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Comparison of ceramic hydroxy- and fluoroapatite versus Protein A/G-based resins in the isolation of a recombinant human antibody from cell culture supernatant

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Abstract

A recombinant human antibody (IgG_1-subtype) was produced in Chinese Hamster Ovary (CHO) cells. Alternatives to the established isolation by Protein A affinity chromatography were investigated. Neither an alternative elution agent (Arginine) nor an alternative affinity ligand (Protein G) resulted in an improvement in yield and/or purity. Subsequently, apatite stationary phases including a novel ceramic fluoroapatite material were tested. By applying a double gradient (first 0 to 1 M NaCl, then 0.01 to 0.4 M phosphate) the culture supernatant was separated into three fractions: the flow through, which contained no active antibody, the NaCl-eluate, which contained the antibody and no other discernible protein contaminants, and a fraction that eluted in the phosphate gradient and contained several proteins, but no active antibody. In case of the hydroxyapatite, retention of the antibody decreased and yield increased when the pH was raised from 6.0 to 8.2 (isoelectric point (p*I*) of the antibody: 8.3), to reach a yield of 71% at pH of 8.2. In case of the fluoroapatite, retention was also found to increase with increasing mobile phase pH, but the yields went through a maximum (of ca. 90%) at a mobile phase pH of 7.0. No traces of contaminants were seen in the corresponding gel. This is the first time that yields of 90% and such high purities have been reported as the result of a single chromatographic step for the antibody in question with either (Protein A) affinity or apatite chromatography.

Keywords: Hydroxyapatite; Fluoroapatite; Comparison studies; Monoclonal antibodies; Bioseparation; Protein A

1. Introduction

Theragnostic antibodies are currently among the most promising compounds on the market and in the pipeline of pharmaceutical biotechnology [1]. As the production scale for such antibodies mounts, product isolation increasingly becomes a challenge. Most established antibody purification protocols include affinity chromatography steps, typically using the generic antibody-specific ligand Protein A [2], a protein naturally found in *Staphylococcus aureus*.

Protein A-based affinity chromatography has many advantages, including the ability to capture the antibody in a highly specific manner even from dilute feed streams. However, there are also some issues that make alternatives to Protein A desirable. Protein A is antibody subtype specific and not all antibodies can be isolated using this ligand. Since Protein A binds the fc-region of the antibodies, molecules lacking this portion, e.g. single chain antibodies or fab-fragments, are also excluded [3]. Elution of the target molecule from the Protein A column generally requires drastic conditions, a step gradient to 0.1 M Glycine–HCl (pH $\leq$ 3.0) is typical. Elution at low pH contributes to virus inactivation, but threatens the yield of biologically active antibody. Rapid neutralisation of the eluting fractions is usually necessary, which is straightforward in case of small (mL-range) columns/fractions, but becomes increasingly difficult as the column/fraction volume increases. Protein A is also toxic, making ligand leakage a problem. Finally, Protein A-columns are difficult to clean and sanitise, due to the sensitivity of the ligand towards elevated pH-values. Several types of recombinant Protein A are available, which are 'improved', e.g. in regard to toxicity, immobilisation chemistry, and chemical stability (especially at elevated pH) [4–6]. Some of the difficulties with Protein A can also be overcome by switching to other generic ligands such as Protein G (other subtype specificity), Protein L (binds the κ-light chain), or low molecular weight Protein A 'mimics' [7–10]. The drastic elution conditions, but also the comparatively

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high price of the Protein A ligand, on the other hand, remain problems in the use of Protein A affinity chromatography at large scale.

Alternatively, procedures have been suggested for antibody purification that rely upon ion exchange chromatography, often followed by size exclusion for polishing. Such methods work well, especially at elevated product titres. However, they lack in specificity and tend to co-purify certain impurities. Another known, albeit rarely used, alternative for antibody purification is apatite chromatography [11]. Some comparisons of hydroxyapatite chromatography with Protein A (Protein G) affinity chromatography can be found in the pertinent literature [12,13]. While the apatite material is granted potential and interesting properties, the reported final product purities and concentration factors are generally below that of the affinity chromatography step.

Apatite, and in particular hydroxyapatite, has been introduced as chromatographic material in the 1950ies by Tiselius et al. [14]. It is an inorganic material naturally found in bones and teeth with the sum formula usually given as $Ca_{10}(PO_4)_6(OH)_2$ [15]. The surface has a negative net charge, which allows the material to attract positively charged molecules (such as proteins at a pH below their isoelectric point (p*I*)) and repulse negatively charged ones by electrostatic interaction [16]. The negative charges of the hydroxyapatite surface are presumably provided by the phosphate groups in the structure (also called P-sites). In addition, hydroxyapatite has been shown to attract molecules that contain certain other groups and even groups bearing negative charges, e.g. carboxylate groups in the case of proteins, presumably due to a chelating interactions with the calcium ions in the apatite structure (also called C-sites) [17].

While P-site interaction can be reduced (for elution) by an increase in ionic strength, C-site interaction is relative indifferent to the ionic strength and best interrupted via the addition of agents to the mobile phase that interact specifically with calcium ions, e.g. ions forming insoluble calcium salts or chelating agents. Standard elution in hydroxyapatite chromatography is therefore by a gradient of increasing phosphate concentration, which will elute all bond molecules, those binding to the P-sites via charge screening and those binding to the C-sites via specific interaction with the calcium ions [18,19]. However, it has also shown to be possible to elute selectively only the P-site binding molecules in a gradient of increasing NaCl concentration [20]. Since proteins usually carry both positive and negative charges that vary in number as a function of the pH, the interaction of such molecules with hydroxyapatite becomes complex. On the other hand, this complexity can be exploited to design highly specific conditions for binding and elution in apatite chromatography.

To date hydroxyapatite chromatography is often considered as a 'last resort' to be used when everything else fails. This is partially due to the complexity of the interaction, which requires more sophisticated method development than, e.g. Protein A affinity chromatography, but also to some of the physicochemical properties of the hydroxyapatite itself. In particular, the sensitivity of hydroxyapatite towards acidic pH (the material easily withstands elevated pH-values and may be sterilised using NaOH) has posed some difficulties. Most suppliers suggest not exposing the material for any time to pH-values below 6.5. This aspect of apatite chromatography has recently been improved by the introduction of a commercially available ceramic fluoroapatite stationary phase (sum formula $Ca_{10}(PO_4)_6F_2$)), which is stable down to a pH of 5 [21].

In this communication, we would like to demonstrate the relative worth of apatite versus Protein A/G-based chromatography for antibody purification, taking a recombinant therapeutic antibody produced by Chinese Hamster Ovary (CHO) cells as example.

2. Experimental

2.1. Materials

Chemicals were from Acros Organics (Geel, Belgium), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Sigma–Aldrich (Seelze, Germany). High purity water was used throughout. All buffers were filtered with a nylon membrane filter (0.2 μm pore size) (Nalgene, Rochester, NY, USA) and degassed prior to use. The ceramic hydroxyapatite (CHT type I, 20 μm particle size, 600–800 Å pore size) and ceramic fluoroapatite (CFT, 40 μm particle size, 600–800 Å pore size) stationary phases were from Bio-Rad (Hercules, CA, USA). The empty Tricorn high performance columns (5 mm × 5 mm) used to pack the apatite columns were from GE Healthcare, Uppsala, Sweden. Protein A/G affinity chromatography was performed on pre-packed HiTrap rProtein A FF 1 mL columns and pre-packed HiTrap Protein G HP 1 mL columns (both GE Healthcare).

2.2. Instrumentation

All chromatographic experiments were performed with an ÄKTApurifier chromatography workstation including fraction collector Frac-950 from GE Healthcare. A dynamic loop (DynaLoop 90, Bio-Rad) was used for sample injection with a maximum sample volume of 90 mL. The Unicorn V4.0 software was used for system control and data analysis.

2.3. Column packing (apatite materials)

For column packing, a 20% slurry of apatite (v/v) was prepared in packing buffer (degassed 200 mM dibasic sodium phosphate, pH 9.5). The slurry was poured into the packing assembly (Tricorn Packing Equipment (GE Healthcare), 10% of the column volume was already filled with buffer). Buffer was pumped through the column with an HPLC pump (K-501, Knauer, Berlin, Germany) at a linear flow rate of 600 cm/h for 10 min.

2.4. Cell line and culture conditions—antibody production

The recombinant human anti-Rhesus D antibody (MDJ8, h-IgG_1-subtype, κ-light chain) was produced by a Chinese Hamster Ovary cell line cultivated in suspension, containing the appropriate expression cassette of the full-length immunoglob-