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Factorial screening of antibody purification processes using three chromatography steps without protein A

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Abstract

Protein A affinity chromatography is often employed as a capture step to meet the purity, yield, and throughput requirements for pharmaceutical antibody purification. However, a trade-off exists between step performance and price. Protein A resin removes 99.9% of feed stream impurities; however, its price is significantly greater than those of non-affinity media. With many therapeutic indications for antibodies requiring high doses and/or chronic administration, the consideration of process economics is critical. We have systematically evaluated the purification performance of cation-exchange, anion-exchange, hydroxyapatite, hydrophobic interaction, hydrophobic charge induction, and small-molecule ligand resins in each step of a three-step chromatographic purification process for a CHO-derived monoclonal antibody. Host cell proteins were removed to less-than-detectable for three processes (cation-exchange–anion-exchange–hydrophobic interaction chromatography, cation-exchange–anion-exchange–mixed cation-exchange chromatography, and cation-exchange–mixed cation-exchange–anion-exchange chromatography). The order of the process steps affected purification performance significantly.

Keywords: Mixed-mode chromatography; Factorial screening; Antibodies

1. Introduction

In the year 2000, monoclonal antibody sales exceeded US$ 2×10^9 [1]. Eleven antibody-based therapeutics have been approved by the US Food and Drug Administration, more than 70 are in clinical trials, and more than 265 are in preclinical development at over 225 companies [1]. Accounting for roughly 20% of the therapeutic products in development in the US, this drug class treats indications including autoimmune disease, infectious disease, cardiovascular disease, transplant rejection, and cancer [2–9].

During purification of pharmaceutical antibodies, impurities including host cell proteins, DNA, antibody variants, and small molecules and potential contaminants including endotoxin and viral particles must be removed [10]. Because many of the indications treated by monoclonal antibodies require high doses and/or chronic administration, economical process-scale production of these molecules is also critical. Yield and throughput are important considerations when designing large-scale purification processes.

Protein A affinity chromatography is a common antibody manufacturing unit operation [11,12], because it selectively and efficiently binds antibodies in complex solutions such as harvested cell culture fluid, and it removes >99.5% of product impurities in a single step with high step yields and high throughput [13–17]. Affinity chromatography also provides significant virus clearance [18,19]. Because it binds a variety of mammalian IgG molecules [20], protein A affinity chromatography also allows process harmonization for multi-product manufacturing.

Key disadvantages of protein A chromatography include cost and resin stability. Protein A resin is over 30 times more expensive than some ion exchange resins, and may account for greater than 35% of the total recovery raw material costs at large scale. Furthermore, the ligand can denature under

Abbreviations: CHOP, Chinese hamster ovary cell proteins (host cell proteins); CV, column volume; HCCF, harvested cell culture fluid; MES, 2-(*N*-morpholino)ethanesulfonic acid

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harsh sanitization conditions [21]. Also, because protein A molecules may cause immunogenic or other physiological responses in humans, ligand leached from the base matrix must be cleared during downstream processing [22].

At Genentech, the most common process includes three chromatography steps: protein A affinity, cation-exchange, and anion-exchange [10,23]. We are interested in determining the feasibility of replacing protein A chromatography with non-affinity chromatography.

A one-to-one comparison of a protein A step with a non-affinity step at the same place in a process is a common and straightforward approach, and antibody capture is a widely researched topic [24–29]. However, this approach sets unfair constraints. For example, a non-affinity step would have to clear >99% of the impurities loaded onto the column to be "comparable" to a protein A step. Furthermore, this approach assumes those impurities not cleared in the non-affinity step could be removed downstream in the process. Because the impurity population removed by a particular step is a function of the separation mechanism of the particular resin chemistry, this assumption may not be valid. It is thus not important to determine whether a non-affinity step can purify antibody to the same degree as a protein A chromatography step, but rather to determine whether the same purification obtained with a process incorporating protein A chromatography can be achieved using a process with no affinity chromatography steps.

With this goal, we have evaluated the purification performance of cation-exchange (S), anion-exchange (Q), mixed-mode ion exchange (ABx), hydroxyapatite (HA), hydrophobic interaction (HIC), hydrophobic charge induction (HCI), and small-molecule ligand (MIM) resins in each step of a three-step chromatographic purification process for a CHO-derived monoclonal antibody. This novel approach spreads the burden of impurity removal over the entire process. Chinese hamster ovary cell proteins (CHOPs) in the product pools were employed as an indicator of product purity. Total host cell protein removal was compared to a traditional three-step recovery process incorporating protein A chromatography (i.e. ProA-S-Q).

2. Experimental

2.1. Chromatography

Cell culture fluid containing a recombinant humanized monoclonal IgG1 antibody, p*I* of approximately 9, and M_r of about 150 000, was obtained from Genentech (South San Francisco, CA, USA). The fermentation broth, containing antibody produced in Chinese hamster ovary cells at a titer of about 1 g/l, was processed by continuous centrifuge to remove cellular debris and 0.22 μm filtered before loading onto any of the capture columns. The protein pools were also 0.22 μm filtered prior to loading onto the next chromatography column. Chromatography was monitored at 280 nm and performed at room temperature using an AKTA 100 chromatography unit from Pharmacia (Uppsala, Sweden).

SP-Sepharose Fast Flow resin (Pharmacia), Q-Sepharose Fast Flow resin (Pharmacia), Bakerbond ABx resin (J.T. Baker, Phillipsburg, NJ, USA), Phenyl-Sepharose Fast Flow resin (Pharmacia), Macroprep Ceramic Hydroxyapatite resin (Bio-Rad Labs., Hercules, CA, USA), MEP HyperCel resin (LifeTechnologies, Rockville, MD, USA), and a prototype small-molecule mimetic resin (ProMetic Biosciences, Cambridge, UK) were each packed into 20 cm × 0.66 cm i.d. Omni glass columns. Some runs were performed in larger columns (20 cm × 5 cm i.d. columns from Pharmacia) to supply sufficient load material for downstream columns. The performance of the larger columns was identical to the smaller columns. The operating conditions for chromatography are presented in Table 1. Columns were run at a flow rate of 100 cm/h, equilibrated with at least three column volumes (CVs) of equilibration buffer, and loaded to 10 g/l (gram antibody per liter column volume). Runs with gradient elution were washed with 3 CVs of equilibration buffer after loading prior to elution. Precipitation was apparent in some pools upon pH adjustment, but the precipitate was easily removed by filtration, and no antibody loss was observed.

Between uses, S, HIC, HCI, and MIM resins were sanitized with three column volumes of 0.5 M NaOH. Columns containing ABx, Q, and HA resins were packed with fresh resin before each use.

2.2. Assays

Antibody concentrations in the harvested cell culture formulation and in the pools generated during the first chromatography steps were determined using a protein A HPLC assay with a 100 mm × 4.6 mm i.d. Poros A/20 column (PerSeptive Biosystems, Framingham, MA, USA). After sample was injected (time = 0 min), the resin was washed in phosphate-buffered saline (PBS) for 3 min. Bound protein was eluted in 2% acetic acid, 100 mM glycine for 2 min, then the resin was regenerated in 20% acetic acid for 0.5 min. Sample volumes of 200 μl were injected, the absorbance was monitored at 280 nm, and the flow rate was held constant at 4 ml/min. Between injections, the column was re-equilibrated with 18 ml PBS.

Antibody concentrations in the pools of the second chromatography steps were determined by absorbance at 280 nm (with absorbance at 320 nm subtracted to correct for light scattering), using a 8453 spectrophotometer with a 10 mm path length flow cell from Agilent (Palo Alto, CA, USA). An extinction coefficient of 1.5 ml/(mg cm) was employed. Antibody concentration was calculated as {[(absorbance at 280 nm) − (absorbance at 320 nm)] × (dilution)}/1.5.

An enzyme-linked immunosorbent assay (ELISA) [30] using goat anti-(host cell protein) antibodies was used to determine CHOP concentrations in all of the pools.

Table 1
Chromatography operating conditions

Resin	Resin type	Mode of operation	Buffers	Load conditioning
SP-Sepharose Fast Flow (Amersham Biosciences, NJ, USA)	Cation-exchange (S)	Bind-and-elute	20 mM MES, 50 mM NaCl, pH 5.5; 10 CVs gradient to 500 mM NaCl	<5 mS/cm pH 5.5
Bakerbond ABx (J.T. Baker, Phillipsburg, NJ, USA)	Mixed-mode ion exchange (ABx)	Bind-and-elute	Same as S	Same as S
Q-Sepharose Fast Flow (Amersham Biosciences, NJ, USA)	Anion-exchange (Q)	Flow-through	25 mM Tris, 50 mM NaCl, pH 8	<7 mS/cm pH 8
Phenyl-Sepharose Fast Flow, low sub (Amersham Biosciences, NJ, USA)	Hydrophobic interaction (HIC)	Bind-and-elute	50 mM MES, 0.8 M Na_2SO_4, pH 6; 3 CVs; 15 CVs gradient to 50 mM MES, pH 6	0.8 M Na_2SO_4 pH 6
Macro-Prep ceramic hydroxyapatite, Type II (Bio-Rad, Hercules, CA, USA)	Hydroxyapatite (HA)	Bind-and-elute	10 mM sodium phosphate, pH 6.8; 10 CVs gradient to 400 mM phosphate, pH 6.8	<3 mS/cm pH 6.8
MEP HyperCel (Life Technologies, Rockville, MD, USA)	Hydrophobic charge induction (HCI)	Bind-and-elute	25 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 7.1, step elute with 50 mM acetate, pH 4	pH > 7
Prototype from Prometic BioSciences Inc. (Burtonsville, MD, USA)	Small-molecule mimetic (MIM)	Bind-and-elute	25 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 7.1, step elute with 0.1 M acetic acid, pH 2.8	pH > 7

CV: column volumes; MES: 2-(*N*-morpholino)ethanesulfonic acid; bind-and-elute columns were washed with 3 CVs of equilibration buffer after loading prior to gradient elution.

For the ELISA (an in-house assay run at Genentech), affinity purified goat anti-CHOP antibodies were immobilized on microtiter plate wells. Dilutions of the pool samples were incubated in the wells, followed by an incubation with peroxidase-conjugated goat anti-CHOP. The horseradish peroxidase enzymatic activity was quantified with *o*-phenylenediamine. Samples were serially diluted in assay diluent so that the absorbance reading fell within the range of the standard curve (1.5–400 ng/ml).

3. Results and discussion

Little process development was performed to establish the operating conditions given in Table 1. Furthermore, all of the columns were loaded to 10 mg antibody per ml resin at a flow rate of 100 cm/h (five column volumes per hour). We chose to evaluate the purification performance of these resins using operating conditions (load, flow rate) less challenging than what is typically employed at large-scale with the hope that we could identify processes that significantly remove host cell proteins. Chromatograms from typical bind-and-elute (Fig. 1) and flow-through (Fig. 2) purification runs show that the antibody eluted as a single peak, and the entire peak was collected. Each step could be optimized to maximize purification performance (for example, by changing elution conditions or pooling criteria), while minimizing operating cost.

Although protein A chromatography followed by cation-exchange chromatography removed more CHOPs than any of the non-affinity processes evaluated after two steps, significant CHOP removal was achieved with several of the non-affinity approaches (Table 2). Five approaches resulted in CHOP levels below 1000 ng CHOPs/mg antibody after the second step.

Of the non-affinity capture steps, the ABx column removed the most CHOPs from harvested cell culture formulation. However, the pools of the two-step processes that employed ABx capture did not contain the lowest CHOP levels. Indeed, step order significantly affected purification performance in several of the non-affinity processes. For example, the ABx-S pool contained 1000 ng CHOPs/mg antibody, while the S-ABx pool contained 140 ng CHOPs/mg antibody. The S-Q pool contained 80 ng CHOPs/mg antibody, while the Q-S pool contained 900 ng CHOPs/mg antibody.

Several hypotheses could explain why step order matters. Binding competition between CHOP species could be occurring. Another explanation is that the resin in the capture (i.e. first) position could be overloaded with impurities. That further purification is observed when applying the pool of the first step to an identical second step supports this hypothesis.

CHOP clearance achieved with a third non-affinity step was evaluated for any of the non-affinity processes yielding <500 ng CHOPs/mg antibody in the antibody pools coming off of the second column. (The <500 ng/mg requirement was chosen arbitrarily to keep the number of experiments required within reason.) Three separate three-step non-affinity processes (S-Q-HIC, S-Q-ABx, S-ABx-Q) removed CHOPs to below the level of CHOP detection (Table 2).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the final pools from each of these processes also showed purities equivalent to those using a traditional three-step process with protein A capture (Fig. 3). It is uncertain whether an extra light, hazy band at

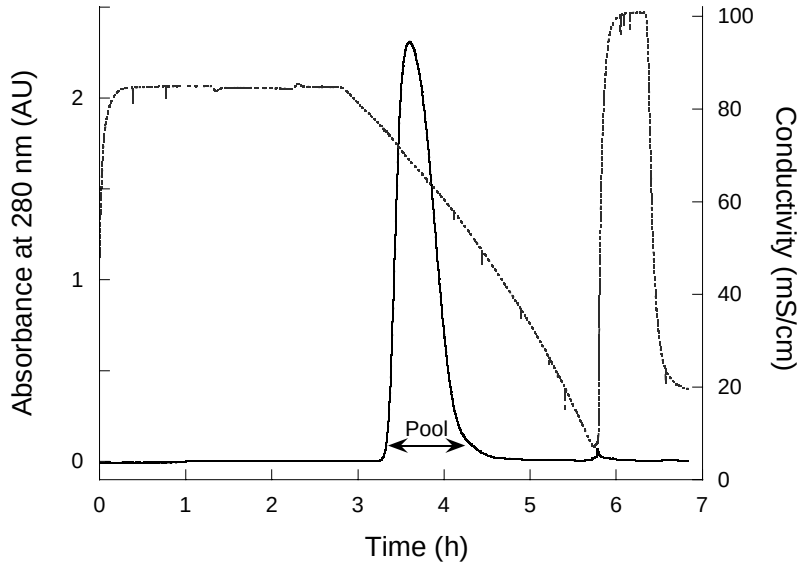

Fig. 1. Typical chromatogram from a bind-and-elute hydrophobic interaction (HIC) step. Load material was a cation-exchange pool [harvested cell cultured fluid (HCCF) purified on a cation-exchange column]. Prior to loading on the HIC column, the cation-exchange pool was conditioned to a final concentration of 0.8 M Na_2SO_4, and the pH of the pool was adjusted to pH 6.0 with NaOH/HCl. The HIC column used Phenyl-Sepharose Fast Flow Low Sub in a 20 cm × 0.66 cm i.d. column at a flow rate of 100 cm/h. The column was equilibrated with 8 CVs of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–0.8 M Na_2SO_4 pH 6.0, loaded to a density of 10 g/l (gram antibody per liter column volume), washed with 3 CVs of 50 mM MES–0.8 M Na_2SO_4 pH 6.0, eluted with a linear gradient from 50 mM MES–0.8 M Na_2SO_4 pH 6.0 to 50 mM MES pH 6.0 over 15 CVs, regenerated with 3 CVs of 0.5 M NaOH, and stored in 4 CVs of 0.1 M NaOH. The pool arrows indicate the area of the chromatogram where the purified antibody was collected during gradient elution.

$\sim 35 \times 10^6$ kDa appears in the S-Q-ABx and S-ABx-Q pool lanes (lanes 4 and 5) of the non-reduced gel; however, no new protein bands appeared in the reduced gel. We can not explain the vertical lines off of the heavy chain bands in the reduced gel. It could be an artifact caused by the buffer front. Nevertheless, the profiles of the antibody from the non-affinity processes were consistent with the profile of the antibody from the traditional protein A process.

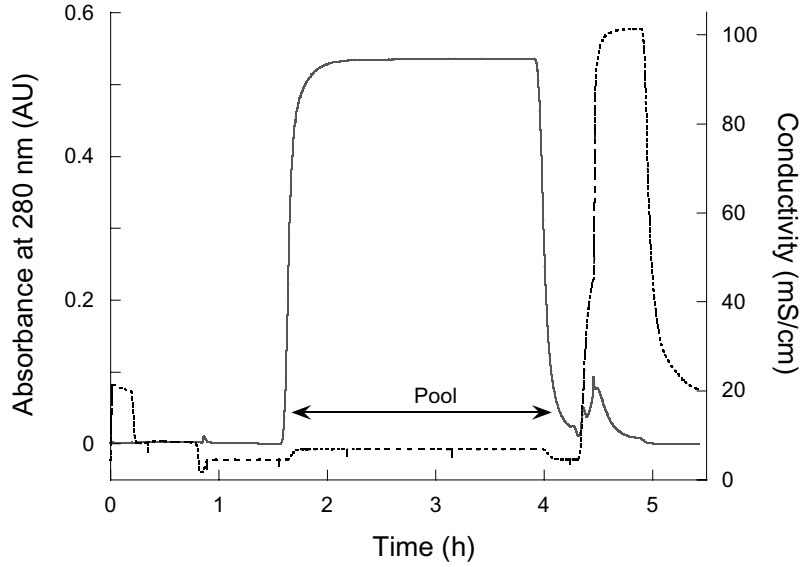

Fig. 2. Typical chromatogram from an anion-exchange (Q) flow-through step. Load material was a cation-exchange pool (HCCF purified on a cation-exchange column). Prior to loading on the Q column, the conductivity of the cation-exchange pool was adjusted to <7 mS/cm using water, and the pH of the pool was adjusted to pH 8.0 with NaOH/HCl. The Q column used Q-Sepharose Fast Flow in a 20 cm × 0.66 cm i.d. column at a flow rate of 100 cm/h. The column was equilibrated with 8 CVs of 25 mM Tris–50 mM NaCl pH 8.0, loaded to a density of 10 g/l (gram antibody per liter column volume), washed with 3 CVs of 25 mM Tris–50 mM NaCl pH 8.0, regenerated with 3 CVs of 0.5 M NaOH, and stored in 4 CVs of 0.1 M NaOH. The pool arrows indicate the area of the chromatogram where the purified antibody was collected during flow-through.

Table 2
CHOP removal over three-step non-affinity purification processes

Step 1	CHOP (ng/mg antibody)	Step 2	CHOP (ng/mg antibody)	Step 3	CHOP (ng/mg antibody)
Q	23,000	Q	14,000		
		HIC	3,000		
		S	900		
		HCI	11,000		
		ABx	1,000		
HIC	26,000	Q	9,900		
		HIC	2,400		
		S	900	Q	22
		MIM	6,000	HIC	20
		ABx	400	S	14
				ABx	13
ABx	6,600	Q	3,100		
		HIC	2,400		
		S	1000		
		HCI	2,800		
		ABx	1,700		
S	**14,000**	**Q**	**80**	Q	30
		HIC	600	**HIC**	**<2**
		S	2,100	S	10
		MIM	1,900	**ABx**	**<2**
		ABx	**140**	**Q**	**<2**
				HIC	6
				S	50
				ABx	28
ProA	**300**	**S**	**30**	**Q**	**<2 (control)**

Only those steps achieving 80% antibody recovery or greater than a three-fold reduction in CHOPs are illustrated. The harvested cell culture formulation loaded onto step 1 contained 220,000 ng CHOPs/mg antibody. CHOP clearance achieved with a third non-affinity step was evaluated for any of the non-affinity processes yielding <500 ng CHOPs/mg antibody after the second step.

Step and process yields of the S-Q-HIC, S-Q-ABx, S-ABx-Q processes were comparable to those of the control protein A process (Table 3). The overall yields for the non-affinity processes ranged from 76 to 88%, while the overall yield for the control protein A process was 85%. How step yield and purification performance are affected by the pooling parameters should be investigated.

Determining whether three-step non-affinity processes should be employed to purify antibodies for the purpose of large-scale drug manufacture requires additional studies. For example, the ability of the S-Q-HIC, S-Q-ABx, and S-ABx-Q processes to remove host cell DNA, small molecules, and antibody variants must be evaluated, and process robustness and process economics must be investigated. Many of these chromatography steps may not provide as much virus clearance as protein A but several alternative methods, such as filtration or chemical inactivation, may be employed [18,19,31,32]. Although most of the resins are stable to sodium hydroxide, resin cleanability must be investigated. For example, we found that harvested cell culture fluid components that bound to the anion-exchange resin in the capture step were not eluted (even after cleaning with 1 M NaOH), leaving the top 5 cm of the column discolored with a dark residue. The purification performance of the S-Q-HIC, S-Q-ABx, and S-ABx-Q processes under more practical operating conditions (e.g. higher flow rates

Table 3
Step and process yields

Process			Overall yield (%)
ProA 97%	S 89%	Q 98%	85
S 96%	Q 100%	Abx 79%	76
S 96%	Q 100%	HIC 89%	85
S 96%	Abx 92%	Q 100%	88

and antibody loads) must also be tested and the process economics evaluated.

In summary, a factorial approach in which any one of seven chromatographic separation mechanisms is tested in each step of a three-step chromatographic purification process has been applied for the purpose of large-scale antibody purification process development. This approach is novel in that, as opposed to making a one-to-one comparison of a protein A step with a non-affinity step at the same place within a process, it examines whether the same purification obtained with a process incorporating protein A chromatography can be achieved using a process with no affinity chromatography steps. To our knowledge, this is the first time such an approach has been applied for the purpose of chromatography process development. Moreover, several three-step processes that do not require a protein A step to remove host cell proteins in harvested call culture fluid to less-than-detectable levels have been identified using this approach. The non-affinity processes could offer such advantages as lower cost, eliminated ligand leaching, and greater resin stability.

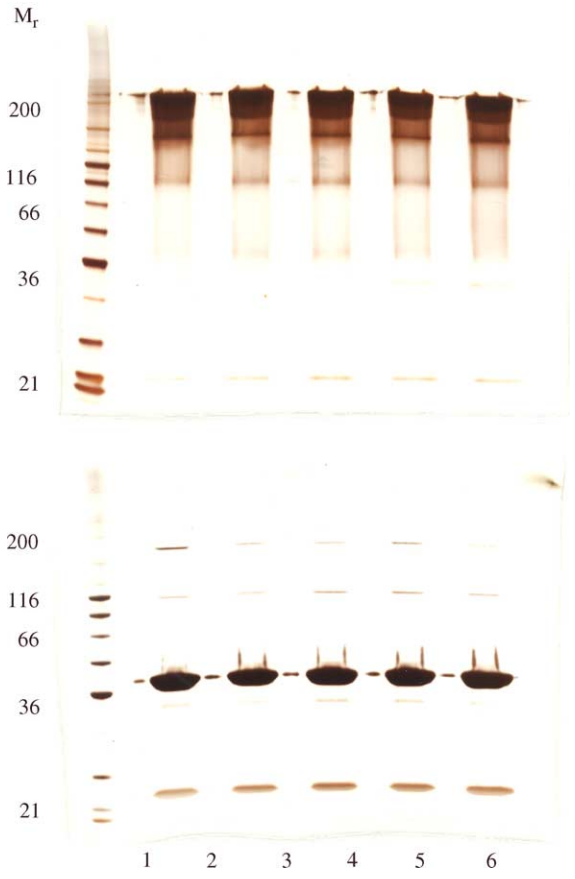

Fig. 3. Silver-stained SDS-PAGE of antibody purified by three-step affinity and non-affinity processes. Non-reduced and reduced gels are shown on the top and bottom, respectively. Lanes: (1) molecular mass standards (200 000, 116 000, 97 000, 66 000, 55 000, 36 000, 31 000, 21 000 and 14 000); (2) bulk antibody (i.e. antibody purified at 12 K scale); (3) ProA-S-Q pool; (4) S-Q-HIC pool; (5) S-Q-ABx pool; (6) S-ABx-Q pool.

4. Conclusions

We have determined that the same host cell protein removal obtained with a process incorporating protein A chromatography can be achieved using a process with no affinity chromatography steps. We identified three non-affinity purification processes (S-Q-HIC, S-Q-ABx, and S-ABx-Q) that remove CHOPs to levels comparable to the traditional protein A process, ProA-S-Q. The order of the chromatography steps within the process significantly affected CHOP clearance.

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