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Advance chromatin extraction improves capture performance of protein A affinity chromatography

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ABSTRACT

Practical effects of advance chromatin removal on performance of protein A affinity chromatography were evaluated using a caprylic acid-allantoin-based extraction method. Lacking this treatment, the practice of increasing loading residence time to increase capacity was shown to increase host protein contamination of the eluted IgG. Advance chromatin extraction suspended that compromise. Protein A ligand leakage from columns loaded with chromatin-extracted harvest was half the level observed on protein A columns loaded with non-extracted harvest. Columns loaded with chromatin-extracted harvest were cleaned more effectively by 50–100 mM NaOH than columns loaded with non-extracted harvest that were cleaned with 250–500 mM NaOH. Two protein A media with IgG capacities in excess of 50 g/L were loaded with chromatin-extracted harvest, washed with 2.0 M NaCl before elution, and the eluted IgG fraction titrated to pH 5.5 before microfiltration. Host protein contamination in the filtrate was reduced to <1 ppm, DNA to <1 ppb, protein A leakage to 0.5 ppm, and aggregates to 1.0%. Caprylic acid and allantoin were both reduced below 5 ppm. Step recovery of IgG was 99.4%. Addition of a single polishing step reduced residual protein A beneath the level of detection and aggregates to <0.1%. Overall process recovery including chromatin extraction was 90%.

1. Introduction

Residual chromatin from dead host cells has been documented to contaminate the soluble fraction of IgG-containing cell culture havests [1,2]. It takes the form of 50–400 nm heteroaggregates, consisting of metastable associations among DNA, histone proteins, and diverse non-histone host proteins. Their histone components cause them to bind strongly to protein A affinity chromatography media, where they reduce dynamic capacity for IgG, inflate contamination of the eluted IgG, promote aggregate formation, and reduce IgG recovery. Several tactical approaches have been developed to manage these burdens. Each works by a different mechanism but all of them exploit the physico-chemical features and behavioral tendencies of chromatin heteroaggregates.

Recently reported methods for advance extraction of chromatin involve co-precpitation of chromatin by allantoin combined with ethacridine or octanoic acid, and then a solid-phase scavenger to remove soluble components [1–3]. These methods increase dynamic binding capacity of protein A as well as antibody recovery and purity while reducing aggregate content. Aggressive pre-elution washes pre-leach contaminants from protein A-bound heteroaggregates so that a lesser mass of dissociable contaminants is available during elution, and fewer leach into the eluted IgG [1,2,4]. Titration of eluted IgG to pH 5.5 at low conductivity has been shown to improve antibody purity over higher and lower endpoint pH values [2,4]. The technique is believed to work by optimizing re-association of leached chromatin hetereoaggregate elements into large particles that enable their removal by microfiltration [2].

These benefits suggest chromatin management might have positive impact on other features of protein A performance. This study particularly characterizes the effects of advance chromatin extraction on protein A performance features that have been previously neglected, such as sample residence time during column loading, ligand leakage into the eluted IgG, and cleaning requirements. The study also evaluates the integrated impact of combining advance chromatin extraction with aggressive pre-elution washes and titration of eluted IgG to pH 5.5 before microfiltration, then

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follow-on polishing with a single multimodal chromatography step.

2. Materials and methods

2.1. Reagents and equipment

Buffers, salts, and reagents were obtained from Sigma–Aldrich (St. Louis, MO), except allantoin, which was obtained from Merck Millipore (Darmstadt, Germany). Chromatography media were packed in XK or Tricorn™ series columns (GE Healthcare, Uppsala, Sweden). Chromatography experiments were conducted on an ÄKTA™ Explorer 100 or Avant 25 (GE Healthcare). Capto™ Adhere was purchased from GE Healthcare. WorkBeads™ 40 TREN high was obtained from BioWorks, Uppsala. UNO sphere™ was purchased from (Bio-Rad Laboratories, Hercules, CA).

For brevity in the discussion, protein A affinity chromatography media employed in the study were coded: PA1, UNOsphere™ SUPRA (Bio-Rad Laboratories, Hercules, CA, USA); PA2, MabSelect™ (GE Healthcare); PA3, MabSelect SuRe™ (GE Healthcare); PA4, Eshmuno A® (Merck, Darmstadt); PA5, Toyopearl™ AF-rProtein A-650 F (Tosoh Bioscience, Tokyo); PA6, Toyopearl AF-rProtein A HC-650 F (Tosoh Bioscience); PA7, MabSelect SuRe™ LX (GE Healthcare). PA1 and PA2 are understood to employ recombinant protein A ligands that conserve the natural IgG-binding domain structure of wild-type Cowan strain protein A. PA3–PA5 are understood to represent recombinants with the modified domain structure to confer alkaline tolerance. PA6 and PA7 represent alkaline tolerant recombinants on chromatography particles optimized for higher capacity.

2.2. Experimental methods

A prospective biosimilar IgG1 monoclonal antibody (Herceptin®) was expressed by mammalian cell culture in Chinese hamster ovary (CHO, DG44, Life Technologies, Carlsbad, CA) cells using a tricistronic vector developed by Ho et al. [5]. Antibody was produced in 5 L BIOSTAT® B stirred-tank glass bioreactor (Sartorius Stedim Biotech) fed batch cultures using protein free medium consisting of an equal ratio of CD CHO (Life Technologies) and HyQ PF (GE Healthcare). Cultures were harvested at 30–50% viability. Pumps were avoided during harvest to minimize potential cell disruption.

Initial clarification of cell culture harvest was performed by centrifugation at 4000 × *g* for 20 min at room temperature, followed by filtration through 0.22 μm membrane (Nalgene® Rapid-Flow Filters, Thermo Scientific, Waltham, MA). The cell culture supernatant (CCS) was then stored at 2–8 °C for short-term usage or −20 °C for long-term storage.

Chromatin extraction was performed by adding octanoic acid to cell-free culture harvest to a final concentration of 0.4%, and allantoin to a final concentration of 1%. pH was adjusted to 5.3 with 1 M acetic acid, and the mixture stirred for 2 h. WorkBeads TREN was pre-equilibrated with 50 mM MES, 150 mM NaCl, pH 5.3 was added at a proportion of 5% (v/v) and mixing continued for 4 h. Solids were removed by centrifugation and/or microfiltration through 0.22 μm membrane and supernatant was either processed immediately or stored at 2–8 °C.

Some experiments employed highly purified IgG as an experimental control to eliminate ambiguity among results. Protein A affinity chromatography was performed with 53 mL of Toyopearl AF-rProtein A-650 F media packed in a XK 26/20 column (10 cm bed), run at linear flow rate of 280 cm/h (volumetric flow rate 25 mL/min). The column was equilibrated with 5 column volumes (CV) of 50 mM HEPES, 120 mM NaCl, pH 7.0. 1.5 L of chromatin-extracted harvest was loaded and the column washed with 3CV of equilibration buffer, then 5 CV 50 mM HEPES, 2 M NaCl, pH 7.0, then 2 CV equilibration buffer. Antibody was eluted with a 5 CV step to 100 mM acetic acid, pH 3.5. IgG was pooled from the point where UV absorbance at 280 nm reached 50 mAU to the point where it descended below that value. The pH of the IgG pool was titrated to pH 5.5 then microfiltered at 0.22 μm. The filtrate was titrated to pH 8.0 and NaCl was added to a final concentration of 1 M. Filtrate containing 1.5 g IgG was loaded onto a 150 mL Capto adhere (XK 26/40), at a linear flow rate of 280 cm/h (25 mL/min). The column was washed with 5 CV of equilibration buffer (50 mM Tris, 1 M NaCl, pH 8.0) and eluted with a 10 CV step to 50 mM MES, 0.35 M NaCl, pH 6.0. IgG was pooled from the point where UV absorbance at 280 nm reached 50 mAU to the point where it dropped below that value. HCP content of IgG purified by this method was consistently below the limit of detection (LoD), DNA was consistently below 1 part per billion (ppb), and aggregates were below 0.1%.

The above process was also used to evaluate relative purification performance of different protein A affinity chromatography media.

Dynamic binding capacity (DBC) at 5% breakthrough was determined for different protein A resins was determined using 4 mL Tricorn 5/10 columns at a linear flow rate of 300 cm/h (4 mL/min, residence time 1 min), 150 cm/h (2 mL/min, residence time 2 min), 75 cm/h (1 mL/min, residence time 4 min), and 50 cm/h (0.67 mL/min, 6 min residence time). The columns were equilibrated with 50 mM HEPES, 150 mM NaCl, pH 7.2 then put off line. The UV monitor was zeroed. Purified IgG was pumped through the inlet line until the UV signal indicated that antibody concentration at the entrance of the UV monitor was the same as concentration of the feed. This UV value was taken to represent 100% breakthrough. The column was put in line and monitored until UV signal indicated at least 5% breakthrough. DBC with cell culture harvest was determined by loading equilibrated feed onto the equilibrated column, collecting the effluent at intervals and checking for IgG breakthrough by analytical SEC. Five per cent breakthrough was judged to have been achieved when the area of the IgG peak in the effluent was 5% of the area of the IgG peak in the feed.

In some protein A chromatography experiments, after loading and washing with equilibration buffer, the resin was further washed with 50 mM Tris, pH 8.0 containing various concentrations of NaCl, then the column rinsed again with equilibration buffer before elution.

2.3. Analytical methods

Total host cell protein (HCP) content was estimated by ELISA with a Generation III CHO HCP kit from Cygnus Technologies Inc. (Southport, NC).

Histone host cell protein content was estimated with a Total H3 Histone kit from Active Motif (Tokyo), or with a PathScan® Total Histone H3 Sandwich ELISA Kit (Cell Signaling Technology Inc., Danvers, MA). Histones were extracted from sample beginning with 1 h incubation in 200 mM hydrochloric acid, 1.5 M NaCl, 0.1% Nonidet™ NP 40, 0.2% ethacridine, followed by filtration through a 0.22 μm membrane filter to remove solids. The filtrate was purified by void exclusion anion chromatography on UNOsphere Q according to [6], in 50 mM Tris, pH 8.0. Total histone values were estimated as 4.5 times the amount of H3 to adjust for the normal distribution of histones in chromatin of living cells: H1(H2a,H2b,H3,H4) 2.

DNA content was measured using a QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories) designed for absolute quantitation of DNA copy number. Samples were prepared according to manufacturer's recommendations. In brief, they were digested by proteinase K (adding 10% v/v of 2 mg/mL proteinase K in 5% SDS to sample) for 16 h at 50 °C, followed by DNA extraction using either a DNA extractor kit (Wako, P/N 295-50201) or QIAamp viral RNA

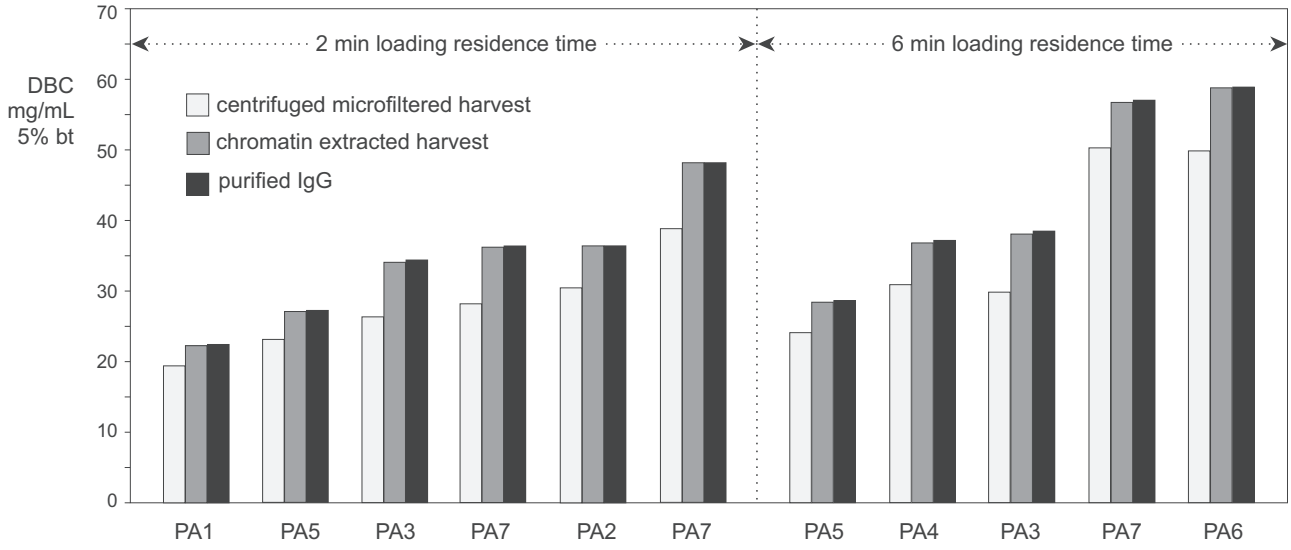

Fig. 1. Influence of chromatin extraction on dynamic capacity of selected protein A affinity chromatography media at different loading residence times.

mini kit (Qiagen, P/N 52906). TaqMan PCR reaction mixture was assembled from a 2 × ddPCR Mastermix, 10 × primer and probes (resDNASEQ® Quantitative CHO DNA Kit, Applied Biosystems, Foster City, CA) and DNA sample in a final volume of 20 μL. Each reaction mixture was loaded into a sample well of an eight channel disposable droplet generator cartridge, then 70 μL of droplet generation oil. Generated droplets were transferred to a 96-well PCR plate, heat-sealed, then placed on a thermal cycler and amplified to end-point by denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s then 60 °C for 1 min. Analysis was performed with QuantaSoft analysis software (Bio-Rad Laboratories). Correlation between DNA copy number and DNA concentration was based on CHO host cell DNA standards from Applied Biosystems (resDNASEQ® Quantitative CHO DNA Kit).

Aggregate content was measured by analytical size exclusion chromatography (SEC) with a G3000SWxl column (Tosoh Bioscience) on a Dionex UltiMate™ 3000 UPLC system (Thermo Scientific) operated at a flow rate of 0.6 mL/min, using a buffer formulation of 50 mM MES, 20 mM EDTA, 200 mM arginine, pH 6.0. Sample injection volume was 100 μL.

The concentration of non-aggregated IgG was monitored by SEC, comparing experimental results with a calibration curve prepared from known quantities of injected purified IgG. This approach avoids the overestimation error by affinity based methods that capture aggregates in addition to non-aggregated IgG.

Reduced and Non-Reduced SDS-PAGE were performed on 4–15% Criterion™ TGX Stain-Free™ Gel (Bio-Rad). Gels were stained with a SilverQuest™ silver staining kit from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions.

Octanoic acid and allantoin content were measured in a single assay. Samples were transferred individually to Amicon ultra centrifugal filter units with a 3 kDa cut-off (Merck Millipore), then centrifuged at 4000 × *g* for 40 min at 22 °C. 1.5 mL of sample filtrate was pipetted into 2 mL HPLC vial with addition of 7.5 μL of 37% HCl. The mixture was vortexed briefly resulting in a clear solution with an approximate pH of 2.8. 40 μL prepared sample was injected onto a Thermo Hypercarb 3 μm 50 mm × 2.1 mm column (Phenomenex) run at 35 °C on a Dionex UltiMate 3000 UPLC System equipped with a diode array detector and Chromeleon 7.2 chromatography data system (Thermo Scientific). Elution buffers were 0.1% formic acid (A) and acetonitrile (B). Flow rate was 0.5 mL/min. The column was washed for 3 min with A, eluted with a 12 min linear gradient to 90% B, maintained at 90% B for 0.5 min, then returned to 100% A over for 7 min. UV absorbance was monitored at 208 nm. The limits of linear quantitation were 50 ng for allantoin and 3.0 μg for octanoic acid.

The assay described above was originally designed to accommodate all fatty acids with aliphatic chains containing 6–12 carbon atoms. A method dedicated to octanoic acid, modeled after Fliszar et al. [7]. achieved higher sensitivity. 20 mL of sample was passed through a 3 kDa centrifugal filter, acidifying it as described above, then applying it to a Strata C18 solid phase extraction cartridge and eluting with 45% acetonitrile, 55% 20 mM phosphoric acid, pH 2.8. Octanoic acid recovery was 96%. 50 μL was injected onto a Kinetix C18 column (1.7 μm particles, 100 mm × 3 mm, Phenomenex). Elution was performed isocratically with 45% 20 mM phosphoric acid, 55% acetonitrile, pH 2.8 at a flow rate of 0.5 mL/min. UV absorbance was monitored at 208 nm. This system was able to detect as little as 500 ng even without concentration, and with 20-fold concentration enabled detection of as little as 25 ng/mL in the original sample.

Other experimental details are described or reiterated for clarity in the following section.

3. Results and discussion

3.1. Chromatin interference with IgG binding

Previous work with PA5 demonstrated that advance chromatin extraction with an ethacridine-allantoin-based method increased dynamic binding capacity (DBC) at 5% breathrough by 15–20% [1]. Loss of capacity with unextracted feed was attributed to accumulation of 50–400 nm chromatin heteroaggregates on external chromatography particle surfaces interfering with access of IgG to the diffusive pores in the media. Fig. 1 shows that caprylic acid-allantoin-based pre-extraction achieved essentially the same result as the ethacridine-allantoin system and did so across all protein A products evaluated. In each case, capacity following chromatin extraction was nearly the same as achieved when loading the respective media with purified IgG.

3.2. Relationship of loading residence time to contamination

Fig. 1 also corroborated the known effect that increasing column residence time during loading increases capacity [8,9]. This

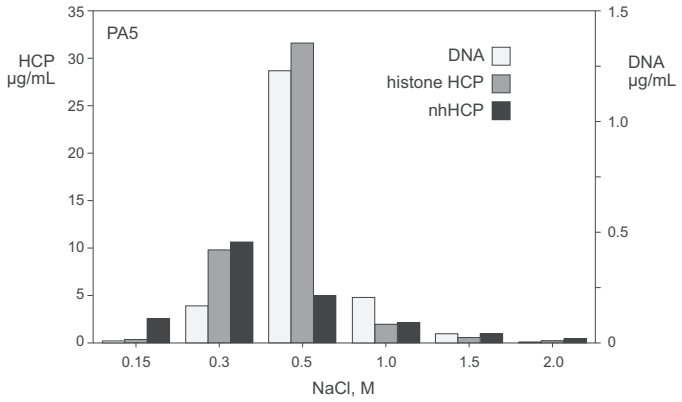

Fig. 2. Host contaminants leached by incremental increases of NaCl concentration from protein A-bound chromatin heteroaggregates. All results at pH 8.0.

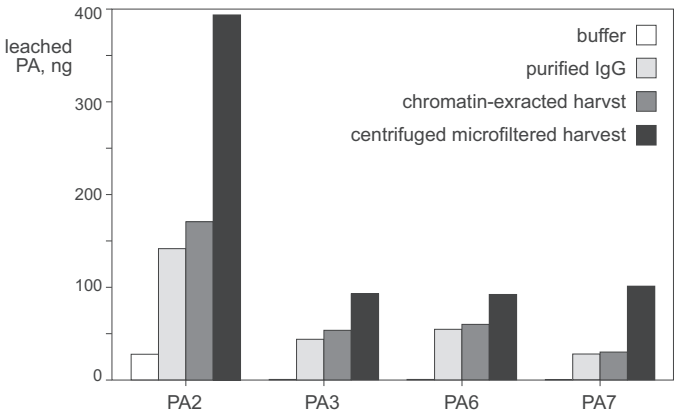

Fig. 3. Leaked protein A ligand in the elution fraction after loading different feed streams.

represents a clear benefit for process economics but a hidden compromise was revealed when columns were loaded with non-extracted feed streams. Loading residence time was varied for a fixed volume of feed equivalent to 100% of the DBC (5% breakthrough) determined at a residence time of 6 min. HCP content of the eluted IgG increased in a roughly linear fashion with 618 ppm at a residence time of 1 min, 731 ppm at 2 min, 801 ppm at 3 min, and 937 ppm at 4 min. Contamination remained substantially unchanged with longer residence time: 951 ppm at 5 min and 931 ppm at 6 min.

When a parallel series of residence time experiments was run with chromatin-extracted feed, HCP values were below 2 ppm at all time points. This highlighted the compound benefit of advance chromatin extraction, not only in reducing HCP contamination several hundred-fold but also enabling loading residence time specifications to be set for maximum capacity without risk of amplifying contamination.

3.3. Compound benefits of chromatin extraction and pre-elution washes with NaCl

NaCl is known to have a strong effect on associations among chromatin elements [10–12]. This creates an expectation that high-salt washes should pre-emptively leach dissociable elements from bound heteroaggregates. Fig. 2 shows the amounts of nhHCP, histone HCP, and DNA leached by a succession of increases in NaCl concentration. Leached nhHCP peaked at 300 mM NaCl, indicating this subset of nhHCP was weakly associated with heteroaggregate elements that remained bound to protein A. Leaching of histones and DNA peaked together at 500 mM NaCl. The higher salt concentration was understood to demonstrate their stronger association with chromatin elements that remained bound to protein A. Leaching of all contaminant classes from 500 mM to 2.0 M NaCl was understood to reflect the same trend.

These results emphasize the linkage between chromatin and the effectiveness of aggressive pre-elution washes. They also suggest combined usage of chromatin extraction and aggressive pre-elution washes should compound their respective benefits, and they do, but the benefit is modest. In one study, advance chromatin extraction without a salt wash enabled protein A to reduce HCP content to 0.8 ppm [1]. Inclusion of a 2 M NaCl wash further reduced it only by an additional 0.3 ppm (to 0.5 ppm). Such results seem not to justify the additional material expense and process time of aggressive pre-elution washes. However, that conclusion ignores their potential utility for eliminating reagents employed to extract chromatin.

Reagents added to process solutions must generally be removed. The octanoic acid and allantoin used in the present experiments are both non-toxic normal human metabolites [13,14], but their reduction to low levels in the final product is still understood to be necessary for therapeutic antibodies. Inclusion of a 2 M NaCl wash reduced octanoic acid beneath its detection limit of 3 ppm (IgG at 10 mg/mL) and reduced allantoin beneath its detection limit of 5 ppm (IgG at 10 mg/mL).

Aggressive wash conditions seem likely to offer parallel benefit for electropositive flocculating agents such as ethacridine [1,15], chlorhexidine [15], polyethyleneimine [15], polybenzallyamine [16,17], and PDADMAC [18]. Protein A has a pI of about 5, making it a *de facto* cation exchanger [19,20]. Elevated salt washes should help to dissociate non-specific interactions that might otherwise permit flocculant carryover into the eluted IgG. Aggressive washing agents have included many other classes of compounds including alcohols [21], glycols [22], surfactants [21–23], and chelating agents [22], which may also be considered to enhance flocculant clearance prior to elution. Any parallel reduction of host cell contamination, even if modest, represents a bonus that can only simplify the remaining task of polishing.

3.4. Impact of advance chromatin extraction on protein A ligand leakage

Leakage of protein A ligands is a complex phenomenon. Previous studies have shown that it involves enzymatic cleavage of random coil sequences between IgG-binding domains; chemical cleavage, including by alkaline hydrolysis in NaOH; dissociation of the immobilization linkage; and breakdown of the solid phase base matrix [22–28]. Leakage has been described as being generally higher during the first few runs performed on a given column [22,25,26]. The present experiments should therefore be understood to represent worst case, since they were conducted exclusively with fresh media.

Fig. 3 shows that advance chromatin extraction reduced ligand leakage by roughly half across all tested protein A media. These included recombinant protein A with wild-type domain and interlinking random coils sequences (PA2), a recombinant analog modified to enhance tolerance to NaOH (PA3), and two recently introduced high capacity NaOH-tolerant media (PA6,PA7). Alkaline-resistant media showed about 5-fold lower leakage than the product based on the wild-type ligand.

Previous studies provided two intepretive guides to possible mechanisms behind these results. Proteolytic clipping of the random coil sequences between IgG binding domains on protein A was a significant contributor to ligand leakage in one case [29]. In

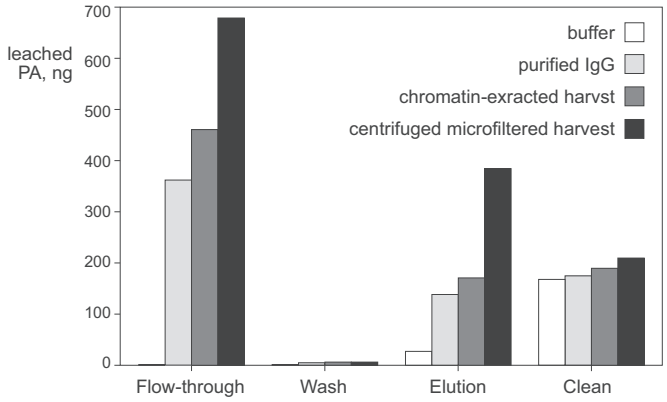

Fig. 4. Protein A leakage at different process stages.

another, sequence modifications induced to enhance alkaline tolerance reduced efficiency of enzymatic cleavage [30]. In the present experiments advance chromatin extraction reduced host protein content of the feed stream by 97.5%. Given the assumption that host cell proteases were eliminated in proportion, it makes sense that protease-mediated ligand leakage should also have been reduced.

To the extent that proteolysis was a factor, it should also have caused leakage during column loading. That was confirmed in Fig. 4 (PA2), but both Figs. 3 and 4 revealed the unexpected influence an additional variable: loading purified IgG also caused sigificant ligand leakage. It seems unlikely that this was caused by residual proteases because that particular IgG preparation contained less than 1 ppm HCP, which should reasonably have corresponded to an even lower protease load. Elution conditions cannot explain it or elution-buffer-mediated leakage would have been greater, and the large increment of leakage with 100 mM NaOH was assumed to result from alkaline hydrolysis.

The results highlight the conspicuous absence of a mechanistic explanation for IgG-mediated ligand leakage but they also reveal a starting point for future investigation. Fig. 3 revealed different degrees of purified-IgG-mediated ligand leakage among that various protein A media. Detailed analysis of their amino acid sequences and structures, and their interactions with IgG might contribute to an understanding of how the system works.

Whatever the mechanism, it seems unlikely to have major practical significance for preparation of therapeutic quality IgG. Even though the phenomonon apparently occurs across all protein A media, its magnitude is so low for NaOH-resistant products that there seems little risk. The ability of polishing steps to further reduce leached protein A increases confidence that it does not represent a safety concern.

3.5. Impact of advance chromatin extraction on cleaning and sanitization

A large subset of chromatin heteroaggregates remain bound to protein A after elution of IgG [1]. In the present experiments, cleaning with 50–100 mM NaOH removed much of it but 250–500 mM was required to remove all of it. As expected, advance chromatin extraction dramatically reduced the amount of material that remained after elution. For comparison, post-elution, 500 mM NaOH removed 20 μg nhHCP from a 3 mL column loaded with 50 mL centrifuged-microfiltered harvest containing 75 mg IgG. With the same amount of IgG in chromatin-extracted feed, 500 mM NaOH released 138 times less nhHCP: 146 ng.

Fig. 5 illustrates the relative amounts of proteins released by 50 mM, 100 mM, 250 mM, and 500 mM NaOH. Results showed advance chromatin extraction enabled 50 mM NaOH to reduce protein contamination to a lower level than cleaning with 250 mM NaOH on columns loaded with non-extracted harvest. This suggested that columns loaded with chromatin-extracted harvests could be washed effectively with 50 mM NaOH, with a likely outcome of increasing the number of cleaning cycles through which the media could retain adequate performance. The reduced accumulation of strongly binding contaminants should also help to minimize interference with continuous chromatography systems in which large cumulative sample loads are applied to relatively small columns over an extended number of cycles without intermediate cleaining.

The large amount of IgG in the NaOH fractions following application of non-extracted harvest is obvious and requires explanation. At low pH, protein A-eluted IgG becomes denatured and has an elevated tendency to form stable associations with chromatin, including the chromatin that remains bound to protein A [2]. This pathway accounts for the loss of about 5% of the applied IgG. Advance chromatin extraction suspends this pathway and the IgG is recovered almost entirely in the elution fraction.

3.6. Integrated process characterization

Recent studies have shown that chromatin heteroaggregate elements that leach during IgG elution remain dissociated to a large

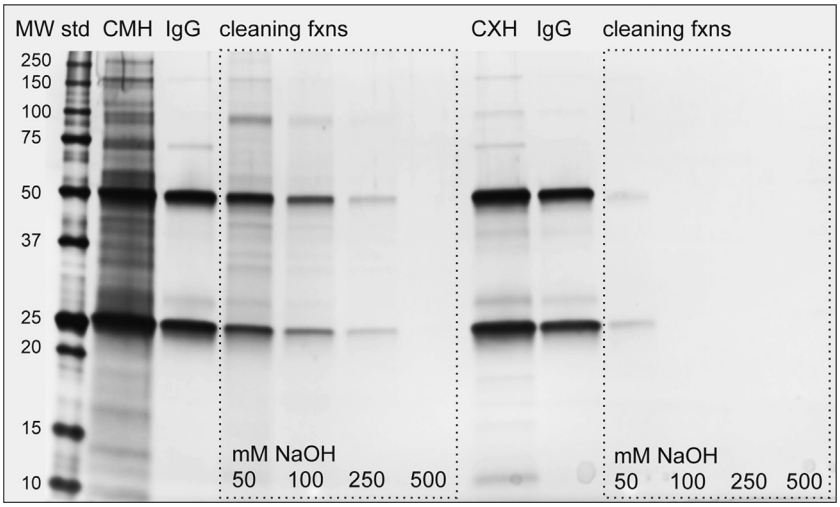

Fig. 5. Reduced SDS-PAGE illustrating relative amounts of protein removed by increments of NaOH after elution. Chromatin extracted *vs.* non-extracted feed streams.

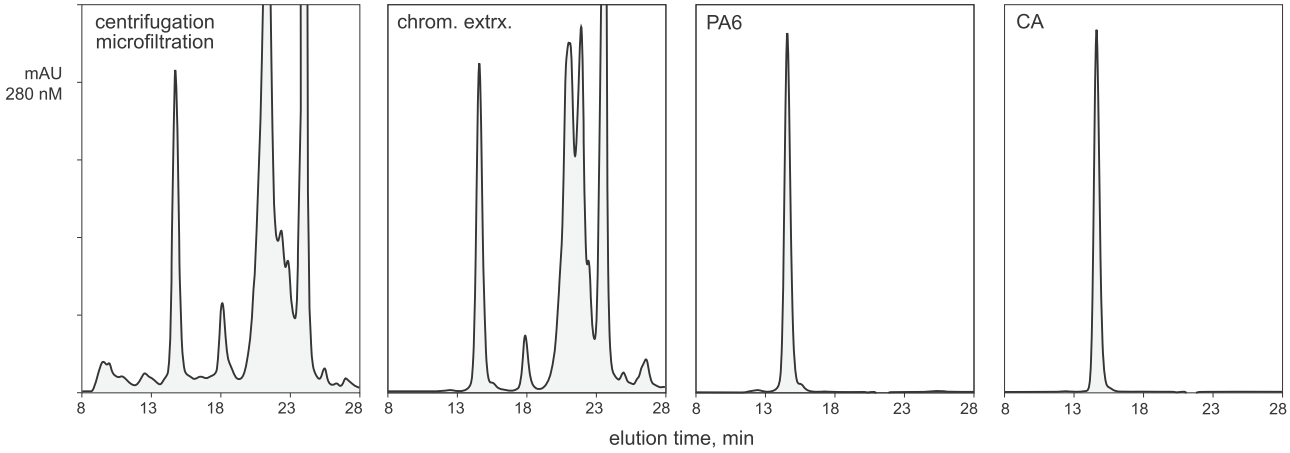

Fig. 6. Purification process summary by analytical SEC. Vertical scale standardized to IgG peak height to facilitate comparison of contaminant content.

Table 1
Comparison of protein A processes with PA6 and PA7.

Analyte/process	Traditional PA		Integrated PA		Improvement
	PA6	PA7	PA6	PA7	
IgG DBC g/L	50	50	57	59	~12–15%
nhHCP ppm	409	459	0.4	0.6	~100-fold
Histone ppm	406	181	0.0	0.0	~400 fold
DNA ppb	361	140	0.02	0.02	~10,000-fold
Aggr.%	4.7	4.0	1.0	1.0	~4-fold
Leached PA ppm	1.3	3.0	0.5	0.6	~2-fold
Step recovery%	94.3	94.5	99.4	99.4	~5%

Table 2
Process summary. Chromatin extraction to PA6 to Capto adhere. The first recovery value in each column is step recovery. The second is cumulative recovery. CFH, centrifuged-filtered harvest. ACX, advance chromatin extraction. PA, post-capture with protein A affinity chromatography. CA, post-polishing with Capto adhere. LoD, below limit of detection.

Analyte/stage	CFH	ACX	PA	CA
nhHCP ppm	414,023	10,104	LoD	LoD
Histone ppm	30,267	LoD	LoD	LoD
DNA ppb	16e6	361	0.02	LoD
Leached PA ppm	–	–	0.5	LoD
Aggr.%	18.8	1.2	1.0	<0.1
Recovery%	100/100	95/95	99/94	96/90

degree as long as elution conditions persist, but re-associate into large turbidity-producing particles when pH is increased [2]. Many of the particles are large enough to be retained by microfilters, permitting easy removal of all the particle-associated contaminants from the soluble IgG. Independent groups of investigators have concluded that titrating the eluted IgG to pH 5.5 supports the greatest reduction of host contaminants in the filtrate [2,4]. The best results require an absence of excess salt. NaCl and arginine both weaken re-association among chromatin heteroaggregate elements, leaving more of them soluble and causing them to reside in the filtrate along with the antibody [2]. The high molar conductivity of citrate should be expected to mediate the same effect.

An integrated protein A protocol was run on the two highest capacity media (PA6, PA7), incorporating advance chromatin extraction, a 2 M NaCl pre-elution wash, and a third element of titrating pH of the eluted IgG to pH 5.5 before microfiltration. Purification performance was compared against protein A loaded with harvest clarified by centrifugation and microfiltration, not including a 2 M NaCl pre-elution wash, and titrating the eluted IgG to pH 8.0 before microfiltration. All performance measures were improved significantly by the integrated process, but contaminant removal most dramatically (Table 1). Reduction of nhHCP was improved more than 100-fold to less than 1 ppm. DNA reduction was improved 10,000-fold to <1 ppb.

Aggregate levels were reduced 4-fold to 1%, leaving them at the upper limit of the acceptable range, but this still suggested a single additional polishing step could achieve clinical quality. Table 2 shows a comprehensive performance profile of the integrated process with PA6 and a final polishing step with Capto adhere. Results with a PA7 process were virtually identical (data not shown).

Fig. 6 illustrates analytical SEC profiles after each process step. Chromatin extraction removed the majority of aggregates (8.5–14 min) and most of the proteins smaller than IgG (15–20 min), leaving a large subset of small-molecule cell culture media components (20–28 min). Protein A eliminated the culture media components and most of the smaller-than-IgG proteins, but no aggregate reduction beyond the chromatin extraction step. The shoulder trailing the non-aggregated IgG peak was previously identified as a product-related impurity containing 2 heavy chains and a single light chain [2]. Polishing by Capto adhere largely eliminated aggregates and impurities.

Capto adhere also reduced protein A leakage beneath the limit of detection. It was used in bind-elute mode to enhance clearance of residual octanoic acid and allantoin. Separate experiments showed that neither additive was retained by Capto adhere when loaded at 1 M NaCl. IgG binding capacity in bind-elute mode was essentially the same as its processing capacity in flow-through mode, but bind-elute aggregate removal was slightly more effective (data not shown).

These results document the ability of advance chromatin extraction to enable IgG purification in two chromatography steps without compromising purity, and highlight another practical benefit for process developers. With clarification by centrifugation and microfiltration, the polishing step of even 3-chromatography-step procedures must do more than just remove aggregates. Persistent contamination by HCP and DNA must also be reduced. The challenge is that all contaminant classes do not all respond the same way to any given set of conditions. One contaminant or impurity class may be reduced effectively while reduction of another is compromised; or IgG recovery may be compromised. When virtually all of the contaminants have been removed before polishing, that step can be focused exclusively on aggregate removal. This potentially qualifies a wider field of aggregate removal tools, limits supporting analysis to nothing more than SEC, and avoids having to compromise aggregate removal in order to achieve parallel reduction of host contaminants.

Even with extraordinary reduction of HCP, DNA, and aggregates, reducing the number of chromatography steps raises a potential concern about compromising virus reduction across a process. Preliminary data suggest that advance chromatin extraction eliminates that risk. Direct treatment of virus cultures with an octanoic acid-allantoin treatment reduced MVM by 5 Log_{10}, and MuLV by 9 Log_{10} [7]. Its impact in the context of industrial processes will need to be validated on an individual product basis, but these results suggest reasonable probability of a favorable outcome.

4. Conclusions

Advance chromatin extraction enabled a wide variety of protein A affinity chromatography media to support dynamic capacities that equaled or closely approached values determined with purified IgG. DBC values obtained with harvest clarified by centrifugation and microfiltration were 10–20% lower across all media. A pre-elution wash with 2 M NaCl eliminated residual octanoic acid and allantoin remaining after the chromatin extraction step. Loading protein A with chromatin-extracted harvest reduced protein A ligand leakage by half. NaOH cleaning of protein A loaded with chromatin extracted harvest required only 50 mM NaOH. Loading with non-extracted harvest required at least 250 mM NaOH to achieve a similar degree of cleaning.

An integrated protein A-based procedure including advance chromatin extraction, a pre-elution wash with 2 M NaCl, and titration of the eluted IgG to pH 5.5 followed by microfiltration reduced contaminant content of the filtrate to <1 ppm HCP, DNA to <1 ppb, protein A leakage to <1 ppm, and aggregates to 1% with an IgG step recovery of 99.4%. Addition of a single polishing step reduced aggregates to <0.1% and all other contaminants beneath their limits of detection, with a step recovery of 96% and overall process recovery, including chromatin extraction, of 90%.

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References

[1] P. Gagnon, R. Nian, J. Lee, L. Tan, S.M. Abdul Latiff, C.L. Lim, C. Chuah, X. Bi, Y.S. Yang, W. Zhang, H.T. Gan, Nonspecific interactions of chromatin with immunoglobulin G and protein A, and their impact on purification performance, J. Chromatogr. A 1340 (2014) 68–78.
[2] P. Gagnon, R. Nian, Y.S. Yang, Q. Yang, C.L. Lim, Non-immunospecific association of Immunoglobulin G with chromatin during elution from protein A inflates host contamination, aggregate content, and antibody loss, J. Chromatogr. A 1408 (2015) 151–160.
[3] P. Gagnon, R. Nian, L. Tan, J. Cheong, V. Yeo, Y.S. Yang, H.T. Gan, Chromatin-mediated depression of fractionation performance on electronegative multimodal chromatography media, its prevention, and ramifications for purification of immunoglobulin G, J. Chromatogr. A 1374 (2014) 145–155.
[4] S. Chollangi, R. Parker, N. Singh, Y. Li, M. Borys, Z. Li, Development of robust antibody purification by optimizing protein-A chromatography in combination with precipitation methodologies, Biotechnol. Bioeng. 112 (2015) 2292–2304.
[5] S.C. Ho, M. Bardor, H. Feng, Y.W. Mariati, Z. Tong, M.G.S. Song, Y.S.Y. Yap, ang, IRES-mediated tricistronic vectors for enhancing generation of high monoclonal antibody expressing CHO cell lines, J. Biotechnol. 157 (2012) 130–139.
[6] R. Nian, C. Chuah, J. Lee, H.T. Gan, S.M. Abdul-Latiff, W.Y. Lee, V. Vagenende, Y.S. Yang, P. Gagnon, Void exclusion of antibodies by grafted-ligand porous particle anion exchangers, J. Chromatogr. A 1282 (2013) 127–132.
[7] K. Fliszar, W. Wuelfing, Z. Li, R. Reed, Profiling of medium chain glycerides used in pharmaceutical formulation development by reversed-phase HPLC, J. Pharm. Biomed. Anal. 40 (2006) 896–900.
[8] R. Hahn, R. Schlegel, A. Jungbauer, Comparison or protein A affinity sorbents, J. Chromatogr. B 790 (2003) 35–51.
[9] R. Hahn, P. Bauerhansl, K. Shimara, C. Wizniewski, A. Tscheliessnig, A. Jungbauer, Comparison or protein A affinity sorbents II. Mass transfer properties, J. Chromtogr. A 1093 (2005) 98–110.
[10] K. Rippe, J. Mazurkiewicz, N. Kepper, Interactions of histones with DNA: nucle-osome assembly, stability and dynamics, in: R. Dias, B. Lindman (Eds.), DNA Interactions with Polymers and Surfactants, John Wiley and Sons, Hoboken, 2008, pp. 135–172.
[11] J.E. Godfrey, T.H. Eickbush, E.N. Moudrianakis, Reversible association of calfthymus histones to form the symmetrical octamer (H2AH2BH3H4) 2: a case ofa mixed-associating system, Biochemistry 19 (1980) 1339–1346.
[12] R.C. Benedict, E.N. Moudrianakis, G.K. Ackers, Interactions of the nucleosomal core histones: a calorimetric study of octamer assembly, Biochemistry 23 (1984) 1214–1218.
[13] V. Morais, H. Massaldi, A model mechanism for protein precipitation by caprylic acid: application to plasma purification, Biotechnol. Appl. Biochem. 59 (2012) 50–54.
[14] I. Benzie, W. Chung, B. Tomlinson, Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical applcation in oxidant:antioxidant balance studies, Clin. Chem. 45 (1999) 901–904.
[15] H.T. Gan, J. Lee, S.M. Abdul-Latiff, C. Chuah, P. Toh, W.Y. Lee, P. Gagnon, Char-acterization and removal of aggregates formed by nonspecific interactions oflgM monoclonal antibodies with chromatin catabolites during cell culture pro-duction, J. Chromatogr. A 1291 (2013) 33–40.
[16] Y. Kang, J. Hamzik, M. Felo, B. Qi, J. Lee, S. Ng, G. Liebisch, B. Shenehsaz, N. Sing, K. Persaud, D. Ludwig, P. Balderes, Development of a novel and efficient cell culture flocculation process using a stimulus responsive polymer to streamline antibody purification, Biotechnol. Bioeng. 110 (2013) 2928–2937.
[17] M. Felo, Y. Kang, J. Hamzik, P. Balderes, D. Ludwig, Industrial application of impurity floculation to streamline antibody purification processes, Pharm. Bioprocess. 3 (2015) 115–125.
[18] T. McNerny, A. Thomas, A. Senczuk, K. Petty, X. Zhao, R. Piper, J. Carvalho, M. Hammond, S. Sawant, J. Bussiere, PDADMAC flocculation of Chineses hamseter ovary cells: enabling a centrifuge-less harvest process for monoclonal antibodies, Mabs 7 (2015) 413–428.
[19] I. Björck, B-Å. Petersson, J. Sjöquist, Some physicochemical properties of protein A from Staphylococcus aureus, Eur. J. Biochem. 29 (1972) 579–584.
[20] J.J. Langone, Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumonococci, Adv. Immunol. 32 (1982) 157–162.
[21] A. Shukla, P. Hinckley, Host cell protein clearance during protein A chromatography: development of an improved column wash step, Biotechnol. Prog. 24 (2008) 1115–1121.
[22] P. Gagnon, Purification Tools for Monoclonal Antibodies, Validated Biosystems, Tucson, 1996, pp. 155–198.
[23] S. Vunnum, G. Vedantham, B. Hubbard, Protein A-based affinity chromatography, in: U. Gottschalk (Ed.), Process Scale Purification of Antibodies, John Wiley and Sons, Hoboken, 2009, pp. 79–102.
[24] P. Füglistaller, Comparison of immuglobulin binding capacities and ligand leakage using eight different protein A affinity chromatography matrices, J. Immunol. Methods 124 (1989) 171–177.
[25] J. Carter-Franklin, C. Victa, P. McDonald, R. Fahrner, Fragments of protein A eluted during protein A affinity chromatography, J. Chromatogr. A 1163 (2007) 105–111.
[26] K. Eriksson, A. Ljunglöf, G. Rodrigo, E. Brekkan, Mab contaminant removal with a multimodal anion exchanger, BioProcess Int. 7 (2) (2009) 52–56.
[27] G. Hale, A. Drumm, P. Harrison, J. Phillips, Repeated cleaning of protein A affinity column with sodium hydroxide, J. Immunol. Methods 171 (1994) 15–21.
[28] R. Hahn, K. Shimihara, F. Steindl, A. Junbauer, Comparison of protein A affnity sorbents III. Life time study, J. Chromatogr. A 1102 (2006) 224–231.
[29] K. Minakuchi, D. Murata, Y. Okubo, Y. Nakano, S. Yoshida, Remarkable stability of an engineered protein A as immunoglobulin affinity ligands: C domain having only one amino acid substitution, Protein Sci. 22 (2013) 1230–1238.
[30] M. Boulet-Audet, B. Byrne, S. Kazarian, Cleaning-in-place of immunoaffinity resins monitored by in situ ATR-FTIR spectroscopy, Anal. Bioanal. Chem. 407 (2015) 7111–7122.