each other than the digests of Prosep vA (lanes 3 and 4), possibly because protein A is immobilized to Prosep via a multipoint attachment, while MabSelect has a single point attachment. The release of intact protein A was not detected from the proteolytic digests of the labeled Prosep vA resin and these results are consistent with the multipoint attachment of the protein A ligand to the controlled pore glass (CPG) base matrix of Prosep vA. Almost intact protein A was released from the MabSelect resin by digest with thermolysin. The release of intact protein A indicates that there is a proteolytic site close to the single thioether linker used to immobilize protein A to the agarose base matrix.

We used the Alexa-Fluor-488 labeled Prosep vA and MabSelect resins to study the mechanism of protein A leaching during the purification of monoclonal antibodies. Chromatograms from typical bind-and-elute protein A affinity chromatography runs using the labeled Prosep vA and Mabselect resins overlay with chromatograms of unlabeled resins (Fig. 2). Purification runs show that the antibody elutes as a single peak with no observed differences in the elution peak shape or width for labeled resins relative to unlabeled resins. Furthermore, labeling of the immobilized protein A did not affect binding of the antibody at the load density used in this study, 14 g/L. The percent recovery of antibody loaded from purification runs of PBS/IgG1 and the HCCF-1 was greater than 94% from both the labeled and the unlabeled Prosep vA and MabSelect resins.

Direct detection of leached protein A in the column eluates from labeled Prosep vA and MabSelect resins was achieved using SDS-PAGE coupled to in-gel fluorescence imaging. The column eluate from both Prosep vA and MabSelect using PBS/IgG1 shows intact leached protein A and two fragments of protein A at about 24 and 28 kDa (Fig. 3a and b). In addition, eluate from Prosep vA has a distinct ladder of bands to about 35 kDa (Fig. 3a, lanes 6 and 8), which may be produced during immobilization. The molecular weight difference of the intact leached protein A from Prosep vA and MabSelect is expected since the engineered protein A ligand immobilized on MabSelect is approximately 8 kDa smaller than the 42 kDa wild type

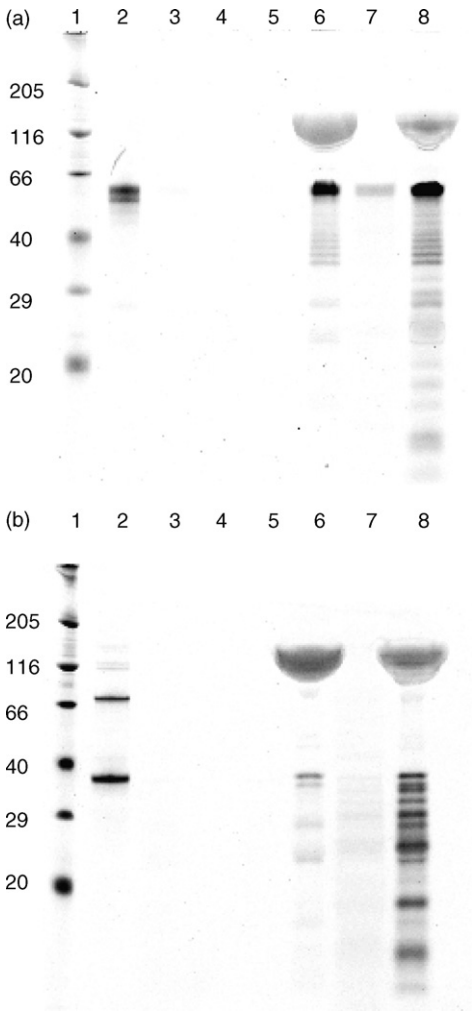

Fig. 3. In-gel fluorescence detection of leached protein A from labeled (a) Prosep vA and (b) MabSelect resins. Column effluents and eluates were prepared in Laemmli sample buffer followed by heating at 80 °C for 4 min. Non-reduced samples were run on a 10% Bis/Tris gel in 1× MES buffer. Fixed gels were scanned on a Typhoon Trio Imager using 532 nm excitation with a 526 SP emission filter. Lanes: (1) FITC-labeled molecular weight marker; (2) protein A-488 reference material; (3) PBS load effluent; (4) PBS column eluate; (5) PBS/IgG1 load effluent; (6) PBS/IgG1 column eluate; (7) HCCF load effluent; (8) HCCF column eluate.

protein A immobilized on Prosep vA. While both the labeled Prosep vA and MabSelect resins leach intact protein A, a direct comparison of the amount of leached protein A from each resin was not made since the percent of the immobilized protein A that is labeled is unknown. Protein A leaching was not detected in the column eluates from either the labeled Prosep vA or MabSelect resins with PBS (containing no antibody), which suggests that leaching of protein A into the column eluates requires binding of IgG1 to protein A during loading. The diffuse protein band present at 150 kDa in the column eluates is IgG1.

The purification of IgG1 from a complex mixture like HCCF leaches predominately fragments of protein A into the column eluates. The column eluate from Prosep vA contains labeled protein A fragments as well as intact protein A (Fig. 3a, lane 8). The leached protein A fragments range in molecular weight from 6 to 40 kDa, and contain unique low molecular weight fragments that are not detected in the column eluates from PBS/IgG1 experiments (lane 6). In addition, the signal intensities for intact protein A in the PBS/IgG1 and HCCF column eluates are similar, and suggest that there are few contributions from the HCCF to the leaching of intact protein A from Prosep vA.

Loading of HCCF over the labeled MabSelect resin leaches primarily protein A fragments into the column eluate (Fig. 3b, lane 8). The molecular weight distribution of the leached protein A fragments is similar to the distribution observed in the column eluates from Prosep vA (Fig. 3a, lane 8). The differences in the SDS-PAGE profiles for leached protein A fragments may reflect the use of different protein A ligands used in Prosep vA and MabSelect or the differing immobilization strategies. The amount of intact MabSelect ligand leaching is similar between the PBS/IgG1 and HCCF samples suggesting that there are few contributions from the HCCF to leaching of intact protein A from MabSelect.

Protein A leaching during chromatography is not limited to the antibody elution phase. SDS-PAGE followed by in-gel fluorescence detection of the HCCF load effluents from the labeled Prosep vA and MabSelect resins shows leaching of protein A also occurs during HCCF loading (Fig. 3a and b, lane 7). Intact protein A leaches from Prosep vA into the load effluent. In contrast, the load effluent from the MabSelect resin contains fragments of protein A.

Protein A leaching during chromatography was also evaluated using HCCF that contain different IgG1s. The three HCCF load materials, HCCF-1, -2, and -3, purified over the labeled Prosep vA similarly leached intact protein A and protein A fragments into the column eluate (Fig. 4a, lanes 3, 5 and 7). The column eluates from the labeled MabSelect resin also contain primarily leached protein A fragments (Fig. 4b, lanes 3, 5 and 7). A comparison of the column eluates for each HCCF load material shows distinct protein A leaching patterns, although there are fragments of protein A that are common to all three HCCF load materials. For example, the protein A fragments in the column eluate of HCCF-3 (Fig. 4b) from the MabSelect resin range in molecular weight from 6 to 35 kDa, similar to the leached protein A pattern of the column eluates of HCCF-1 and HCCF-2 (Fig. 4b). These results suggest that there may be sites on the protein A ligand that are particularly susceptible to fragmentation. However, in the column eluate of HCCF-3 (Fig. 4b, lane 7) there are unique fragments of protein A between 20 and 29 kDa that are not detected in the column eluates of HCCF-1 and HCCF-2 (Fig. 4b, lane 3 and 5). Furthermore, the column eluate of HCCF-3 contains fragments of protein A that have greater signal intensity when compared to the same molecular weight fragments detected in the column eluates of HCCF-1 and HCCF-2. These results would support that there are distinctive contributions from different HCCF to protein A leaching.

The leaching of protein A fragments into the HCCF load effluent and the column eluate during chromatography supports a mechanism for the degradation of the immobilized protein A ligand by components such as proteases in the HCCF. EDTA, a general inhibitor of metalloproteases and possibly an acceptable process additive, was added to HCCF-1, -2 and -3 load material prior to loading on labeled Prosep vA and MabSelect resins and

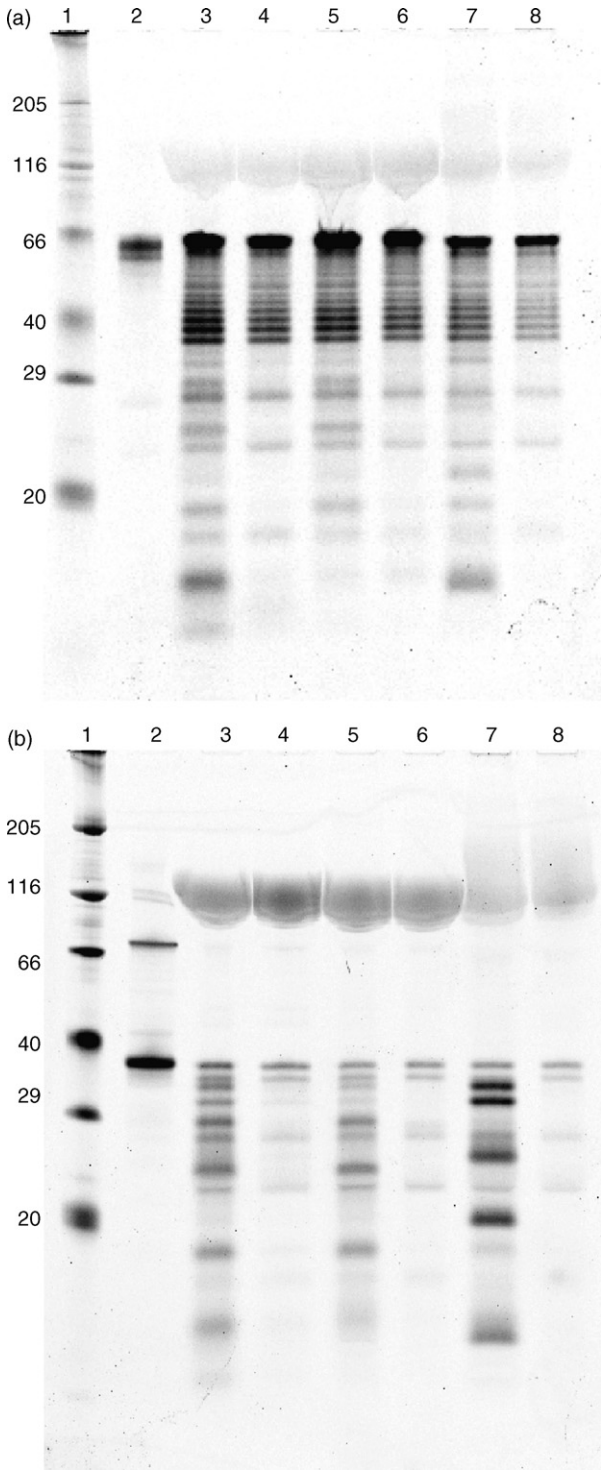

Fig. 4. In-gel fluorescence detection of leached protein A from HCCF-1, -2, and -3 from labeled (a) Prosep vA and (b) MabSelect resins. Protein A pools were prepared in Laemmli sample buffer followed by heating at 80 °C for 4 min. Non-reduced samples were run on a 10% Bis/Tris gel in 1× MES buffer. Fixed gels were scanned on a Typhoon Trio Imager using 532 nm excitation with a 526 SP emission filter. Lanes: (1) FITC-labeled molecular weight marker; (2) protein A-488 reference material; (3) HCCF-1 column eluate; (4) 5 mM EDTA added to HCCF-1, column eluate; (5) HCCF-2 column eluate; (6) 5 mM EDTA added to HCCF-2, column eluate; (7) HCCF-3 column eluate; (8) 5 mM EDTA added to HCCF-3, column eluate.

its effect on the leaching of protein A fragments was evaluated. The addition of 5 mM EDTA to HCCF-1, -2, and -3 decreases the levels of leached protein A fragments detected in the column eluates from Prosep vA (Fig. 4a, lanes 4, 6, and 8). Likewise, addition of 5 mM EDTA to HCCF-1, -2, and -3 also decreases leached protein A fragments in the column eluates from MabSelect (Fig. 4b, lanes 4, 6, and 8). In contrast, the addition of EDTA did not inhibit or decrease the leaching of intact protein A from Prosep vA or MabSelect resins. The EDTA inhibition of protein A fragment leaching supports a mechanism for the degradation of immobilized protein A by proteases in the HCCF.

The elution of intact protein A and protein A fragments along with IgG1 during protein A affinity chromatography may have significant implications for downstream process chromatography of pharmaceutical antibodies. In particular, subsequent chromatography steps may have difficulties in removing some fragments of leached protein A. To generate a model system of protein A fragments, we digested intact protein A with thermolysin. After digestion with thermolysin, we separated the thermolysin-protein A mixture by SEC. The chromatogram has five main peaks (Fig. 5). The peaks range in concentration from 30 to 48 ng/mL by A280, and from <0.04 to 36 ng/mL as determined by protein A ELISA. The two late eluting peaks (at about 105 min and 115 min) have less than detectable activity in the ELISA. The masses of the peaks range from 1040 to 15 660 Da. In all of the peaks, the mass spectrometer found more than one fragment mass, but we show only the mass of the most abundant fragment (Table 1). We could not get mass spectrometer or sequencing data for peak E (which elutes at about 115 min). Fragments larger than about 6 000 Da are active in the protein A ELISA, which suggests that the epitope is still intact in these fragments. The concentration of protein A fragments measured by spectrometry correlates well to the concentration measured by ELISA, which suggests that none of the thermolysin generated fragments had abnormally high activity in the protein A ELISA (Table 1).

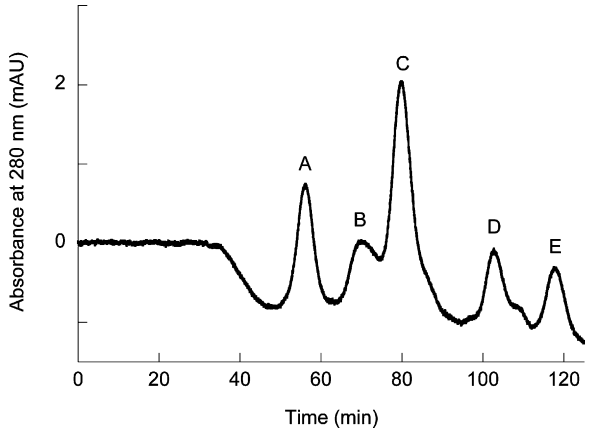

Fig. 5. SEC separation. 800 μg of wild type protein A was incubated with 20 μg of thermolysin for 5 min. This mixture was injected onto a 16 mm × 60 mm Sephacryl-200 SEC column. The column was run at a flow rate of 1 mL/min in phosphate buffered saline. Peaks that eluted during SEC were collected and analyzed by spectrophotometry, protein A ELISA mass spectrometry, and sequencing. Intact protein A (undigested) elutes at about 35 min.

Table 1
Quantitative results from five peaks in Fig. 5

Peak	Concentration byA280 (ng/mL)	Concentration by ELISA (ng/mL)	Mass (Da)	N-terminal sequence
A	42	36	15660	FNKEQQNAFY
B	40	30	7920	LNESQAPKAD
C	48	15	6590	LKDDPS(Q)ST N
D	30	<0.04	1040	FIQS LN(A)DQRN LAK VLNMPN
E	30	<0.04	–	–

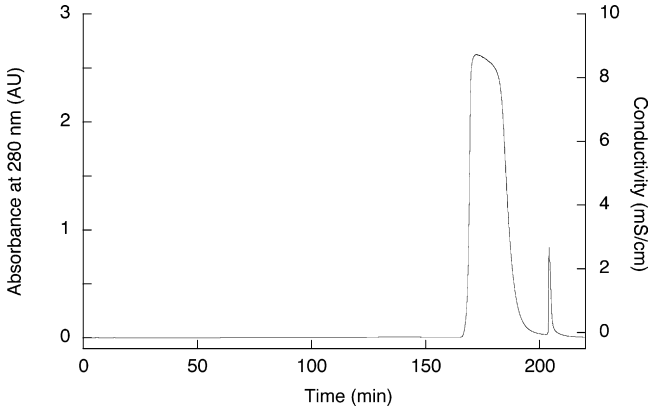

Fig. 6. Cation-exchange chromatogram. A 0.66 cm × 30 cm SP-Sepharose Fast Flow column run at 150 cm/h was equilibrated with 5 CVs of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, loaded to 40 g/L (g antibody per L column volume), washed with 5 CVs of 25 mM HEPES pH 8.0, eluted with 5 CVs of 25 mM HEPES, 30 mM NaOAc pH 8.0, regenerated with 5 CVs of 0.5 M NaOH, and stored in 5 CVs 0.1 M NaOH. The pool was collected from an absorbance of 0.5 to 0.5 OD during the elution phase.

To determine if protein A fragments are more difficult to clear by downstream processes, we spiked the 15 660 Da fragment into a protein A pool and loaded it onto a cation exchange column, using the optimized cation exchange conditions for one of our antibody processes (Fig. 6), similar to a previously described method [23]. We found that when the protein A fragment is spiked to a level of 130 ng/mg (ng fragment/mg antibody), it was reduced to 110 ng/mg in the cation exchange pool. In comparison, when we spiked 100 ng/mg of intact protein A into the same feedstock, protein A was <2 ng/mg in the cation exchange pool. This demonstrates that the 15 660 Da protein A fragment is more difficult to remove by cation exchange chromatography than intact protein A.

4. Discussion

In this study, we used fluorescently labeled Prosep vA and MabSelect affinity resins which allowed for the direct detection of leached protein A in column eluates by SDS-PAGE and fluorescence imaging. In our experiments using the labeled resins, we determined that the column eluates contain a complex mixture of intact protein A and protein A fragments. The leaching of intact protein A and fragments of protein A support two mechanisms for protein A leaching during chromatography.

We first propose that there is a distinct mechanism for leaching of intact protein A from Prosep vA and MabSelect resins. In our studies, chromatography runs performed using PBS/IgG1 leached intact protein A into the column eluate, indicating that leaching of intact protein A is not due to proteolysis.

In Prosep vA, the immobilized protein A ligand is attached to the CPG matrix by multiple covalent bonds through lysine side chains on protein A. The release of intact protein A from Prosep vA by proteolysis would require cleavage at each point of attachment to the CPG without proteolytic action on additional regions of protein A. Intact protein A was not released upon treatment of labeled Prosep vA with the protease thermolysin. The leaching of intact protein A from Prosep vA is likely from the IgG1 absorption of non-specifically bound or non-covalently bound protein A on the CPG, which then co-elutes with antibody during the elution phase. Alternatively, given that CPG has an increased solubility at basic pH, it is also possible that a small portion of the base matrix with the attached protein A ligand dissolves due to the pH cycling that occurs during chromatography. As a result, intact protein A is released and the protein A can be absorbed by IgG1 during chromatography.

Leaching of the intact protein A from MabSelect likely occurs because of physical or chemical breakdown of the agarose base matrix or through extraction of non-covalently immobilized protein A by IgG1 during loading. Similar to Prosep vA, intact protein A was not released from the MabSelect resin upon treatment with thermolysin.

In the PBS/IgG1 column eluates from both Prosep vA and MabSelect, we also detected a small amount of low molecular weight fragments. It is probable that these fragments are protein A related impurities not removed during the purification of protein A from *Staphylococcus aureus* (Prosep vA) or recombinant source (MabSelect). These fragments are likely immobilized during the manufacturing of the resins. Since these fragments co-elute with the antibody during the elution phase, the fragments probably have some affinity for IgG1.

The second mechanism for protein A leaching during chromatography is degradation of the immobilized protein A ligand by proteases in the HCCF. In our studies, chromatography runs performed with HCCF leached fragments of protein A, as well as intact protein A, into the column eluate. In addition, fragments of protein A also leached into the load effluents. Leaching of fragments into the load effluent is similar to what Balint et al. also report for the proteolytic cleavage of protein A from an extracorporeal immunoadsorbent matrix by proteases present in plasma and serum samples during therapeutic apheresis [25]. In their study, release of protein A peptides from the solid matrix into plasma or serum upon contact with the immunoadsorbent

matrix was inhibited by addition of a protease inhibitor cocktail to the plasma or serum samples.

It is probable that the protein A fragments released from the resin during HCCF loading bind to nearby IgG1 already bound to the resin or IgG1 in the HCCF load. The protein A fragments remain bound to IgG1 during the wash phases and are subsequently leached along with antibody into the column eluate during the elution phase. This suggests that the population of protein A fragments detected in the column eluates retains the ability to bind the IgG1 Fc-region or VH chain of the Fab domain. By contrast, protein A fragments that are detected in the load effluent may no longer bind or only weakly bind to IgG1.

Protein A fragment leaching is inhibited by the addition of EDTA to HCCF. The addition of EDTA to the HCCF likely inhibits a metalloprotease. The addition of EDTA to the three HCCF load materials tested decreased protein A fragment leaching in all cases. While each HCCF appears to contain proteases sensitive to the addition of EDTA, the distinct protein A leaching patterns in the column eluates suggest different proteases may be involved. It should also be considered that the different IgG1 in the HCCF may bind fragments of protein A with varying affinity.

Our initial work with cation-exchange chromatography and a model fragment of protein A suggests that clearance of protein A fragments in downstream processes may be problematic. The clearance mechanisms of protein A fragments require further study to understand what chromatography parameters can achieve the protein A clearance levels required for pharmaceutical antibodies.

In summary, we used fluorescently labeled protein A affinity resins which allowed for the detection of leached protein A in column eluates using fluorescence imaging. Protein A is leached during chromatography as intact protein A and fragments of protein A, which leads us to speculate that there are two contributing mechanisms to the leaching of protein A. Our preliminary studies suggest leaching of protein A fragments may be caused by degradation of the immobilized protein A ligand by proteases, some of which may be metalloproteases, in the HCCF. Lastly, cation exchange chromatography did not provide rigorous clearance of a model fragment of protein A, suggesting that clearance of protein A fragments may be more difficult than clearance of intact protein A.

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