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Short communication

Multimodal chromatography: Characterization of protein binding and selectivity enhancement through mobile phase modulators

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ABSTRACT

The unique selectivity of mixed mode chromatography resins is driving increasing utilization of these novel selectivities into bioprocess applications. There is a need for improved fundamental understanding of protein binding to these stationary phases to enable the development of efficient and robust purification processes. A panel of four monoclonal antibodies and two model proteins were employed to characterize protein interaction with a mixed-mode chromatographic resin comprising a hydrophobic ligand with cation-exchange functionality. Binding of these proteins was studied as a function of salt concentration and pH in the presence of various mobile phase modulators. This knowledge was applied towards screening mobile phase modulators that could selectively decrease host cell protein levels during monoclonal antibody purification.

1. Introduction

Multimodal chromatography (a.k.a. mixed-mode chromatography) refers to chromatographic stationary phases that combine more than one mechanism of interaction into the design of their ligand. Given the significant interest in evolving the downstream process platform for monoclonal antibodies [1], a number of applications for mixed-mode chromatography have emerged, particularly modes involving a combination of hydrophobic and electrostatic interactions. The hydrophobic nature of mixed-mode resins has been exploited for aggregate removal during post-Protein A polishing [2,3]. The salt independent binding behavior of mixed-mode resins was used to directly bind mAb cell culture supernatant to resins that combine hydrophobic and electrostatic interactions [4,5]. A variety of new generation mixed mode resins have been developed and used for capture of mAbs from cell culture supernatant [6,7]. Several case-studies have described the development of mixed-mode chromatographic steps for mAbs [8–12].

Fundamental understanding of how proteins interact with multimodal resins is still improving [13–15]. A connection is needed between some of the fundamental characterization of protein binding and the development of practical separation steps for preparative protein purification.

The work presented here connects the fundamentals of protein binding to mixed-mode chromatographic resins to the development of truly selective separations using selective mobile phase modulators as components of a wash buffer.

2. Materials and methods

2.1. Materials

The four monoclonal antibodies used in this study were expressed in CHO cells and captured by Protein A chromatography. Molecular weights for the mAbs ranged from 145 kDa to 155 kDa and basic isoelectric points were between 8.0 and 8.5. Lysozyme (Fisher BioReagents) has a molecular weight of 23 kDa and a pI of 9.6 while RNase (MB Bio) is 16 kDa and has a pI of 8.9. Capto MMC mixed mode resin was obtained from GE Healthcare (Uppsala, Sweden). Buffer salts and mobile phase modulators were obtained from Sigma (St. Louis, MO).

2.2. Equipment

All Capto MMC chromatographic experiments were performed on an AKTA Avant chromatographic system from GE Healthcare (Uppsala, Sweden). For the HCP ELISA assay a microtiter plate shaker (ThermoScientific) and a Molecular Devices (Sunnyvale, CA) SpectraMax 96-well plate reader were used. Reverse phase HPLC

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experiments were performed on an Agilent 1100 Series HPLC using a POROS R2/10 column for titer determination.

2.3. Methods

2.3.1. Gradient elution studies

For each experiment, 2.5 mg protein was injected on to a Capto MMC column pre-equilibrated with the appropriate equilibration buffer depending on the pH of the experiment. A 15 CV NaCl gradient was used for elution with the mobile phase modulator present in both buffers. Mobile phase modulators included: 5% ethylene glycol, 50 mM arginine, 50 mM sodium thiocyanate, 1 M urea and 1 M ammonium sulfate.

2.3.2. Log k′ vs. log salt concentration studies

The retention factors (k') for mAb1, RNase and lysozyme were determined under isocratic conditions at various sodium chloride concentrations in the presence of no modulator, 1 M urea, 5% ethylene glycol and 50 mM arginine at pH 7.0.

2.3.3. HCP ELISA assay

The CHO Host Cell Protein 3rd Generation kit for CHO HCP detection purchased from Cygnus Technologies (Southport, NC) was used to determine HCP levels in each eluate sample.

3. Theory

The retention of proteins under linear loading conditions on a chromatographic surface relates to the fundamental thermodynamics of the interaction between the protein and the stationary phase. The equilibrium constant (K) for this interaction can be described in terms of the Gibbs free energies for the dominant interactive forces as:

$$\log K = \frac{-\Delta G^{\circ}_{es}}{2.3RT} + \frac{-\Delta G^{\circ}_{h\phi}}{2.3RT} \quad (1)$$

where ΔG°_{es} and $\Delta G^{\circ}_{h\phi}$ are the Gibbs free energies for retention by electrostatic and hydrophobic interactions, respectively, T is the absolute temperature and R is the universal gas constant.

The retention factor (k') can be related to the equilibrium constant by the equation:

$$k' = \phi K \quad (2)$$

where ϕ is the phase ratio (i.e. ratio of stationary and mobile phase volumes).

Melander et al. [16] developed this formalism further to describe the dependency of linear retention factor on a mixed mode sorbent as a function of salt concentration as:

$$\log \quad k' = A - B \quad \log \quad (c_{\text{salt}}) + C(c_{\text{salt}}) \quad (3)$$

where c_{salt} is the mobile phase salt concentration in molar units and A, B and C are constants.

Eq. (3) is used in this work to characterize the binding of proteins to mixed-mode stationary phases under linear retention conditions in the presence of various mobile phase modulators.

The retention factor (k') can be determined under isocratic elution conditions as:

$$k' = \frac{t_r - t_0}{t_0} \quad (4)$$

where t_r is the retention time for the protein and t_0 is the retention time for an unretained tracer on the column.

4. Results and discussion

Capto MMC is a mixed-mode resin with cation-exchange functionality combined with hydrophobic moieties in its ligand structure. This resin will be further studied in this paper.

4.1. Characterization of protein binding – linear salt gradient experiments

To gain a fundamental understanding of how molecules interact with the Capto MMC mixed mode resin, we performed gradient elution studies with six different test molecules (four mAbs, RNase and lysozyme). Sodium chloride was used as the eluting agent for these experiments. In addition, a variety of mobile phase modulators were included in both the starting and ending buffers to study their impact on protein binding. Their effects could be studied by comparing the salt concentration at peak maximum on the linear salt gradients in the presence and absence of the mobile phase modulator.

Fig. 1A shows the amount of NaCl required for each protein to elute in the presence of each modulator at different pH conditions. Despite all six proteins having isoelectric points between 8.0 and 9.6, the concentration of NaCl required for elution varied widely. Examining the trend at pH 7.0 first, lysozyme adsorbed to the resin with the highest affinity as 2.2 M NaCl was needed for product elution, while only 300 mM NaCl was required for mAb2 and mAb3 to elute. This variation in elution conditions depicts the variability in how molecules can interact with this resin and serves to demonstrate the contribution of both hydrophobic and electrostatic interactions to protein binding on the Capto MMC ligand.

The addition of mobile phase modulators also impacts how molecules bind to the chromatographic surface (Fig. 1B). Urea yielded the largest effect on RNase and lysozyme retention with a significant decrease in affinity in both cases. Urea also decreased the affinity for the mAbs to the Capto MMC resin. On the other hand, sodium thiocyanate caused the greatest decrease in affinity for mAb1, mAb2 and mAb3. For mAb4, arginine produced the greatest decrease in retention. In all cases but one, the addition of mobile phase modulators reduced the binding affinity to the resin. The addition of 5% ethylene glycol to the buffer resulted in mAb4 having a slightly stronger retention. The other five proteins had similar or decreased retention in the presence of 5% ethylene glycol. Lysozyme and all four mAbs bound very strongly to the resin in the presence of 1 M ammonium sulfate and did not elute during the salt gradient (denoted as ∞). Clearly, for these proteins, it is possible to switch the mixed-mode resin into a hydrophobic binding mode in the presence of a high concentration of a kosmotropic salt. In contrast, RNase flowed through the column under these conditions. This indicates, that while hydrophobic interactions are important on Capto MMC, decreasing them alone does not change protein binding to the stationary phase significantly. This points towards the existence of a synergistic effect of electrostatic and hydrophobic interactions on this resin. pH also has an impact on protein binding to this mode of chromatography. Each protein was found to adsorb more strongly to the resin at pH 6.0 than at pH 8.0 (Fig. 1A). This is expected based on the cation-exchange properties of the Capto MMC resin, which typically decrease as pH is increased beyond pH 6.0 and as the proteins approach their pI.

These experiments display how protein:ligand interactions can be altered by the addition of mobile phase modulators and changes in pH. Such differences in how each target protein interacts with the Capto MMC ligand yield the possibility for using these mobile phase modulators to create specificity for the target protein during a mixed-mode chromatography step.