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Isoform separation of proteins by mixed-mode chromatography

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ABSTRACT

Mixed-mode chromatography uses a multimodal functional resin, mainly composed of electrostatic and aromatic/hydrophobic groups. Here we have tested 2 mixed-mode resins, anion-exchange Capto adhere and cation-exchange Capto MMC, using 2 model proteins, i.e., an Fc-fusion etanercept, and bovine serum albumin (BSA). When etanercept was produced in Chinese hamster ovary cells, a large amount of misfolded species was generated. A novel technology to achieve effective separation of the misfolded or aggregated species has been developed in this study using these mixed-mode columns and elution conditions that combine pH change and NaCl or arginine at different concentrations. Etanercept, which has been purified by Protein-A chromatography, was bound to the Capto MMC or Capto adhere columns under various conditions and eluted by modulating the pH and salt or arginine concentration. The misfolded species occurred in the fractions at higher salt or arginine concentrations, most likely reflecting stronger electrostatic and hydrophobic interactions of the misfolded species with these mixed-mode resins.

Another model protein, BSA, containing several oligomeric species, was also subjected to Capto adhere or Capto MMC chromatography using either NaCl or arginine gradient elution, with a greater recovery by arginine gradient. The oligomers were effectively separated on these mixed-mode columns using either gradient elution, eluting in later fractions similar to etanercept misfolded species.

1. Introduction

Misfolding and aggregation frequently occur during production of recombinant proteins [1–10]. For example, refolding of disulfide-containing proteins often lead to generation of misfolded species. These species must be removed from the final product through downstream processes. Since these species are made of the same protein, they have similar physical properties, including electrostatic charges and hydrophobicity, and therefore could be difficult to be separated from the native species. Although the misfolded species may be effectively separated by reverse-phase chromatography, use of low pH and organic solvent can denature the proteins and may cause aggregation of the purified proteins in the subsequent processes. Even when misfolding is negligible during production of pharmaceutical proteins, e.g., in the case of mammalian secretory expression, aggregation can still occur during purification [1,5,11]. Separation of these non-native species under non-denaturing conditions has been done using aqueous chromatography employing ion exchange (IEC) [10,12–14], hydrophobic interaction (HIC) [15–17] and hydroxyapatite [18–22] chromatography. We have tested here mixed-mode chromatography for separation of misfolded as well as aggregated species.

Mixed-mode chromatography was developed to accommodate loading samples containing varying amounts of salts [23,24]. Mixed-mode resins are composed of various functional groups, mainly of electrostatic and hydrophobic natures. For example, Capto MMC from GE is a cation-exchange mixed-mode resin and Capto adhere also from GE is an anion-exchange mixed-mode resin. We used 2 model proteins, etanercept that has been shown to misfold [25–27] and BSA that has been shown to aggregate [28,29]. Etanercept has been described to form varying degree of misfolding, when expressed in Chinese hamster ovary cells, depending on the cell culture or refolding conditions [25]. Analytical HIC showed 3 peaks of Protein-A purified etanercept corresponding to a clipped, native and misfolded forms eluting in that order during descending ammonium sulfate gradient [25–27]. Bovine serum albumin (BSA) is known to form soluble oligomers, comprising monomers, dimers, trimers, tetramers, etc. [28–30]. This paper reports binding and elution behaviors of these two model proteins in Capto adhere and Capto MMC mixed-mode chromatography.

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2. Materials and methods

Etanercept was expressed in conditioned medium (CM) of recombinant CHO cells transfected with a gene coding for the human tumor necrosis factor receptor p75 extracellular domain fused to the N-terminus of human Fc receptor [31,32]. The CM was first subjected to Protein-A chromatography using HiTrap rProtein-A columns (GE Healthcare) equilibrated with 10 mM Na phosphate, 0.1 M NaCl, pH 7.0. After washing the column with 1 M arginine (arginine hydrochloride simply referred to as arginine hereafter), 0.1 M citrate, pH 6.0, the bound proteins were eluted in stepwise with pH 4.2, 3.7 and 3.0 containing 1 M arginine and 0.1 M citrate. Alternatively, the column was processed without arginine in descending pH with 0.1 M citrate. In the presence of 1 M arginine, etanercept was eluted at pH 4.2 with a majority of both contaminating proteins and misfolded etanercept appearing below this pH, although some misfolded species co-eluted with the native etanercept. The Protein-A eluate was used to test the ability of Capto MMC and Capto adhere columns to separate misfolded species. Two different sizes of the HiTrap Capto MMC and Capto adhere columns from GE, 1 and 5 ml, were used. About 5–15 mg protein was loaded per 1 ml resin. Capto adhere chromatography was carried out mostly on Capto MMC-purified samples.

The eluted fractions from Protein-A and mixed-mode chromatography were analyzed by sodium dodecyl sulfate or native polyacrylamide gel electrophoresis (SDS–PAGE or native-PAGE) and also by HIC. Both SDS–PAGE and native-PAGE were carried out using 6% Tris–Glycine gel (Life Technology). The native gel, running buffer and 2× sample buffer are all at pH 8.1. Electric field was applied from cathode to anode, meaning that only negatively charged protein at pH 8.1 will enter the gel. SDS–PAGE was done under non-reducing conditions so that disulfide-linked aggregates could be observed. HIC was carried out using TSKgel butyl-NPR equilibrated with 1.8 M ammonium sulfate, 0.1 M sodium acetate, pH 6.0. The bound proteins were eluted with a descending ammonium sulfate gradient.

Fatty acid-free BSA was purchased from Sigma–Aldrich. It was dissolved in 20 mm acetate, pH 4.0 and loaded onto Capto MMC or Capto Adhere column. The bound BSA was eluted as described in the results.

3. Results

3.1. Protein-A-etanercept

Expression of etanercept in recombinant CHO cells resulted in secretion of the Fc-fusion protein in the supernatant of the CM. Characterization of the expressed etanercept showed highly heterogeneous nature, comprising both correctly folded and misfolded species, when analyzed by analytical HIC [25–27]. Thus, we have examined the ability of the Capto MMC and Capto adhere chromatography to separate the folded, native etanercept from the misfolded/aggregated species. Enrichment and partial purification were achieved by applying the CM first to Protein-A chromatography as described below.

The CM was loaded onto Protein-A column and the bound proteins were eluted with low pH solvent, either 1 M arginine in 0.1 M citrate or 0.1 M citrate alone. Fig. 1A shows the elution profile of the CM from Protein-A with low pH buffers containing 1 M arginine, while Fig. 1B shows the native-PAGE analysis of the eluted fractions. The CM (lane-1) showed a broad smearing band, indicating highly heterogeneous nature of the loading sample. The band corresponding to etanercept was not observed in the flow-through fraction (FT, lane-2), although FT appeared to contain high

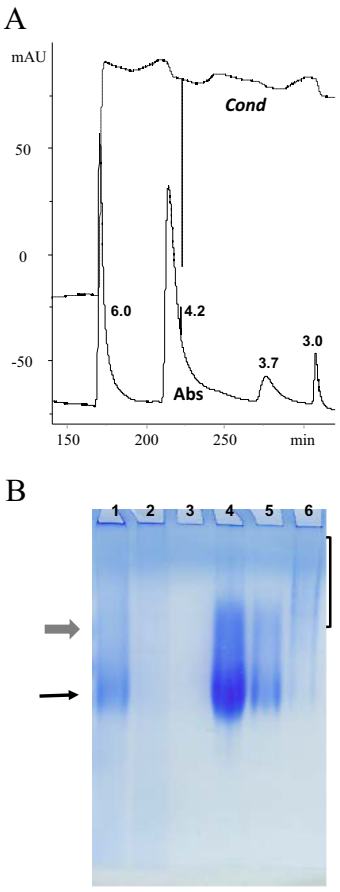

Fig. 1. Protein-A chromatography of etanercept CM. (A) Elution profile of Protein A with 1 M arginine, 0.1 M citrate at indicated pH. Solid, UV absorbance (Abs); dotted, conductivity (*Cond*). (B) Native-PAGE: lane-1, load; lane-2, FT; lane-3, pH 6.0; lane-4 pH 4.2; lane-5, pH 3.7; lane-6, pH 3.0.

molecular weight or basic proteins (see the staining at the top of the gel): positively charged basic proteins will not enter the gel. After the loading, the column was washed with 1 M arginine at pH 6.0. A large absorbance peak was observed with this wash (peak marked 6.0) and contained no protein bands (Fig. 1B, lane-3). Although no protein bands were observed at pH 6.0, the observed large UV absorbance indicates elution of pigments. After reaching baseline absorbance (Fig. 1A), the bound proteins were then eluted from the Protein-A column with 1 M arginine, 0.1 M citrate, pH 4.2, resulting in a sharp elution peak (Fig. 1A, marked pH 4.2). Native-PAGE analysis of this peak (lane-4) showed an intense band of etanercept (shown by thin arrow), along with smearing low mobility bands (thick arrow). Although the nature of the low mobility bands is not clear, it could be misfolded species of etanercept, as it bound to Protein-A and hence should contain the Fc domain. These species are tentatively termed "misfolded species" throughout the paper. Slow mobility may be ascribed to a larger hydrodynamic size or aggregation. Although its nature is not clear, we followed its separation from the major species. The remaining bound proteins were eluted from the Protein-A column with a buffer at pH 3.7 and then 3.0, both containing 1 M arginine. These low pH solutions resulted in elution of small peaks as shown in Fig. 1A (marked 3.7 and 3.0). The pH 3.7 peak contained a small amount of etanercept (lane-5), while the pH 3.0 peak contained mainly low mobility species, corresponding to the misfolded etanercept (lane-6, shown by bracket). Although a similar elution pattern was observed with 0.1 M citrate elution in the absence of arginine, a lower pH (e.g., 3.8 instead of 4.2) was required to elute