High-Throughput Ion Exchange Purification of Positively Charged Recombinant Protein in the Presence of Negatively Charged Dextran Sulfate

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Product quality analyses are critical for developing cell line and bioprocess producing therapeutic proteins with desired critical product quality attributes. To facilitate these analyses, a high-throughput small-scale protein purification (SSP) is required to quickly purify many samples in parallel. Here we develop an SSP using ion exchange resins to purify a positively charged recombinant growth factor P1 in the presence of negatively charged dextran sulfate supplemented to improve the cell culture performance. The major challenge in this work is that the strong ionic interaction between P1 and dextran sulfate disrupts interaction between P1 and chromatography resins. To solve this problem, we develop a two-step SSP using Q Sepharose Fast Flow (QFF) and SP Sepharose XL (SPXL) resins to purify P1. The overall yield of this two-step SSP is 78%. Moreover, the SSP does not affect the critical product quality attributes. The SSP was critical for developing the cell line and process producing P1. © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:516–520, 2014

Keywords: ion exchange chromatography, small-scale purification, high-throughput analytics, product quality analysis, bioprocess development

Introduction

Cell line and bioprocess development play a major role in production of biologics with appropriate product quality attributes, such as protein aggregation, sialylation, and α-galactosylation.[1–5] These tasks require product quality analyses of many samples generated from clone screening and process optimization studies. To perform these analyses, a high-throughput small-scale purification (SSP) is required for purifying cell culture samples from cell line and process development in a short period of time.

Here we need an SSP for developing cell line and process producing a positively charged recombinant growth factor P1 (∼11–17 kDa). This task is complicated by the presence of negatively charged dextran sulfate (∼5 kDa). Dextran sulfate has been used for various applications, including improving cell growth,[6,7] increasing cell productivity,[8] and reducing cell clumping.[9] Thus, the SSP developed in our study can be used for various applications in cell line and process development, especially when the protein of interest is positively charged. In our study, dextran sulfate was supplemented to the cell culture process at 0.25 g/L to improve its performance. With typical P1 harvest concentration of less than 0.7 g/L, these values translate to a mol ratio of greater than 3 mol dextran sulfate to 1 mol of P1, where 1 mol dextran sulfate consists of ∼36 mol of R-SO_3^-. As a result, the dextran sulfate strongly interacted with P1 and disrupted the interaction between P1 and several chromatography resins that we have tried previously.

To solve this problem, we developed a two-step SSP to purify P1 using 96-well plates and 96-well filter plates. The first step used anion exchange QFF resin in flow-through mode to remove the dextran sulfate, and the second used cation exchange SPXL in bind and elute mode to purify P1 from the QFF flow-through (Fig. 1). This SSP had an overall yield of 78 %. Moreover, the SSP did not affect several product quality attributes critical for our development of cell line and process producing P1.

Materials and Methods

Two-step protein P1 small-scale purification (SSP) using QFF and SPXL resin

First, 8 mL SPXL resin (GE, 17-5073-01) was washed with HPLC water (4x volume, twice) and 50 mM phosphate buffer, 125 mM NaCl, pH 7.0 (4x volume, three times) by centrifugation at 3,000 rpm, 5 min. After washing, 3x volume of the buffer was added such that the slurry contains 25% SPXL resin. QFF (GE, 17-0510-01) was prepared similarly with water and 75 mM sodium carbonate/bicarbonate

Additional Supporting Information may be found in the online version of this article.

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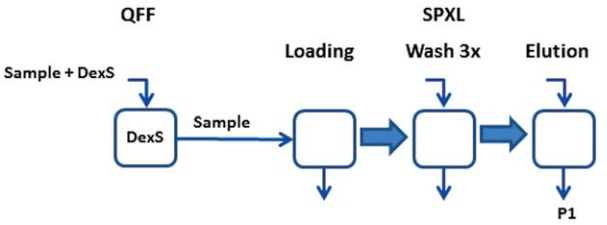

Figure 1. A two-step SSP is developed for purifying protein P1 from cell culture samples supplemented with dextran sulfate (DexS). QFF resin captures DexS. The P1 is collected in the QFF flow-through and purified by SPXL resin in bind and elute mode.

buffer, 500 mM NaCl, pH = 9.0. The washed QFF and SPXL slurry should be kept at 4°C and used within 1 week.

The first step of SSP used QFF resin in flow-through mode to remove dextran sulfate. Totally, 400 μL of the washed QFF slurry (100 μL resin) was added to a 2 mL 96-well plate (Fisher Scientific, 278743), and centrifuged at 3,000 rpm, 5 min. ~300 μL buffer was then removed by Caliper Sciclone (Perkin Elmer). Cell culture samples were mixed with 5 M NaCl to increase the concentration by 500 mM NaCl, and mixed with 5 M NaOH to increase the pH to ~9.0. 1.2 mL of the conditioned sample was then added to the resin, and mixed for 15 min using an orbital shaker (Fisher Scientific, 260100F). The mixture was then transferred to a 96-well lysate clearing plate (Promega Wizard SV96, A2241). The dextran sulfate bound to QFF resin, while P1 was collected in QFF flow-through in a 96-well plate. The QFF flow-through was then mixed with 3.6 mL of 50 mM phosphate buffer, pH = 7.0 to reduce the conductivity to less than 20 mS/cm and pH to ~7.0.

The diluted QFF flow-through was then purified by SPXL in bind and elute mode. In the loading step, 267 μL of the washed SPXL slurry (67 μL resin) was added to three wells of a 2 mL 96-well plate, and centrifuged at 3,000 rpm, 5 min. Totally, 200 μL buffer was removed from each well by Caliper Sciclone. 4.8 mL of the diluted QFF flow-through was equally distributed to the three wells, and mixed for 15 min using the shaker. The mixture from the three wells was then transferred into one well of a 96-well lysate clearing plate. 3 × 1.2 mL of 50 mM Tris buffer, 210 mM NaCl, pH = 8.5 was used in the washing step. 1.2 mL of 50 mM Tris, 500 mM NaCl, pH = 8.5 was used in the elution step, and the elution was collected in a 2 mL 96-well plate. In all of these steps, the liquid flowed through the membrane by gravity.

Protein P1 quantification and % yield calculation

Protein P1 in cell culture samples and purified samples were quantified using our in house ELISA assay. An in house antibody against protein P1 was used as the capture protein. % yield of QFF and two-step SSP was calculated using Eqs. 1 and 2, respectively

$$\%\ yield\ \text{of}\ QFF = \frac{mass\ of\ P1\ in\ QFF\ flow-through}{mass\ of\ P1\ in\ QFF\ load} \times 100\% = \frac{concentration\ of\ P1\ in\ QFF\ flow-through \times QFF\ flow-through\ volume}{concentration\ of\ P1\ in\ QFF\ load \times QFF\ load\ volume} \times 100\% \quad (1)$$

$$\%\ yield\ of\ SSP = \frac{mass\ of\ P1\ in\ SPXL\ elution}{mass\ of\ P1\ in\ QFF\ load} \times 100\% = \frac{concentration\ of\ P1\ in\ SPXL\ elution \times SPXL\ elution\ volume}{concentration\ of\ P1\ in\ QFF\ load \times QFF\ load\ volume} \times 100\% \quad (2)$$

SDS-PAGE and Western Blot

13 μL samples were mixed with 2 μL 1 M DTT (Sigma, 646563) and 5 μL LDS sample buffer (Invitrogen, NP0002) for denaturation at 70°C, 10 min. This mixture was separated by NuPAGE 4–12% Bis-Tris gel (Invitrogen, WG1402BX10) and NuPAGE MES SDS running buffer (Invitrogen, NP0002) at 200 V, 35 min. Wet transfer (30V, 1 h) was used for western blot. Blocking buffer (Thermo Fisher, 37536) was used for 1 h blocking. An in-house mouse anti-P1 antibody was used as the primary antibody and a goat anti-mouse polyclonal antibody (Abcam, Ab97023) as the secondary antibody. The membrane was developed using the LumiGLO Peroxidase Chemiluminescent Substrate (KPL, 54-61-00) and X-ray film in a dark room.

Size exclusion chromatography (SEC)

Zenix SEC-300 (3 μm, 300 Å, 7.8 x 150 mm, Sepax Technologies, 213300-7815) with an isocratic neutral mobile phase and a UV detection were used for SEC.

Sialic acid quantification

Purified P1 samples were hydrolyzed by mild acid hydrolysis to release the sialic acid,[10,11] and analyzed by an HPLC using an ion-exclusion column, an isocratic mobile phase, and a UV detection.[12]

Results and Discussions

In order to purify the positively charged P1 in cell culture samples containing the negatively charged dextran sulfate, we developed a two-step SSP using QFF and SPXL (Fig. 1). SPXL was chosen to purify P1 because of the high pI of protein P1. The removal of dextran sulfate by QFF was very important because the dextran sulfate strongly bound to P1 and disrupted the interaction between P1 and SPXL, causing significant loss of P1 in the SPXL flow-through and wash. Consequently, the yield was very low (2 ± 1%, SD, $n = 2$) (Supporting Information Figure 1).

We first optimized QFF purification by varying NaCl concentration from 200 to 500 mM at pH = 9.0 (Fig. 2 and

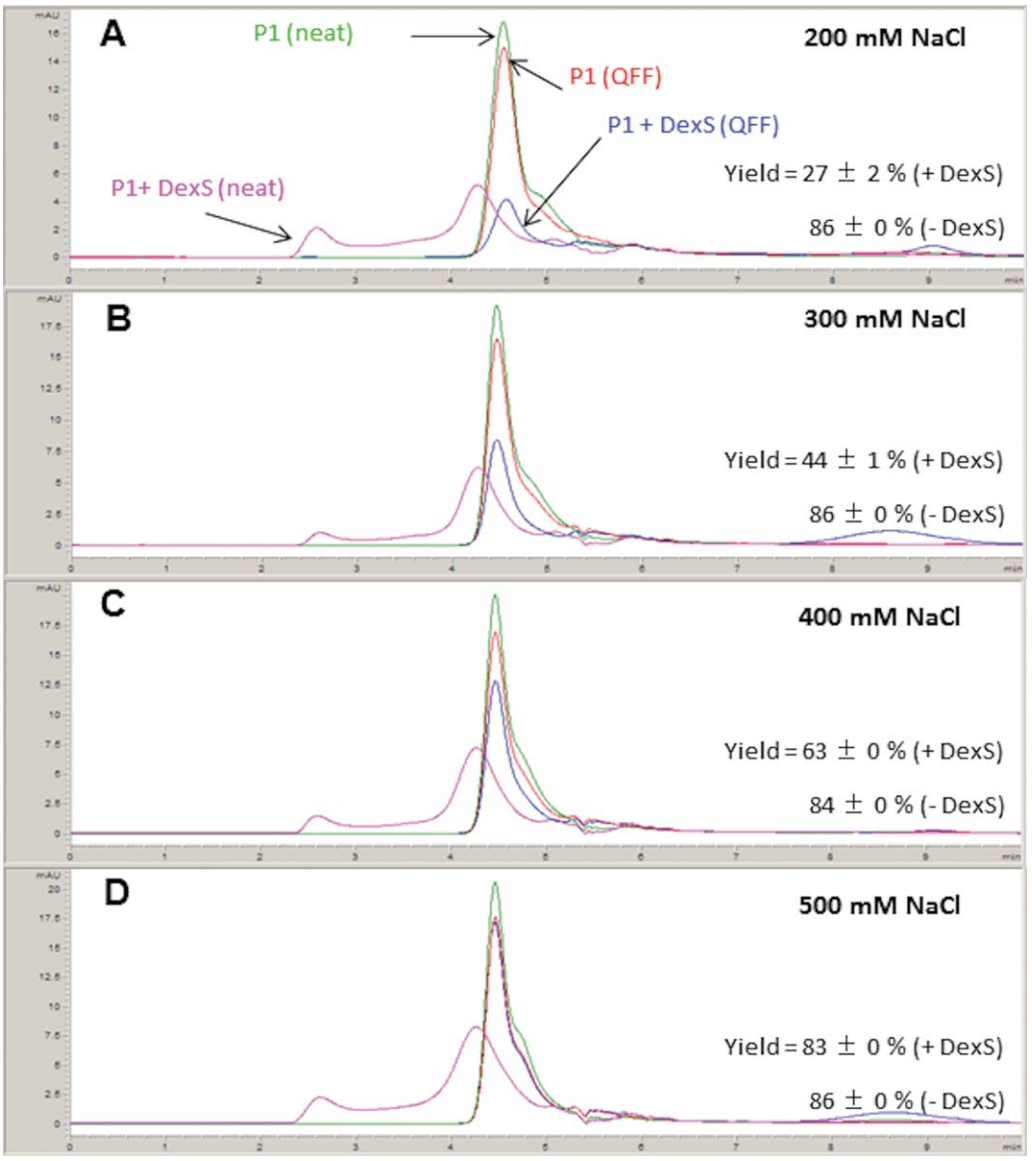

Figure 2. Optimization of QFF purification by adjusting NaCl concentration. Solutions of P1 in 75 mM carbonate-bicarbonate buffer, pH = 9.0, (A) 200 mM, (B) 300 mM, (C) 400 mM, and (D) 500 mM NaCl were mixed with dextran sulfate (DexS), purified by QFF, and the flow-through (blue) was analyzed by SEC. Neat pure P1 (green), pure P1 processed by QFF purification (red), and neat mixture of pure P1 and DexS (pink) were controls for each NaCl concentration. The yield of QFF purification of pure P1 mixed with DexS (+DexS) and pure P1 mixed with water (-DexS) for all conditions are indicated in the figures.

Supporting Information Table 1). The performance of QFF resin in removing the dextran sulfate from a mixture of pure P1 and dextran sulfate was analyzed by SEC. The SEC profiles of QFF flow-through (Fig. 2A–D, blue) were similar to that of pure P1 (Fig. 2A–D, green), indicating that the QFF purification removed dextran sulfate. Otherwise, we would observe high molecular weight complexes of P1 and dextran sulfate as shown by the SEC chromatograms of unpurified dextran sulfate and pure P1 mixtures (Fig. 2A–D, pink). Nonetheless, the yield was only $27 \pm 2\%$ (SD, $n = 2$) at 200 mM NaCl, indicating that there were significant amount of P1 and dextran sulfate complexes bound to QFF. As the NaCl concentration was increased from 200 to 500 mM, the yield increased from $27 \pm 2\%$ to $83 \pm 0\%$ (SD, $n = 2$). The yield of QFF purification of pure P1 without dextran sulfate was ~86% regardless of the NaCl concentration (Fig. 2A–D, red). These results indicate that the interaction between dextran sulfate and P1 was disrupted by 500 mM NaCl, causing the dextran sulfate to bind to QFF while P1 remained in the QFF flow-through. Thus, 500 mM was the optimum NaCl concentration.

We then combined the QFF purification with the SPXL purification that we have optimized (Supporting Information Fig. 2) and found that P1 in QFF load was recovered in the QFF flow-through and SPXL elution (Fig. 3A and B Lanes 2, 3, and 9). Moreover, no significant loss of P1 was observed in SPXL flow-through and washes (Fig. 3A and B Lanes 5, 6, 7, and 8). In addition, no impurity was observed in the SPXL elution (Fig. 3A and B Lane 9). We also stripped QFF and SPXL resin with 50 mM Tris, 2 M NaCl, pH = 8.0, and found that there was no detectable P1 left bound to the QFF and SPXL resins (Fig. 3A and B, Lanes 4 and 10). The overall SSP yield was $78 \pm 3\%$ (SD, $n = 2$). These results demonstrated that the SSP successfully purified P1 in the presence of dextran sulfate.

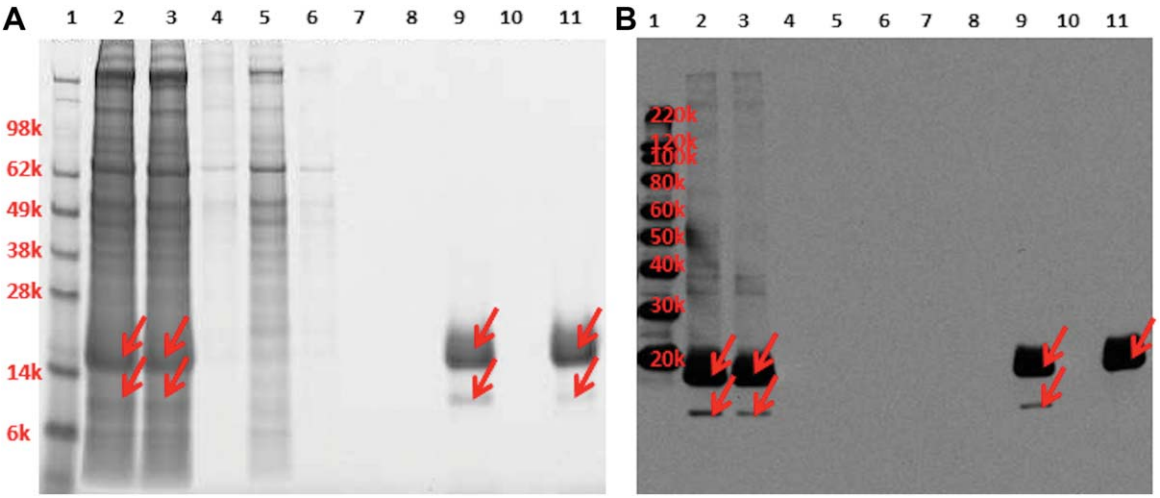

Figure 3. **The two-step SSP successfully purified P1 in cell culture samples containing dextran sulfate. (A) Reduced SDS-PAGE and (B) Western Blot were used to analyze the performance of the SSP. Lane 1 is the ladder, Lane 2 is the QFF load, Lane 3 is the QFF flow-through, Lane 4 is the QFF strip, Lane 5 is the SPXL flow-through, Lanes 6, 7, and 8 are the first, second, and third SPXL washes respectively, Lane 9 is the SPXL elution, Lane 10 is the SPXL strip, and Lane 11 is pure P1 control. Red arrows indicate P1.**

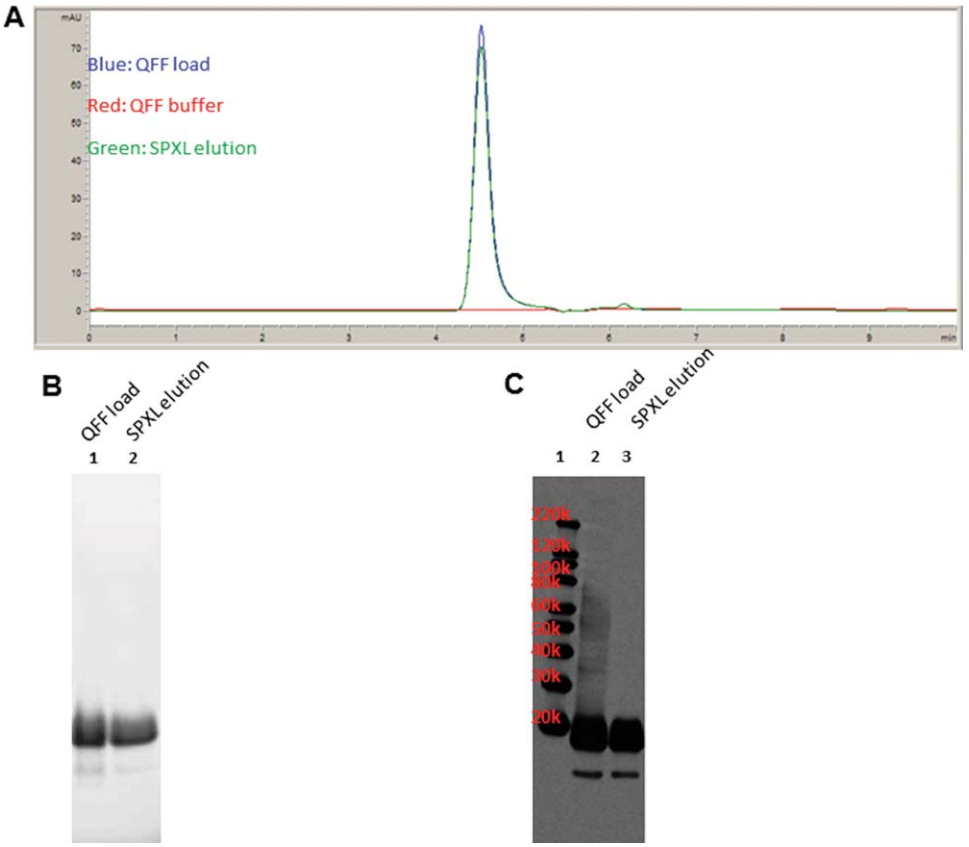

Figure 4. **The two-step SSP did not have any significant effect on product quality attributes of P1. (A) The SEC profiles of QFF load (blue) and SPXL elution (green) were similar. QFF buffer blank (red) was processed as a control. The SSP recovered not only the glycosylated form (14–17 kDa) but also nonglycosylated form (11 kDa) of (B) a mixture of pure P1 and dextran sulfate as shown by reduced SDS-PAGE, as well as (C) P1 in cell culture samples containing dextran sulfate as shown by Western Blot.**

We also studied whether the SSP affected product quality attributes critical for P1 bioactivity and safety. Here we performed the SSP on pure P1 dissolved in 75 mM carbonate-bicarbonate buffer, 500 mM NaCl, pH = 9.0. We found that the total sialic acid content of QFF load and SPXL elution were identical at 2.4 ± 0.0 mol sialic acid/mol P1 (SD,

$n = 2$). Similarly, NGNA level of QFF load and SPXL elution were identical at $6.8 \pm 0.0\%$ (SD, $n = 2$). The SSP also did not affect P1 aggregation level (Fig. 4A). Moreover, the SSP recovered not only the glycosylated (~14–17 kDa), but also the non-glycosylated (~11 kDa) forms of pure P1 (Fig. 4B) and P1 in cell culture samples (Fig. 4C). These results show that the SSP does not affect the product quality attributes. Moreover, we have scaled-up the SSP to purify 6 mL of samples using disposable PD-10 columns (GE) and a Visiprep SPE vacuum manifold (Supelco). We found that the two-step purification successfully purified P1 from samples containing dextran sulfate (Supporting Information Fig. 3). The results indicate that the process can be scaled-up for large scale purification processes.

Overall, the SSP can purify protein P1 from cell culture in the presence of dextran sulfate with ~78% yield. More importantly, the two-step SSP does not affect the critical product quality attributes. The SSP is also high-throughput. To support high-throughput product quality analyses in our lab, typically ~450 μg of protein is required. If the cell culture samples contain ~300 μg/mL P1, the SSP can purify 72 samples within a day. If the cell culture samples contain higher concentration of P1, the throughput will be higher than 72 samples/day. In contrast, if we use column purification, it will take ~72 days to purify these 72 samples. This throughput is too low for purifying large number of samples generated in cell line and process development. Thus, the SSP is critical for developing the cell line and process producing P1.

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