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Expanded bed protein A affinity chromatography of a recombinant humanized monoclonal antibody: process development, operation, and comparison with a packed bed method

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Abstract

We show that expanded bed protein A affinity chromatography using Streamline rProtein A media is an efficient method for purifying a recombinant humanized monoclonal antibody from unclarified Chinese hamster ovary cell culture fluid and that it provides purification performance comparable to using a packed bed. We determined that the dynamic capacity of the expanded bed media is related to flow rate (measured in column volumes per hour) by a power function, which allows a high capacity at a low flow rate. At 250 cm h^{-1} with a 25 cm bed height (10 column volumes h^{-1}), the dynamic capacity is 30 g l^{-1}. The yield and purity (measured by the amount of host cell proteins, DNA, SDS-PAGE, and turbidity) of the antibody purified by expanded bed is comparable to the yield and purity obtained on a standard packed bed method using Prosep A media.

Keywords: Recombinant antibody; Preparative chromatography; Expanded bed

1. Introduction

Recombinant humanized monoclonal antibodies have important therapeutic applications including the treatment of several forms of cancer (Carter et al., 1992; Anderson et al., 1996; Baselga et al., 1996; Bodey et al., 1996; Longo, 1996). Producing these proteins at large scale requires efficient bioprocess methods. A common bioprocess method for purifying antibodies is protein A affinity chromatography (Kagel et al., 1989), which selectively binds antibodies in complex solutions such as clarified cell culture fluid (Ey et al., 1978; Surolia et al., 1982; Lindmark et al., 1983; Reis et al., 1984). However, a packed bed protein A affinity column must have cells and cell debris removed from the cell culture fluid prior to loading, often by tangential flow filtration (van Reis et al., 1991). The ability to load unclarified cell culture fluid onto the protein A affinity column

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would eliminate a unit operation and thus could improve production efficiency and reduce production cost.

Expanded bed chromatography has been used to recover proteins directly from unclarified feedstocks such as yeast and bacteria homogenates, mammalian and hybridoma cell culture fluid, and transgenic milk (Frej et al., 1994; Hansson et al., 1994; Batt et al., 1995; Chang et al., 1995; Thommes et al., 1995; Chang and Chase, 1996; Frej, 1996; Johansson et al., 1996; Noppe et al., 1996; Pessoa et al., 1996; Thommes et al., 1996; Degener et al., 1998; Raymond et al., 1998). In this paper we show that expanded bed protein A affinity chromatography can be used to efficiently purify a recombinant humanized monoclonal antibody from unclarified Chinese hamster ovary cell culture fluid.

We first determined the effect of flow rate and bed height on the dynamic capacity of Streamline rProtein A media, and used this data to suggest a large-scale production scenario. The dynamic capacity depends on many factors, including the flow rate, bed height, type of protein A affinity chromatography media, the antibody concentration in the load, temperature, and the conductivity and pH of the load (Katoh et al., 1978; Tu et al., 1988; Fuglistaller, 1989; Kamiya et al., 1990; Schuler and Reinacher, 1991; Van Sommeren et al., 1992). This paper focuses on flow rate and bed height, primarily because they are easily varied in bioprocess applications. While changes in pH, conductivity, or temperature could denature, precipitate, or otherwise affect the antibody, changing the flow rate and bed height would have little impact on the antibody itself. Using an efficient operational protocol for expanded bed chromatography, we then compared the yield and purity of antibody purified using the expanded bed technique to the yield and purity of antibody purified by a standard packed bed technique using Prosep A media.

2. Materials and methods

2.1. Materials and instruments

Streamline media and columns were from Pharmacia Biotech (Uppsala, Sweden). Poros columns, and BioCAD instruments were from PerSeptive Biosystems (Framingham, MA, USA). Prosep A media was from BioProcessing (Consett, England). SDS-PAGE materials were from Novex (San Diego, CA, USA). The Mini-ultrasette device was from Filtron (Northborough, MA, USA). The turbidimeter was from Hach (Loveland, CO, USA). The DNA Threshold Assay Kit was from Molecular Devices (Sunnyvale, CA, USA).

Load material for preparative chromatography was either clarified cell culture fluid (clarified by tangential flow filtration, removing cells and cell debris) or unclarified cell culture fluid from Chinese hamster ovary cell culture that contained a recombinant humanized monoclonal antibody at a concentration of 0.5 g l^{-1}–0.75 g l^{-1}. The antibody was a fully humanized IgG1 with a molecular weight of 145 400, a pI of 8.8, and an extinction coefficient of 1.5. The unclarified cell culture fluid contained approximately 5% wet volume of solids. This load material was obtained from Genentech (South San Francisco, CA, USA).

2.2. Bed height

In this paper, the column volume (CV) and the bed height were always calculated using the settled bed height.

2.3. Preparative chromatography

Expanded bed preparative chromatography used Streamline rProtein A media (protein A immobilized on metal-core agarose media). Expanded bed experiments used a 2.5 cm inner diameter Streamline column. The column was equilibrated with 5 CV and washed with 7 CV of 25 mM Tris pH 7.2. The column was eluted with five CV of 25 mM sodium citrate pH 2.8, and regenerated with five CV of 1 mM sodium hydroxide pH 11. If storage was required, the column was stored in 50 mM sodium acetate pH 5.5, 2% benzyl alcohol. This preparative chromatography was run on a BioCAD.

Packed bed preparative chromatography used Prosep A media (protein A immobilized on controlled pore glass media). Packed bed experiments

used a 20 cm length column loaded to 20 g l^{-1}. Four buffers were used. Buffer A was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1; buffer B was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M tetramethylammonium chloride pH 5.0; buffer C was 25 mM sodium citrate pH 2.8; and buffer D was 2 M guanidine HCl, 10 mM Tris, pH 7.5. The column was equilibrated with five CV of buffer A, loaded, washed with 3 CV of buffer A, washed with 3 CV buffer B, washed with 3 CV of buffer A, eluted with 5 CV of buffer C, and regenerated with 3 CV of buffer D. Chromatography was performed at a flow rate of approximately 500 cm h^{-1}. Load material was clarified cell culture fluid.

2.4. Dynamic capacity

Dynamic capacity was measured at 1% breakthrough using an on-line assay. The on-line assay used a 30 mm length × 2.1 mm diameter Poros A/M (immobilized protein A, 20 μm particle size) column at room temperature. Buffer A was 8 mM sodium phosphate, 2 mM potassium phosphate, 3 mM potassium chloride, 137 mM sodium chloride, pH 7.2, and buffer B was 8 mM sodium phosphate, 2 mM potassium phosphate, 3 mM potassium chloride, 137 mM sodium chloride, pH 2.2. The assay was run on a BioCAD/RPM, and used two flow rates, one for run (5.8 ml min^{-1}) and one for purge (50 ml min^{-1}). On the BioCAD instrument, purging takes the column out of the flow path, allowing the increased purge flow rate to rapidly fill the flow path with buffer. After purging, the flow rate is decreased and the column is then placed back in line. The BioCAD/RPM software automatically integrates the antibody peak and displays the result immediately after the assay is finished. Detection was at 280 nm. The assay directly sampled the preparative column effluent using an in-line sampling T, with an injection volume of 500 μl. The method was: purge 5 ml buffer A; run 3 ml buffer A; inject, run 2.5 ml buffer A; purge 5 ml buffer B; run 2 ml buffer B. The assay was 2.0 min long.

When the load material was unclarified cell culture fluid, effluent from the preparative column was directed through a Mini-Ultrasette 0.3 μm tangential flow filtration device. The syringe pump on the BioCAD drew 2 ml through the membrane and into a 500 μl injection loop, and the clarified effluent was injected onto the on-line assay.

2.5. Analysis

Yield was determined as the percent of loaded antibody that eluted in the purified pool. Antibody concentration in the pool was determined by absorbance at 280 nm. Turbidity was measured on a Hach 2100N turbidimeter. DNA was measured using the Molecular Devices DNA Threshold Assay Kit. The amount of host cell proteins (Chinese hamster ovary proteins) was determined by ELISA using goat anti-(host cell proteins).

SDS-PAGE was performed using a 8–16% Tris–Glycine 1.0 mm × 12 lane gel and the Silver Express staining kit from Novex. Samples were run under reduced [5 μg of protein $(lane)^{-1}$] and nonreduced [2.5 μg of protein $(lane)^{-1}$] conditions.

3. Results and discussion

An efficient recovery method requires sufficient dynamic capacity for a reasonably sized column to purify a batch in a reasonable amount of time. In a study using various bed heights, linear flow rates, and load material in both packed and expanded beds, the dynamic capacity of a recombinant humanized monoclonal antibody on Streamline rProtein A media was related to the flow rate measured in CV h^{-1} by a power function (Fig. 1). Bed height and linear flow rate both affect the dynamic capacity; together bed height and linear flow rate determine the residence time. The inverse of residence time is flow rate in CV h^{-1}, calculated as the linear flow rate (measured in cm h^{-1}) divided by the bed height (measured in cm). A flow rate measurement in CV h^{-1} is independent of scale and is also independent of the aspect ratio of the column.

The Streamline column has a flow distributor plate as well as a screen to help evenly distribute flow into the expanded bed. The expanded bed

experiments in Fig. 1 using a 14 cm bed height and loading clarified cell culture fluid were performed with the screen in place, with a 2.75-fold expansion at 300 cm h^{-1} in equilibration buffer. When running the column with a 25 cm bed height, the screen was removed because unclarified cell culture fluid deposited debris on the screen that eventually increased the backpressure beyond the pressure limit of the Streamline column. Without the screen, the column was run with only the bottom flow distributor to evenly distribute flow. For the 25 cm bed height with no screens, flowing at 300 cm h^{-1} in equilibration buffer resulted in a 2.4-fold expansion. Despite this change in expansion, no change in dynamic capacity was observed when loading either clarified or unclarified cell culture fluid.

Flow rates above 375 cm h^{-1} produced an unstable interface at the top of the expanded bed between the Streamline media and the mobile phase. Above 425 cm h^{-1}, fines began migrating out of the bed. For Streamline rProtein A media that has not been elutriated, 375 cm h^{-1} may be the upper limit of linear flow rate for a robust process with no instabilities in the expanded bed. This limit helps define the range of operating conditions for the column. While a higher linear flow rate on a high bed height could improve production rate, we did not investigate the possibility of elutriating the Streamline media to allow these higher linear flow rates.

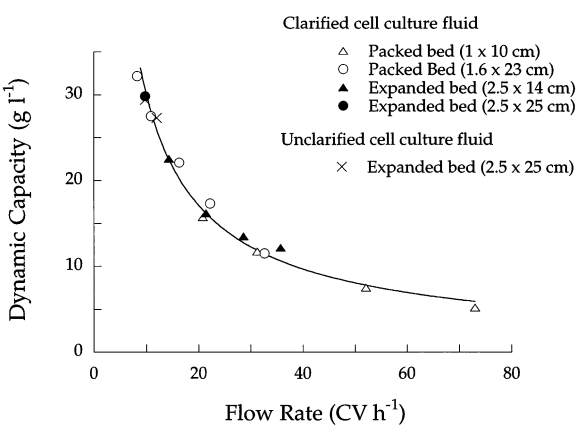

Fig. 1. The effect of flow rate on the dynamic capacity of Streamline A. Flow rate is measured in column volumes (CV) h^{-1}, and dynamic capacity is measured in grams of antibody per liter of column volume (g l^{-1}) at 1% breakthrough, with column volume for both calculated based on settled bed height. Column dimensions are measured in inner diameter × settled bed height. Load material is either clarified or unclarified cell culture fluid. Power function fit: $y = 199.5*x\,\hat{}\,(-0.8200)$, $R^2 = 0.982$.

The dynamic capacity was the same when loading clarified or unclarified cell culture fluid, and when running a packed or an expanded bed. This result suggests that for future development, optimal conditions could be found by loading clarified cell culture fluid onto a packed bed, which would accurately predict the dynamic capacity of the expanded bed loading unclarified cell culture fluid. This would significantly decrease the experimental complexity associated with obtaining and holding unclarified cell culture fluid, and running an expanded bed. The longest time the unclarified cell culture fluid was held during these studies was 36 h.

The power function relationship between dynamic capacity and flow rate (Fig. 1) means that at low flow rates (long residence times), dynamic capacity becomes very large. While packed bed chromatography using Prosep A media is typically run at 20 CV h^{-1} or higher, for Streamline rProtein A media lower flow rates (CV h^{-1}) are necessary to achieve comparable production rates.

Operating conditions for an efficient expanded bed process can be determined from the data in Fig. 1. At a bed height of 25 cm and a linear flow rate of 250 cm h^{-1} (10 CV h^{-1}), the dynamic capacity is 30 g l^{-1}. To prevent possible yield loss during loading due to antibody breakthrough, the column was loaded to 25 g l^{-1} (83% of the dynamic capacity). With an antibody concentration in the load of 0.63 g l^{-1}, a 5 CV equilibration, a 7 CV wash, a 5 CV elution, and a 5 CV regeneration, the total cycle time was 6.5 h (Fig. 2). Without screens, the bed expanded 2.2-fold in equilibration buffer and 2.5-fold when loading unclarified cell culture fluid. The column was regenerated with 1 mM NaOH pH 11, which eluted a small peak (Fig. 2). The expanded bed was stable during all phases of the process, with no channels observed and a clear interface between the top of the bed and the mobile phase.

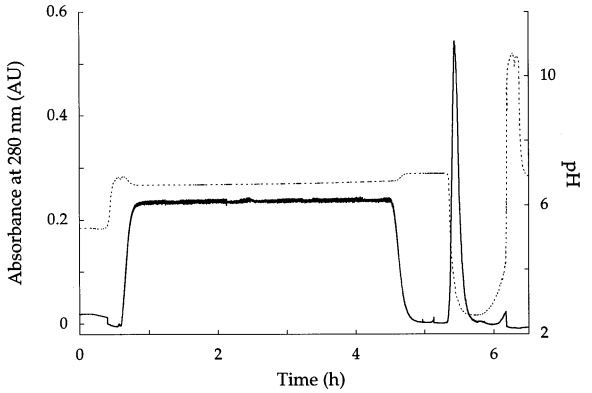

Fig. 2. Expanded bed protein A affinity chromatogram. This run uses a 2.5 cm inner diameter × 25 cm settled bed height Streamline rProtein A column. The flow rate was 250 cm h^{-1} and the column was loaded to 25 g l^{-1} (grams of antibody per l of column volume) with unclarified cell culture fluid. The column was regenerated with 1 mM NaOH pH 11. Absorbance is the solid line, and pH is the dashed line.

Because the screens had to be removed, the entire process in Fig. 2 was performed in expanded bed mode. While this may suggest that the eluted antibody would be more dilute in the collected pool, we found that the antibody concentration in the eluted pool was only slightly lower in the expanded bed (9.6 g l^{-1}) compared to the packed bed (11.6 g l^{-1}) using Prosep A (Table 1). This equates to a concentration factor of 18 for the packed bed and a concentration factor of 15 for the expanded bed.

Running the entire process with the bed expanded does not significantly affect the process time or the production rate for several reasons. The volumes (calculated by settled bed height) required for equilibration (5 CV), wash (7 CV), elution (5 CV), and regeneration (5 CV) did not increase significantly over the volumes required for a packed bed process. Also, the majority of the process time is spent loading, washing, and equilibrating, which would have to be done with the bed expanded regardless of the presence of screens. Finally, running the entire process with the bed expanded eliminates the time required to allow the bed to settle, move the flow adapter down and then back up, and allow the bed to expand again.

This small-scale process could translate into an efficient large-scale process. For a 6 kg batch of antibody and an 80 cm diameter × 25 cm height column (125 l CV), it would require two cycles to purify the antibody for a total process time of about 13 h. Thus, the expanded bed process could be run in a reasonable amount of time using a reasonably sized column. Reducing the CV to 70 l (60 cm diameter × 25 cm height) would increase the process time to about 26 h, but would reduce the cost of the column. In this study, unclarified cell culture fluid was held for 36 h with no detrimental effects on the expanded bed chromatography, which suggests that holding the load material for 26 h would not pose any significant operational problems.

The expanded bed process was cycled three times loading unclarified cell culture fluid. These expanded bed runs were compared to a packed bed run using clarified cell culture fluid from the same cell culture run. During the three expanded bed runs, yield, protein concentration, and purity were stable (Table 1), with low S.D.s for the three runs. The yield for both expanded bed and packed bed was $>90\%$ (Table 1), which indicates that little or no antibody flowed through the expanded bed column during load, and that al-

Table 1
Comparison of yield and purity of antibody purified by expanded bed method loading unclarified cell culture fluid on Streamline rProtein A media and a standard packed bed method loading clarified cell culture fluid on Prosep A media[a]

	Packed bed	Expanded bed
Yield (%)	93	97 ± 2
Antibody concentration (g l^{-1})	11.6	9.6 ± 0.2
Host cell proteins (mg g^{-1})	0.7	3.1 ± 0.3
DNA (ng mg^{-1})	0.2	4.7 ± 2.3
Turbidity (Neph)	50	180 ± 20

[a] For both methods, values are measurements of the purified antibody pools eluted with low pH. Values for the expanded bed method are the average ± 1 S.D. for three chromatography runs. The amount of host cell proteins is measured in mg of host cell proteins per gram of antibody. The amount of DNA is measured in ng of DNA per mg of antibody.

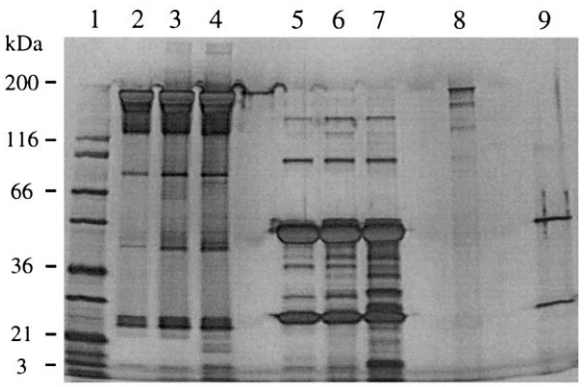

Fig. 3. Silver-stained SDS-PAGE comparing antibody purified by expanded and packed bed methods, nonreduced (lanes 2–4, 8) and reduced (lanes 5–7, 9). Lane 1: molecular weight markers, 3–200 kDa, labeled to the left of the gel. Lanes 2 and 5: purified antibody standard (purified by protein A affinity chromatography in packed bed, then cation and anion exchange chromatography). Lanes 3 and 6: antibody purified by packed bed. Lanes 4 and 7: antibody purified by expanded bed. Lanes 8 and 9: high pH regeneration peak from expanded bed.

most all the loaded antibody was recovered by the low pH elution.

SDS-PAGE (Fig. 3) shows that the purity of the antibody from the expanded bed method was consistent with the purity of the antibody from the packed bed method. There is a band in the antibody purified by expanded bed at a molecular weight of ≈ 15 kDa (lane 4) that is not present in the antibody purified by packed bed (lane 3), but (as discussed later) this new band should not be a significant concern. The high pH regeneration eluted some antibody as well as some contaminant proteins (lanes 8 and 9).

A quantitative measurement of purity (by ELISA) revealed that the presence of host cell proteins increased fourfold in the antibody purified using the expanded bed method when compared to that using the packed bed method (Table 1). The amount of host cell proteins in the load was ≈ 950 mg g^{-1} (mg host cell proteins per gram antibody). For the expanded bed purification, this equates to a 300-fold clearance in host cell proteins, as compared to a 1300-fold clearance for the packed bed process. Therefore, the expanded bed process removed 99.67% of the host cell proteins, while the packed bed process removed 99.92% of the host cell proteins. This slight decrease in removal of host cell proteins for the expanded bed process will most likely not affect the final purified antibody because the complete antibody purification process has both cation exchange and anion exchange columns downstream of the protein A affinity chromatography step, which together provide a significant reduction of host cell proteins. The removal of host cell proteins downstream can be seen in Fig. 3, where lanes 2 and 5 are a purified antibody standard, and much of the contaminant protein has been removed.

If the removal of host cell proteins downstream is not sufficient, several modifications to the expanded bed process might help decrease the amount of contaminant proteins in the purified pool. The volume of the wash between the load phase and the elution phase could be increased, which may wash out more unbound material that has diffused into the pores and ends up in the elution pool. An intermediate wash between the load phase and elution phase could contain chemicals such as 1 M NaCl that may remove nonspecifically bound proteins. Finally, a column regeneration with 2–6 M guanidine HCl, 6 M urea, or a weakly ionic detergent, may better clean the column between uses.

The amount of DNA increased 23-fold in the expanded bed pools compared to the packed bed pool (Table 1). The amount of DNA in the clarified cell culture fluid is ≈ 9700 ng mg^{-1} (the amount of DNA in unclarified cell culture fluid was not measured). Thus the amount of DNA in the purified antibody pools represents a 99.998% DNA removal (48 500-fold) for the packed bed, and a 99.950% DNA removal (2000-fold) for the expanded bed. As with host cell proteins, this decrease in DNA removal for the expanded bed should not be a concern because the downstream cation exchange and anion exchange columns provide significant DNA clearance. If these columns do not provide sufficient DNA clearance, the expanded bed process could be modified with a longer wash volume or an intermediate wash that may provide more DNA removal.

Some particulate matter elutes with the pool in expanded bed, increasing the turbidity of the antibody pool (Table 1). The higher turbidity may indicate that a filter with a larger area than that required for the packed bed process will be needed, which may affect the economics of the expanded bed process.

This study has shown that a recombinant antibody may be efficiently purified from unclarified Chinese hamster ovary cell culture fluid, but several questions still remain. One important factor in production economics is the lifetime of the column. Because the load material for the expanded bed is unclarified, it may irreversibly foul the column quickly. If the expanded bed column can be used for significantly less cycles than the packed bed column, the expanded bed column would have to be replaced more often, and the total process cost for the expanded bed may be higher than the cost for the packed bed even though a unit operation (clarification of cell culture fluid) has been removed. Also, the suggestion that the higher levels of host cell proteins and DNA can be effectively cleared downstream of the protein A affinity chromatography operation will need to be confirmed. The carryover of antibody, host cell proteins, and DNA into a blank run needs to be determined. Finally, more data on a variety of recombinant humanized monoclonal antibodies is needed to determine if this is a good general method for antibody purification.

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