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A potential generic downstream process using Cibracon Blue resin at very high loading capacity produces a highly purified monoclonal antibody preparation from cell culture harvest☆

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Abstract

The use of a dye-ligand chromatography for the purification of monoclonal antibody (MAb) from cell culture and other feed streams has been largely overlooked in large scale production. Cibracon Blue dye (CB), a polycyclic anionic ligand, interacts with protein through a specific interaction between the dye, acting as a mimic of NAD^+ and $NADP^+$, or through non-specific electrostatic, hydrophobic, and other forces. In this paper, a CB resin was used to effectively and efficiently separate an IgG_4 MAb from host and process impurities following the capture of the MAb on a Protein-A (PA) column. The CB unit operation, challenged at ≤180 g MAb/L of resin with the PA eluate, reduced BSA (1–2 log), host cell protein (HCP; 2–3 log), MAb oligomer (31–85%), fragment (from ∼0.8% to <0.1%), and other undesired MAb species. Purity, as measured by non-reducing (NR) SDS-PAGE, was improved 33–85%, to 92–99.5% overall (>99% by reducing SDS-PAGE). A facile three column scalable production scheme, employing CB as the second column in the process was used to generate highly purified MAb from cell culture harvest derived from two media of very different compositions. Free CB dye was ≤1 ng/mg in MAb preparations purified through the three column process and then concentrated and buffer exchanged into the appropriate buffer using tangential flow filtration (TFF).

Keywords: Generic downstream process; Cibracon Blue resin; Monoclonal antibody; Purification of cell culture harvest

1. Introduction

Triazine dyes, first developed in the 1950s for the textile industry, have been widely used for large scale enzyme production but have found limited application in biopharmaceutical production [1]. This lack of usage in the pharmaceutical industry is principally due to a concern of toxicity associated with dye leakage resulting in dye presence in drug [2,3]. Furthermore, early resins also often lacked the chemical and mechanical stability that would allow them to be sanitized in NaOH and operated for multiple cycles before replacing the adsorbent [4].

Cibracon Blue (CB), a synthetic polycyclic dye, binds to a wide variety of proteins [1,5]. The interaction is either through a strong affinity association between protein and CB due to its mimicry of nucleotide cofactors, as in case of oxidoreductases, transferases, kinases and dehydrogenases [6], an interaction of dye at the enzyme active site resulting in competitive inhibition of substrate binding [7,8,9], or an interaction distal from the active site through multiple interaction involving hydrogen bonding, Van der Waals forces, and hydrophobic, and ionic bonding [10]. Albumin and blood coagulation factors interact through this latter mechanism [11]. Triazine "designer" dyes have also been developed, based on computer modeling of protein–dye interaction, and used for the large scale purification of yeast recombinant human albumin [12].

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The separation of a protein of interest from other materials on CB can be accomplished by manipulating pH and ionic strength [13], through the addition of detergents, chelating agents or dipole modifying agents [3], and in the case of an affinity interaction, the inclusion of NADP+, ATP, AMP [14], or enzyme substrate [15], to elute bound enzyme after removing impurity. The choice of anions, and the presence of divalent metal ions, also affects dye–protein interaction [16–18].

The focus in this paper was to develop a facile and scalable CB unit operation with high loading capacity, throughput, and recovery that effectively reduced product and process impurity in antibody preparations post Protein-A (PA) chromatography. In the CB operation described here, monoclonal antibody (MAb) weakly interacts with the resin and flows through the column whereas impurities are either bound to the resin, or occur at the distal end of flow through, which is not collected as part of the product pool. This flow through design resulted in a high MAb loading capacity ($\leq$180 g/L) as the resin binding components (impurities) are at a relatively low level, and also minimized dye leaching into the product pool. Since PA resin is typically used as the capture step for many commercial antibody processes, CB was examined as the second column operation. A downstream process consisting of harvest clarification, three columns and tangential flow filtration (TFF) was developed that resulted in a highly purified MAb preparation from two media of very different compositions (with and without BSA; Fig. 1). This process for purifying MAb from cell culture harvest has potential as a "generic" or "platform" system applicable to a number of MAbs.

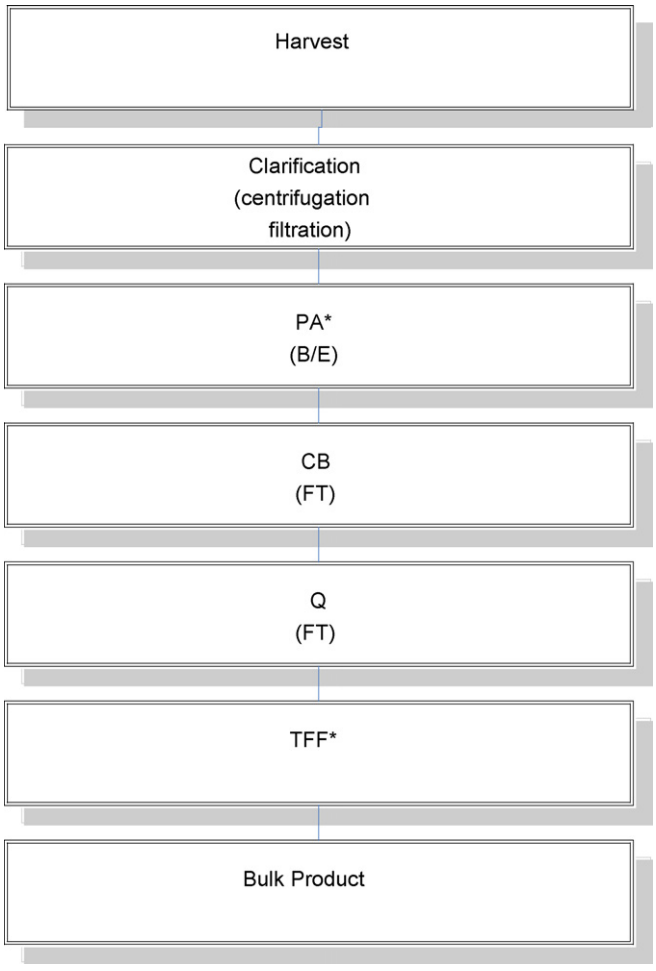


Fig. 1. Flow diagram of a proposed downstream process for purifying MAbs from cell culture harvest. The asterisks refer to the likely addition of viral inactivation technology post the PA unit operation (low pH inactivation of enveloped virus) and generally before, or after, TFF (nanofiltration) for a production downstream process. Nanofiltration removes virus from the product feed stream by physical exclusion and absorption to the filter matrix.

2. Experimental

2.1. Materials

NS0 cell harvest was clarified by an SA-1 centrifuge (Westfalia Separator, Oelde, Germany) followed by 0.2 μm absolute filtration (Millipore Corp., Billerica, MA, USA). rmp PA-Sepharose FF and Q Sepharose-FF resin (GE Healthcare, Piscataway, NJ, USA), and Trisacryl Plus LS resin (CB resin; Pall Corp, Northborough, MA) in 0.1N NaOH were packed at 300 cm/h × 1.6 cm, or 5 cm, × 10 cm diameter XK columns (GE Healthcare) which were then attached to an automated chromatography unit (AKTA^{TM} 100 Explorer from GE Healthcare, Piscataway, NJ, USA) or a manual system. The manual system consisted of a peristaltic pump (Watson Marlow 101 U/R or 600 series, Medford, MA, USA), in-line UV-1 monitor (2 mm or 5 mm cell path length from GE Healthcare, Piscataway, NJ, USA), in-line conductivity meter (EC 09101-00, Cole-Palmer Co., Chicago, Illinois, USA) The absorbance and conductivity traces were taken using a Kipp and Zonen BD-12 chart recorder (Bohemia, NY, USA). Tangential flow filtration occurred on a Pellicon II plate and frame system containing 0.1 m^2 of 30 kDa BioMax membrane (both from Millipore Corp., Billerica, MA, USA), associated 0.375″ tubing, pump (Watson Marlow Series 600, Medford, MA, USA) pressure gauges (three 0–60 psig in line, Anderson Instrument Co., Fultonville, NY, USA) and three 3/4″ diaphragm valves (ITT, Lancaster, PA, USA).

The MAb concentration was determined on a PA ID cartridge (Applied Biosystems, Foster City, CA, USA) on an Agilent 1100 Series HPLC system (Agilent Corp., Palo Alto, CA, USA). The MAb fragment and oligomer content were determined by gel permeation (GP) on a 7.8 mm × 30 mm TSK-GEL G3000 SW_{xl} column [ToSoHaas, Montgomeryville, PA, USA]) on an Agilent 1100 Series HPLC system. The residual PA (rPA) and host cell protein (HCP) ELISA were commercial kits produced by Cygnus Corp (San Francisco, CA, USA) and Repligen Corp (Cambridge, MA, USA), respectively. After sample digestion with Proteinase K (Roche Applied Sciences, Indianapolis, IN, USA), SDS extraction (Waco Pure Chemicals, Osaka, Japan) and heat denaturation, DNA was determined using the Threshold system (Molecular Devices, Sunnyvale, CA, USA).

SDS-PAGE was performed on Novex® 12% Tris-glycine gels (Invitrogen Corp, Carlsbad, CA, USA) in a Novex® system according to the gel and system recommendations. Proteins were separated under reducing (R; dithiothreitol), or non-reducing conditions (NR), then stained with Coomassie Blue

R-350 and then quantified using the Pharmacia ImageMaster™ (GE Healthcare, Piscataway, NJ, USA).

SDS-PAGE and gel permeation standards were purchased from Invitrogen (Mark 12 MW Standards, Invitrogen Corp., Carlsbad, CA) and Biorad (Bio-Rad Labs, Hercules, CA, USA), respectively.

2.2. Purification of NS0 cell culture harvest

Columns were qualified by height equivalent to a theoretical plate (HETP; ≤0.09) and asymmetry factor (Af; 1.0 ± 0.2) by standard methodology using a 1.5% (v/v, of column) injection of 2 M NaCl, 0.1 M NaOH at the column inlet.

NS0 cells, containing the MAb plasmid of interest, were inoculated into glass bioreactors (Bellco Glass, Vineland, NJ, USA), filled with 36 L of either a proprietary media with BSA, or a second proprietary media that did not contain BSA. Harvest was collected from the bioreactors after several days. The clarified material was loaded at 20–25 g MAb/L onto an rmp PA-Sepharose FF column previously equilibrated with 20 mM succinate, pH 6.0. Succinate anion was chosen for the PA buffers based on previous studies which showed higher MAb recovery with this ion versus buffers constructed with citrate, phosphate, acetate, or glycine (results not shown). The column was then washed with that same buffer until the A_{280} nm was ≤0.05 units, and the bound MAb eluted with 20 mM succinate, pH 3.60. The pool, 3–4 column volumes (cv), was adjusted to pH 6.0 and the desired conductivity for loading onto the CB column (e.g. 12 mS/cm ± 0.5 mS/cm for a CB column equilibrated into 20 mM succinate, 100 mM NaCl, pH 6.0) using 0.2N NaOH and 2 M NaCl, respectively. NaOH (0.2N) and NaCl (2 M) were used to minimize volume expansion during the adjustment of the pH and conductivity, respectively. Material pre and post pH and conductivity adjustment were comparable by NR SDS-PAGE, GP-HPLC and MAb concentration (A_{280} nm; result not shown).

Salt concentrations (NaCl) from 50 mM to 1000 mM in pH 6.0 succinate buffer were chosen for study on the CB operation. The CB column was first rinsed with several column volumes of buffer until the pH and conductivity of the effluent matched that of the buffer. After equilibration the column was loaded with protein, and then washed with the same buffer until the absorbance at 280 nm was ≤0.2 units. The MAb containing column flow through and wash were analyzed by the methods described below. The column was then sanitized with several column volumes of 0.1N NaOH and stored in 50 mM succinate, 2 M NaCl, pH 4.0. The operation was performed at 200 cm/h.

In some cases the CB eluate was further subjected to purification on a Q Sepharose-FF column (XK column, 1.6 cm or 5 cm × 10 cm). This column operation was performed with the same buffer used for chromatography on the CB column. The MAb containing flow through from this column was concentrated by tangential flow filtration (TFF) on a 50 kDa membrane (Millipore Pellicon II membrane).

The Q unit operation was placed into the process upon completion of the development of the CB unit operation. The desired operating buffer on the CB column was determined to be 20 mM succinate, 100 mM NaCl, pH 6.0, and this buffer was subsequently used for both Q and CB unit operations. The Q column was equilibrated into this buffer, loaded with unadjusted (pH or conductivity) CB pool, washed with the same buffer until the A_{280} nm was ≤0.05 units. The flow through and wash contained the MAb. The column was then sanitized with 0.5 M NaOH, 1 M NaCl, and stored in 0.1N NaOH. The operation was performed at 200 cm/h.

Q column pools were concentrated to 7 g/L on the TFF system and then buffer exchanged into phosphate buffered saline, pH 7.2 (PBS). The retentate containing the MAb was collected followed by a 1.5 system volume rinse using PBS. The system was rinsed with 10 system volumes of water, followed by 10 system volumes of 0.1N NaOH, and then stored in 0.1N NaOH.

The same strategy described above for determining the appropriate salt and pH conditions on the CB unit operation would be employed to adapt the CB unit operation for the separation of additional MAbs of interest from cell and process impurities. The salt concentration and pH would be adjusted such that the MAb of interest flows through the column and impurities are retained. Those buffer conditions will be dependent on the physical and chemical characteristics of the antibody such as its p*I* and hydrophobicity. The subsequent Q unit operation would likely use the same buffer used on the CB column. TFF, post the Q unit operation, would be performed as described above except that the solution for buffer exchange would be specific for the antibody of interest such that it maximized the stability of that molecule (see Fig. 1 for a proposed processing scheme).

2.3. Sample analysis

Samples were analyzed for MAb amount (A_{280} and/or Protein-A HPLC), general purity (reduced and non-reduced SDS-PAGE), cellular and process impurity (HCP, BSA, rPA, DNA) and undesired forms of the MAb (oligomer and fragment).

MAb concentration in the PA column eluate, and subsequent unit operations, was determined at A_{280} using an extinction coefficient of $E^{1\%}{}_{A280} = 14.10$. The MAb concentration in the harvest and PA eluate was determined by PA HPLC. The column was equilibrated with 10 mM sodium phosphate, 150 mM NaCl, pH 7.2, samples and MAb standards injected, the column washed to baseline with equilibration buffer, and bound MAb eluted with 12 mM HCl, 150 mM NaCl, pH 2.0 (detection at 280 nm). MAb concentration was determined by comparing the sample elution peak area to the elution peak area for MAb standards (0.25–2 mg/mL).

The molecular weight (mw) of the components in MAb preparations separated by SDS-PAGE was determined by comparison of the sample Rf (distance [cm] from the stacking/running gel interface to the band of interest) to a curve composed of Rf values for several mw standards. The standard Rf values were plotted versus the log of their mw.

GP-HPLC determination of monomeric (intact), oligomeric, and other forms of MAb was accomplished under isocratic conditions in 40 mM sodium phosphate, 150 mM NaCl, pH 7.2 (0.5 mL/min). The mw of the sample components was deter-

mined by comparing their retention time with the retention time of protein standards plotted versus log mw of these standards. The percentage of each MAb form was determined from its area under the peak versus the total peak area of all MAb forms.

The PA and HCP ELISA were commercial kits performed according to the manufacturer procedure and recommendations. The BSA ELISA uses an affinity purified anti-BSA MAb coating antibody (Sigma Chemical, St. Louis, MO, USA) and a peroxidase labeled rabbit anti-BSA conjugate detection antibody (MP Biomedicals, Irvine, CA, USA). Samples and BSA standards in PBS (1–50 ng/mL) were added (100 μL) to the individual wells of 96 well ELISA plates (Corning Life Sciences, Acton, MA, USA) following the coating process. The wells were washed with PBS containing 0.05% Tween-20 after the coating, sample addition, and detection stages. Bound BSA was detected with the peroxidase substrate *ortho*-phenylenediamine (OPD, Sigma Chemicals, St. Louis, MO, USA). The substrate was added to the wells (100 μL) for 20 min before the reaction was stopped with the addition of 4.5 M sulfuric acid. The A_{490} nm for each well was determined using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). The amount of BSA in the sample was determined from the BSA standard curve.

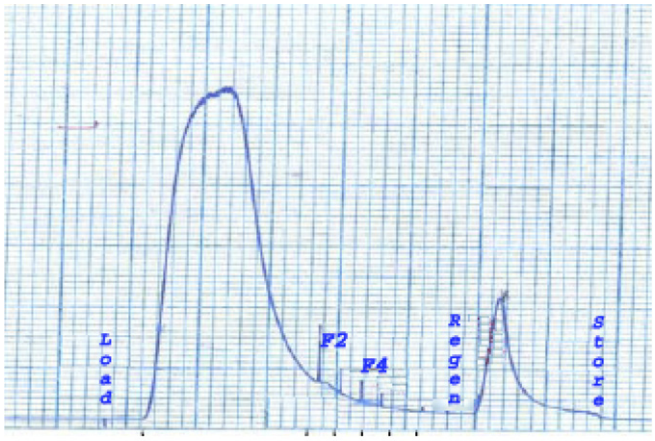
DNA was determined following sample digestion with Proteinase K (Roche Applied Sciences, Indianapolis, IN, USA), SDS extraction (Waco Pure Chemicals, Osaka, Japan) and DNA heat denaturation. Samples, positive and negative controls, and DNA standards were assayed using the Threshold system (Molecular Devices, Sunnyvale, CA, USA).

CB was determined by a competitive ELISA (Ciphergen Biosystems, Cergy-Saint-Christophe, France). In this assay, anti-CB rabbit antibody competes with a hemoglobin–dye conjugate, absorbed onto the well, for CB present in the sample [19]. The anti-CB antibody is specific for the anthaquinone portion of the dye and detects both free CB and CB with part, or all, of the resin attachment spacer arm. The anti-CB antibody hemoglobin–dye conjugate complex was quantified with peroxidase labeled anti-rabbit IgG followed by a peroxidase substrate. Duplicate blanks, controls, standards, and triplicate test samples, were tested in the same ELISA plate. The 100% inhibition point (positive control) was generated by adding excess CB (10 μg/mL) to the sample, and 0% inhibition (negative control) by not adding CB to the sample. The standard and sample values were expressed as a percentage of the positive control absorbance ($x = 1 - \{$(sample average value/negative control value) $\times$ 100$\}$). The standards were plotted as the percentage of inhibition (y-axis) versus the log of dye concentration (x-axis). CB in an unknown sample was then calculated based on its percentage inhibition in relationship to the standard curve.

3. Results

CB resin has a strong affinity for albumin, a major component in one of the production media, and therefore a major impurity in the chromatography feed stream. CB resin was examined for its effectiveness in the reduction of albumin and other impurities.

The initial utility of using CB for albumin reduction was determined by spiking purified MAb, in 20 mM succinate buffer, pH 6.0, with albumin (1:10, w/w), and loading this material (10 mg/mL) onto a CB column. The CB column was previously equilibrated into this same buffer. Succinate ion was chosen because it effectively buffers at pH 6.0 (pK_{a2} of 5.57), a pH that is slightly below this MAb's pI range (6.2–6.8) that would likely result in minimal antibody binding to the resin. A series of step elutions with increasing NaCl (250 mM, 400 mM, 600 mM, and 1000 mM) in 20 mM succinate, pH 6.0 was performed and the protein was collected and then analyzed by non-reducing SDS-PAGE. The MAb (~160 kDa) was present in 250 mM NaCl whereas albumin (~66 kDa) was not detected until 600 mM NaCl (result not shown). The large increase in NaCl required to elute BSA, versus MAb, suggested that a unit operation could be designed to retain BSA and allow MAb to flow through. Additional experimentation with a 7 cv NaCl linear gradient (50–300 mM) in 20 mM succinate, pH 6.0, determined an antibody elution range of 80–100 mM NaCl (result not shown).



Fraction #	Protein (% total load)	Intact MAb (% total MAb)
Load	100	94.2
1	84	99.4
2	2	99.0
3	1	98.9
4	< 1	98.7
5	< 1	98.6
Regeneration	nd	~50

Fig. 2. Reduction of MAb oligomers on the CB unit operation. A MAb preparation, containing ~6% oligomer, was adjusted with constant stirring to pH 6.0 (0.2N NaOH) and to 100 mM NaCl (2M NaCl). This material was loaded (20 mg/mL) at 200 cm/h onto a CB column (1.6 cm × 10 cm) equilibrated into 20 mM succinate, pH 6.0, 100 mM NaCl. The column was then rinsed with this same buffer until the A_{280} was <0.05 units. One major fraction, several small fractions at the tail end of the flow through, and the sanitization material, were collected and analyzed for the presence of intact MAb (~160 kDa) and oligomeric antibody (≥320 kDa) by GF-HPLC. For the sanitization sample, a sample of the effluent was collected as the A_{280} rapidly rose, but while the pH was ~7–8. On the chromatogram below, the chart recorder setting was 1 mm/min and the full scale absorbance (280 nm) setting was 10.

Subsequent studies employed ≥100 mM NaCl in 20 mM succinate, pH 6.0 and the development of an operation in which this MAb flowed through the CB column. The separation of oligomer, and other undesired MAb forms, from intact MAb, was then studied using an oligomer enriched MAb preparation. The chromatogram and the analysis of fractions from this separation on CB are shown in Fig. 2.

A review of the data collected from this chromatography run on CB resin shows a 90% decrease in oligomer in Fraction 1 in comparison to the CB load (0.6% versus 5.8%). The sanitization samples contained about 50% oligomer indicating that most oligomer remained bound to the resin.

Additional experiments were performed, with the oligomer enriched MAb preparation, to examine the effect of NaCl concentration (100 mM and 150 mM) and load amount (10 mg/mL and 20 mg/mL), on MAb recovery and oligomer reduction (results not shown). Although MAb recovery was similar for these experiments performed in 20 mM succinate, pH 6.0 (±3%), oligomer were slightly reduced at 100 mM NaCl versus 150 mM NaCl (Fraction 1 of 0.6% and 1.2%, respectively). 100 mM NaCl was chosen for further study.

Next, the CB unit operation was challenged with material derived from clarified cell culture harvest. The analysis of this chromatographic separation is shown in Table 1 (reduction of BSA, HCP, and oligomer), Fig. 3 (SDS-PAGE), and Fig. 4 (GF-HPLC).

The mean recovery for the three CB operations was 81 ± 2.6%. The CB unit operation effectively reduced BSA (≥1.5 log), HCP (≥3.5 log), oligomer (≥44%), and increased antibody purity by 48–66% (Table 1). The reducing SDS-PAGE profiles were similar for PA and CB eluates (Fig. 3(A), lanes 2 and 3 versus lanes 4 and 5). The oligomer and half antibody, that are present in greater abundance in the non-reduced PA pools in comparison to the non-reduced CB pools (Fig. 3(B), lanes 2 and 3 versus lanes 4 and 5), are reduced, along with intact MAb, to heavy and light chains, exclusively.

The half antibody apparent in NR SDS-PAGE (Fig. 3(B), ~80 kDa) appears to be intact under physiological conditions upon analytical ultracentrifugation (result not shown). Presumably, the intact MAb is dissociated by SDS due to the absence of disulfide bonding between the two antibody heavy chains [20,21]. Since the amount of half antibody in the PA pool can be reduced by CB unit operation, it is likely that minor structural differences exist between half and intact antibody, possibly related to the absence of the disulfide bond.

The load onto the CB resin was then increased to 120 mg/mL ($n = 3$) and 180 mg/mL ($n = 2$) using PA product pools derived from clarified harvest. CB pool purity was then tested at these higher load amounts. The analysis of this chromatographic separation is shown in Table 2 (reduction of BSA, HCP, and oligomer and improvement in SDS-PAGE purity).

The recovery of MAb at loads of 120 mg/mL and 180 mg/mL of MAb were 83.1 ± 1.1%, and 84.3 ± 2.2%, respectively. The recovery values, and the reduction of HCP and BSA, were comparable at 120 g/L and 180 g/L load and similar to a ≤30 g/L load. The oligomer reduction (%) was slightly higher at 120 g/L and 180 g/L loads (65–81%) versus 30 g/L loads (44–64%). NR SDS-PAGE purity was slightly less at 120 mg/mL and 180 mg/mL load (92.0–94.5%) than 30 mg/mL load (94-9-96.8%). The inverse relationship between these two outputs may be related to a greater oligomer percentage in the PA pools used at the higher loads, or may reflect chromatographic performance with increasing load amount. Dye was not detected in the 5 mg/mL preparation derived from CB2 (≤1 ng/mg MAb).

The PA and CB unit operations were then challenged with harvest derived from a second medium of very different composition than the original medium. The analysis of this chromatographic separation is shown in Table 3 (reduction of HCP,

Table 1
Analysis of PA and CB pools derived from clarified harvest

Unit operation	BSA (ng/mg IgG)	HCP[a] (ng/mg IgG)	Oligomer (GP-HPLC) (% total IgG)	NR SDS-PAGE (% monomer)
PA1	26.8	4484	3.2	90.5
CB1	1.8	<0.93	1.3	96.8
Reduction	1.2 log	>3.68 log	59%[b]	
Improvement				66%[c]
PA2	46.5	3563	3.2	91.7
CB2	1.5	<0.84	1.8	96.3
Reduction	1.5 log	>3.63 log	44%[b]	
Improvement				48%[c]
PA3	33.5	2559	4.4	85.1
CB3	<0.34	<0.90	1.6	94.9
Reduction	>2.0 log	>3.46 log	64%[b]	
Improvement				66%[c]

Three separate batches of clarified harvest were processed through the PA unit operation (25 g/L load) and then onto a CB column (30 g/L) equilibrated into 20 mM succinate, 100 mM NaCl, pH 6.0. The column was washed with this same buffer till the baseline was 0.1 A_{280} units (Fig. 4). A single pool was collected at the point of A_{280} inflection to 10% of the baseline at the distal end of the flow through. The data analysis is presented in Fig. 2. SDS-PAGE analysis (non-reduced [NR] and reduced [R]) and GF-HPLC chromatograms of the PA and CB eluates are shown in Figs. 3 and 4, respectively.

[a] HCP (host cell protein).

[b] % reduction in oligomer: (% PA oligomer − % CB oligomer/% PA oligomer) × 100.

[c] % improvement in purity: (% CB purity − % PA pool purity)/(100 − % PA purity) × 100.

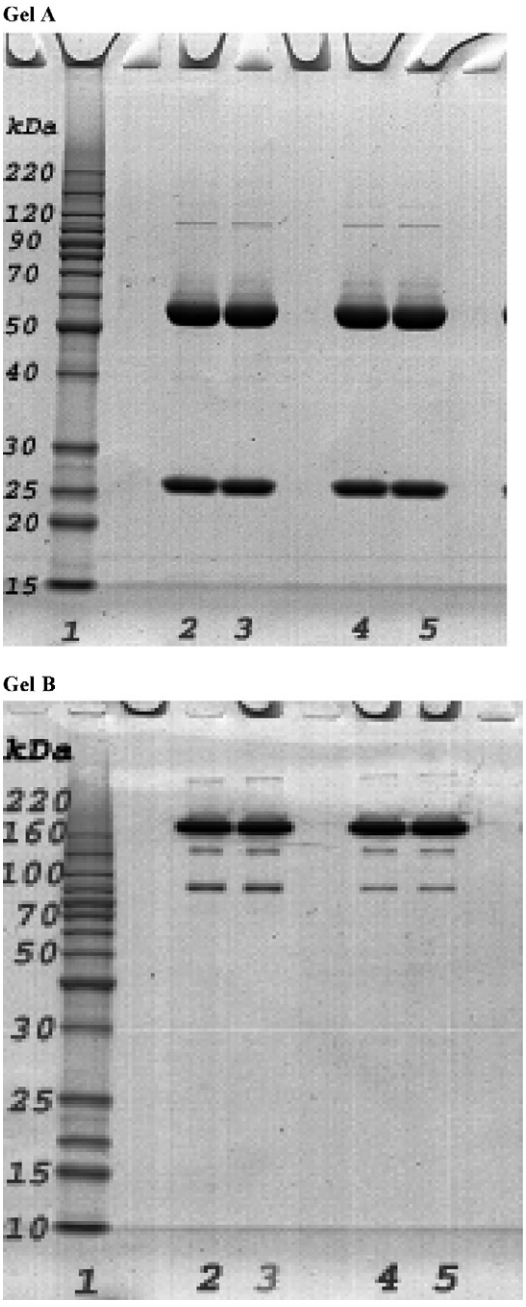


Fig. 3. Reducing (A) and non-reducing (B) SDS-PAGE of PA and CB product pools. Samples derived from two of the harvest were separated in gels A and B as described in *Conditions*. Lane 1 is mw standards; lane 2 and 3 are PA eluates 1 and 2, respectively; lane 4 and 5 are CB eluates 1 and 2, respectively. Equal amounts of sample (5 μg) were loaded in each lane.

and oligomer and improvement in SDS-PAGE purity). BSA was not measured as this material was not present in the cell culture medium.

Substantial impurity reduction was realized on the CB unit operation from this second, compositionally different, medium that did not contain BSA.

Lastly, MAb was purified from clarified harvest using a process consisting of PA-CB-Q-TFF, as described in *Conditions* and diagrammed in Fig. 1. The purity was determined for eleven lots, eight produced from BSA containing harvest ($n = 6$ at

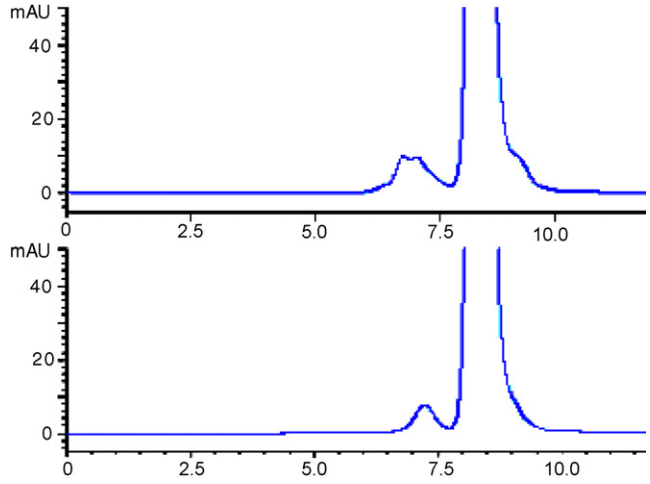


Fig. 4. GF-HPLC analysis of PA1 and CB1 eluates. Samples (20 μg) of PA1 (top chromatogram) and CB1 (bottom chromatogram) eluates were separated by GP-HPLC as described in *Conditions*. The peak to the left is oligomer, the right (main) peak is monomeric IgG, and the inflection at the tail end of the main peak is predominantly antibody fragment. The chromatograms were enlarged to enhance the oligomer peak and main peak tail inflection.

30 mg/mL load and $n = 2$ at 120 mg/mL), and three produced from harvest absent of BSA ($n = 3$ at 30 mg/mL load). One set of columns was used to process harvest containing BSA and a second set for harvest without BSA. Residual PA was not detected in the preparations (<0.29 ng/mg). The lots from BSA containing media showed a very low level of HCP (1.2 ± 1.2 ng/mg), BSA (0.56 ± 0.33 ng/mg), DNA (1.4 ± 0.2 pg/mg), oligomer ($0.90 \pm 0.54\%$,) and a high purity by non-reducing SDS-PAGE ($96.1 \pm 2.6\%$). The lots purified from media without BSA also showed very low levels of HCP (0.67 ± 0.06 ng/mg), oligomer ($0.42 \pm 0.09\%$), DNA (<1.2 pg/mg) and a high purity by non-reducing SDS-PAGE ($96.8 \pm 0.76\%$). Four of the 11 lots were tested for residual CB dye. Dye was not detected in three lots ($\leq$1 ng/mg) whereas one showed a detectible, but not quantifiable amount ($\sim$1 ng/mg).

4. Discussion

A CB unit operation was facile and effective for antibody purification, reducing several logs of BSA, HCP, and undesired forms of the antibody. The CB operation also resulted in an SDS-PAGE purity increase of 33–85% and a reduction in oligomer of 44–82%. MAb fragments were detected at 0.6–0.7% in the PA pool but were not present in purified MAb prepared following clarification by the PA-CB-Q-TFF process (result not shown).

There are several distinct advantages in product throughput using the processing scheme outlined in Fig. 1. The high column loading capacity on the CB resin reduces column volume and a single buffer, 20 mM succinate, 100 mM NaCl, pH 6.0, is used for the equilibration, and collection of MAb from the CB and Q unit operations. Furthermore, the CB column pool is applied directly (without any adjustment) to the Q unit operation. The Q column further reduces albumin from a mean of 2.95–0.53 ng/mg (for those lots prepared from media containing BSA) and also reduces MAb

Table 2
Analysis of CB pools from high MAb CB loads (120 g/L and 180 g/L resin)

Unit operation	IgG load (mg/mL resin)	BSA (ng/mg IgG)	HCP[a] (ng/mg IgG)	Oligomer (% total IgG)	NR SDS-PAGE[c] (% monomer)
PA1	25	227	3583	14.1	88.6
CB1	120	6.06	4.64	4.9	94.5
Reduction		1.57 log	2.89 log	65%[b]	
Improvement					52%[c]
CB2	120	5.10	5.03	3.9	92.9
Reduction		1.65 log	2.85 log	72%[b]	
Improvement					38%[c]
PA2	25	77[d]	1463[d]	5.9	81.9
CB3	120	0.32	0.36	1.1	92.0
Reduction		2.38 log	3.61 log	81%[b]	
Improvement					56%[c]
CB4	180	6.1	4.5	nd[e]	93.4
Reduction		1.6 log	2.90 log	21%[b]	
Improvement					42%[c]
CB5	180	8.1	6.1	4	92.4
Reduction		1.45 log	2.77 log	72%[b]	
Improvement					33%[c]

The chromatography conditions were as described previously (Fig. 2). Harvest was purified through the CB unit operation. PA1 eluate was used as the load for five of the six CB unit operations (all except CB3). CB1 was applied onto a Q column, the Q flow through concentrated to 5 mg/mL, buffer exchanged into PBS, and then tested for residual CB dye (as described in *Conditions*).

[a] HCP (host cell protein).

[b] % reduction in oligomer: (% PA oligomer − % CB oligomer/% PA oligomer) × 100.

[c] % improvement in purity: (% CB purity − % PA pool purity)/(100 − % PA purity) × 100.

[d] Two PA pools were combined for PA2.

[e] Not determined, sample storage error.

oligomer. MAb prepared using this processing scheme showed high purity by non-reducing SDS-PAGE (92.0–99.5%) and low amounts of HCP (<0.42–3.43 ng/mg), BSA (<0.53–1.2 ng/mg [purified from media containing BSA]), DNA (<1.5 pg/mg), MAb oligomer (0.34–1.6%), and MAb fragments (<0.1%).

Table 3
CB pool purity from a second cell culture medium

Unit operation	HCP[a] (ng/mg IgG)	Oligomer[b] (% total IgG)	NR SDS-PAGE[c] (% monomer)
PA1	2369	1.8	81.8
CB1	1.1	0.46	95.9
Reduction	3.33 log	74%	
Improvement			77%
PA2	1960	1.4	81.8
CB2	6.0	0.33	97.2
Reduction	2.51 log	76%	
Improvement			85%
PA3	1928	1.8	84.8
CB3	1.4	0.33	97.1
Reduction	3.14 log	82%	
Improvement			81%

The clarified harvest was separated on a PA and CB column as described previously in Fig. 2. The CB column was loaded at 30 mg/mL.

[a] HCP (host cell protein).

[b] % reduction in oligomer: (% PA oligomer − % CB oligomer/% PA oligomer) × 100.

[c] % improvement in purity: (% CB purity − % PA purity)/(100 − % PA purity) × 100.

Further reduction of oligomers and other undesired MAb forms are possible at the expense of decreased recovery on the CB unit operation (as these materials are concentrated toward the tail end of the CB pool). Oligomer might be further reduced by fractionation of the Q column flow through and subsequent pooling of only those fractions with low amounts of oligomer. Oligomer was reduced in the Q pool (0.34–1.6%) versus the CB pool (0.33–4.9%) suggesting that some oligomer remains bound to the Q resin and that segregation of monomer and oligomer is occurring during this unit operation.

The process described here (Fig. 1) should be amenable to several MAb by adjusting the pH (within a range of 5.5–8.5) and conductivity (within a range of 0–500 mM NaCl [or other salt]) of the CB and Q buffers to reflect the physical and chemical characteristics of that MAb, and thereby effect its separation from host and cell culture impurities. Many MAb have isoelectric points between pH 5.5–8.5, and albumin, a major impurity in some media, binds very tightly to the CB resin within that pH range. This process has another potential advantage of processing MAb from different media, since media changes often occur during the time period from early through late clinical trials. A potential limitation is the relatively large buffer tanks that are necessary to collect the CB and Q pools, due to volume expansion at each of these steps. However, a TFF system could be placed between these steps if facility volume constraints existed.

Commercial CB resins are available that are mechanically stable to relatively high flow rate and stable to base sanitization. These resins are used in several commercial processes such as the production of albumin from Cohn fraction I, Recombulin®

(yeast derived recombinant human albumin), and Thyrogen® (thyroid stimulating hormone) [11,12,22,23].

Dye leaching from CB resins appears to be relatively low. Resin leachate levels under typical operating conditions are in the picogram to nanogram level, six to nine orders of magnitude lower than the safe limit [24]. Dye leakage decreases with repeated column usage and occurs through matrix degradation rather than disruption of the dye–matrix bond [25–27]. CB dye is also reported to be non-toxic to cells and chromosomes (according to WHO guidelines), non mutagenic, non genotoxic, and unlikely to be carcinogenic, or antigenic [28,29]. The small amount of leached dye would likely be further reduced by subsequent downstream processing. CB dye was not detected in three of the four tested purified MAb preparations from the PA-CB-Q-UF process ($\leq$1 ng/mg). The low amount detected in one material ($\sim$1 ppm) may be an artifact based on clearance studies performed on the Q unit operation that showed >5 log of clearance (data not shown).

There will be MAbs which are not readily adaptable, or applicable, to this processing scheme (Fig. 1). This might be due to antibody instability in the appropriate buffers for the PA, CB and Q operations, or a lack of separation between MAb and unwanted MAb forms and host cell impurities on the CB unit operation (after testing a wide range of buffer conditions). The MAb p*I*, its tendency to aggregate and other factors will affect its chromatographic characteristics. In these cases, orthogonal types of chromatography, hydrophobic interaction, ion-exchange, metal chelate, or hydroxyapatite should be examined. However, "difficult antibodies" that, for example, show limited stability, or solubility, tend to present development and production challenges on whatever mode of chromatography is employed.

In summary, the CB operation described in this paper is a facile and effective means of purifying MAb preparations derived from different media sources (with and without BSA), such that the resulting preparation contains ppm levels of impurities, and low levels of undesirable MAb forms. A downstream process consisting of PA-CB-Q-TFF appears to be a relatively simple, adaptable and effective means of obtaining highly purified MAb from different cell culture sources.

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