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Purification of an Fc-fusion biologic: Clearance of multiple product related impurities by hydrophobic interaction chromatography

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Abstract

An hydrophobic interaction chromatography step was developed for the large-scale production of an Fc-fusion biologic. Two abundant product-related impurities were separated from the active monomer using a Butyl resin and a simple step-wash and step-elution strategy. Capacity and resolution of the HIC step was optimal when sodium sulfate was employed as the lyotropic salt and pore size of the Butyl resin was 750 Å. Factorial analysis identified critical parameters for the Butyl chromatography and an operating window capable of delivering high product quality and yield over a broad column loading range.

Keywords: Hydrophobic interaction chromatography; Butyl; Fc-fusion; Biologic; Manufacturing

1. Introduction

Hydrophobic interaction chromatography (HIC) is a useful technique for large-scale purification of recombinant proteins [1–9]. It exploits the reversible interaction of protein hydrophobic patches with the hydrophobic ligand of a chromatography sorbent under mild conditions in the presence of a lyotropic salt [10–14]. Key considerations for the development of a manufacturing scale-purification process are efficiency, robustness, cost of goods and waste management. Furthermore, recent improvements in protein titers achieved using mammalian cell expression systems have increased the demand for high capacity unit operations, including chromatography steps that minimize column cycling and maximize throughput [15–19]. In accordance with regulatory guidance, the design space of a biologics manufacturing process is routinely explored by experiments performed according to factorial design. Process specifications are thus set based on modeling and statistical analysis of data [20–23]. This approach minimizes the number of experiments required while building an accurate model of the process and establishing quality by design.

Here we describe the development of a preparative HIC step appropriate for manufacturing scale production, in which two major product-related impurities were separated from an antibody Fc-fusion protein by the application of simple step gradients. The feed for the HIC step (neutralized eluate from a protein A affinity capture step) consisted of the product, termed active monomer (61% of the total), two related monomeric, disulfide-scrambled species, termed Inactive-1 (5%) and Inactive-2 (13%), dimer and higher molecular weight forms (termed aggregate; 21%). The target for the HIC step was to reduce the level of Inactive-1 and Inactive-2 to less than 1% of the total protein with a product yield acceptable for a manufacturing scale production (>50%). As the product was expressed in mammalian cells, reduction of host cell protein and DNA impurities was also required of the step. Furthermore, the HIC step followed a protein A affinity chromatography step, and therefore clearance of protein A leachate was sought. In addition, HIC chromatography may also be effective for the clearance of 20 nm, non-enveloped viruses that, unlike enveloped viruses, are not chemically inactivated (by low pH or detergent) in other processing operations. Here, the capacity of the HIC step was optimized by resin and salt screening experiments while critical chromatography parameters were identified by a factorial design approach. The conditions chosen for the HIC chromatography are discussed in the context of process robustness, economy and waste management.

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2. Experimental

2.1. Laboratory scale chromatography

Chromatography was performed using an AKTA Explorer 10 (10 mm path-length flow cell) with Unicorn software (GE Healthcare). Columns were 0.5 cm (Pharmacia HR5/5), 0.66 cm (Omnifit) or 2.6 cm (GE Healthcare) in diameter. Feedstock for HIC chromatography was clarified chinese hamster ovary cell culture medium purified by protein A affinity chromatography, stored at 4 °C. HIC load material was adjusted to ambient temperate (22–24 °C) prior to the addition of load adjustment solution at ambient temperature with gentle stirring. Load adjustment volume was 165% or 100% with adjustment solution containing 4.0 M NaCl or 1.2 M Na_2SO_4, respectively. Column load materials were filtered (0.22 μM, Millipak 20 or Millex-GV) immediately prior to the start of each chromatography experiment. Chromatography was performed at ambient temperature (22–24 °C) and the column was incubated at 24 °C using an ECHOtherm Chiller/Heater (#C030; Torrey Pines Scientific, CA). Tosoh Bioscience HIC resins were defined prior to use by washing once with water then twice with HIC equilibration buffer. Resins were packed in HIC equilibration buffer. Resins employed were Butyl 650 M or 750 M (Tosoh Bioscience), SOURCE 30PHE or Phenyl Sepharose 6 Fast Flow Low Sub (GE Healthcare).

2.2. Analysis of column intermediates by high pressure liquid chromatography (HPLC)

HPLC analysis was performed using a Waters 600S controller, 717plus autosampler and Millennium software. Analysis of intermediates for the level of Inactive-1/Inactive-2/active monomer by HIC–HPLC was performed using an Applied Biosystems HP2/20 column (2.1 × 30, #1-4522-12) with a salt gradient elution strategy operated at a flow rate of 1.0 mL/min. Analysis of intermediates for high molecular weight/monomeric/low molecular weight forms by size exclusion HPLC (SE-HPLC) was performed under isocratic conditions using a Tosoh Biosciences G3000SW_{XL} analytical column with a TSK_{gel} guard column (#08543). Measurement of Absorbance at 280 nm was performed using a Synergy 2 Platereader (Biotek Instruments Inc., VT) and UV transparent 96-well plates (#3635, Corning).

2.3. Statistical analysis

Design of experiments and statistical analysis was performed using JMP software version 6.0 (SAS). To identify critical parameters for the Butyl HIC step, a three-factor, two-level full factorial screening design with two center points was employed with full resolution of main effects and two-way interactions (10 chromatography experiments in total). The factors examined were column load amount (18 g/L and 26 g/L), wash (0.46 M and 0.54 M) and elution step (0.17 M and 0.23 M) sodium sulfate concentration. The responses measured were the level of Inactive-1 and Inactive-2 in the eluate, the yield of active monomer and the amount of protein in the wash fraction. The regression analysis was performed using a standard least squares fitting personality.

2.4. Analysis of process-related impurities in Butyl intermediates

Host cell protein (HCP) was measured using custom antibodies generated against mammalian HCPs and enzyme linked immunosorbent assay (ELISA) format utilizing electrochemiluminescence (ECL) for detection. Host cell DNA was detected using a quantitative polymerase chain reaction assay and custom primers homologous to mammalian DNA sequences. Protein A leachate was detected by ELISA using antibodies directed against recombinant protein A.

3. Results and discussion

A purification process was sought capable of separating an Fc-fusion drug candidate from two related but inactive forms at manufacturing scale. During the initial characterization of the drug candidate its purification was achieved by a protein A affinity capture, to concentrate the feedstock, followed by a hydrophobic interaction chromatography (HIC) step to separate active product from the two related inactive species (termed Inactive-1 and Inactive-2). However, the initial HIC step employed a resin with poor pressure-flow characteristics and a continuous gradient elution strategy combined with fractionation to achieve the required separation (not shown), both of which were considered undesirable for large-scale production. To identify a purification strategy appropriate for drug manufacturing nine HIC resins obtained from four different vendors were screened for pressure-flow characteristics compatible with large-scale production (for example, backpressure less than 0.3 MPa at a linear flow velocity of 100 cm/h) and separation of the active and inactive forms via a simple step gradient-wash and step-elution strategy. In this initial screen (not shown) a Butyl sorbent was identified that resolved the product-related species into three fractions (Fig. 1). Using a step gradient strategy, Inactive-1 was recovered in an intermediate wash fraction while active monomer and aggregate were each recovered in the elution fraction. Surprisingly, Inactive-2 remained bound during the elution step and was removed by stripping the column with water. Quantitation of product-related species present in the process intermediates was performed using two different high pressure liquid chromatography (HPLC) assays (Fig. 2). A conventional size exclusion HPLC assay was employed to quantitate the amount of aggregate and monomeric species, while a custom HIC–HPLC assay, capable of resolving the monomeric active and inactive species, was additionally employed to provide an overall estimate of active monomer yield.

Sodium chloride at 1.6 M supported a dynamic binding capacity of 8.0 g of protein per liter of the Butyl resin. In contrast, ammonium sulfate supported a higher binding capacity but with poor resolution of protein species (not shown). However, ongoing improvements to the fermentation process increased the feedstock titer from an initial level of 300 mg/L in early