

Recombinant human erythropoietin separation using a cation-exchange multimodal adsorbent

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Abstract: This work deals with the capture of human recombinant erythropoietin (rhEPO) from a mixture of proteins in a concentrated postcultivation supernatant. Cation-exchange multimodal adsorbent Capto MMC ImpRes was selected as potential chromatographic separation material. Its equilibrium properties were investigated in batch adsorption experiments. The effect of pH in the range of 5.5–7.5 and NaCl concentration in the range of 0–300 mM on the adsorption of rhEPO and contaminant proteins was examined. Optimal conditions found in these equilibrium experiments were applied to rhEPO adsorption in a chromatographic column. Several experiments were carried out at different elution conditions to optimize the rhEPO yield and selectivity.

Keywords: erythropoietin, multimodal adsorbent, selectivity, elution chromatography

Introduction

Erythropoietin is a human hormone involved in the formation of red blood cells. Recombinant human erythropoietin (rhEPO) is a commercially available drug used to treat anaemia and other diseases. rhEPO produced by mammalian cells is secreted into the growth medium and subsequently isolated and purified to the required purity.

During purification, all foreign proteins, viruses and toxins have to be removed because they can cause an undesirable immune response. Different chromatographic methods are used for rhEPO purification (Adamíková et al., 2019). Size-exclusion, ion-exchange, hydrophobic, affinity and multimodal interactions between protein molecules and adsorbent are the most commonly applied in rhEPO purification.

Multimodal chromatography is the novel process separation mode which was originally developed for monoclonal antibodies purification. Multimodal adsorbents contain several different functional groups, which allows simultaneous binding of proteins through different interactions. Multimodal adsorbents offer different selectivity compared to classical ion exchangers and have larger operating range in terms of adsorption buffer conductivity (Holstein et al.). Nowadays, there are several applications of multimodal adsorbents for recombinant proteins purification, but applications for rhEPO purification are rare (Koticha and Chaudhari, 2011; Bandi et al., 2017). Our main goal was to determine optimal conditions of rhEPO binding to adsorbent Capto MMC ImpRes

from a real mixture and to find appropriate elution of bound proteins.

Materials and methods

Adsorbent

Multimodal adsorbent Capto MMC ImpRes (GE Healthcare, Uppsala) has a matrix consisting of agarose chains. Its ligand is shown in Fig. 1. This ligand can provide ionic interactions (1), hydrogen bonds (2), hydrophobic interactions (3) and thiophilic bridges (4) with molecules.

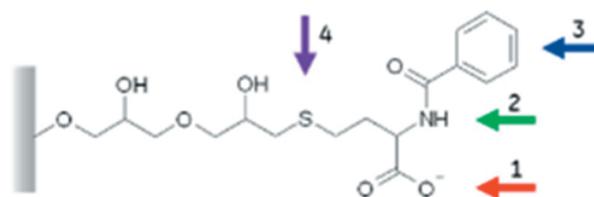


Fig. 1. Structural formula of ligand Capto MMC ImpRes.

rhEPO post-culture supernatants

Two different batches of a post-culture supernatant containing rhEPO produced by human embryonic kidney cells 293 were used (Molnár et al., 2019). The first one was concentrated and desalted using different membrane filtration processes (Molnár et al., 2019). The final rhEPO concentration and total protein concentration were 0.05 g/l and 0.8 g/l, respectively. This protein solution was used in batch equilibrium experiments and separation runs S1–S5 (Table 1). The second batch contained 0.08 g/l

of rhEPO and 2.1 g/l of total proteins and was used in the separation run S6 (Table 1). This post-culture supernatant was not treated via membrane processes. Only pH was set by concentrated HCl and 300 mM NaCl was added into the supernatant before adsorption.

Batch adsorption

Adsorbent in the amount of 25 mg was placed into a syringe filter, washed three times with 1 ml of distilled water and five times with 1 ml of 50 mM citrate phosphate buffer. The equilibrated adsorbent was suspended in 1 ml of adjusted rhEPO-containing solution and the suspension was stirred for 20 hours at the frequency of 80 rpm using a rotary shaker. The content of rhEPO and total proteins in the solution before and after the adsorption were determined and the adsorbed rhEPO/total protein amount was calculated from the material balance.

Column separation

System ÄKTA FPLC™ (GE Healthcare) was used for rhEPO separation from the rhEPO concentrate. Chromatographic column Tricorn (GE Healthcare) was filled with the adsorbent Capto MMC ImpRes. The column adsorbent volume was 1.198 ml when the bed height and diameter were 6.1 cm and 0.5 cm, respectively.

Tab. 1. Eluent composition in separation runs.

Run	Eluent*
S1	Shock-wave gradients: B1 → B2 → B6
S2	Shock-wave gradients: B3 → B4 → B6
S3	Shock-wave gradients: B5 → B6
S4	Linear gradient B1/B5 → shock-wave gradient B6
S5	Shock-wave gradients: B7 → B8 → B6
S6	Shock-wave gradients: B3 → B4 → B6

*Symbols B1–B8 refer to different buffers specified in Table 2.

Tab. 2. Elution buffers.

Buffer code	Buffer type*
B1	Tris, pH 7
B2	Tris/1 M NaCl, pH 7
B3	Tris, pH 8
B4	Tris/1 M NaCl, pH 8
B5	Tris, pH 9
B6	Tris/1 M NaCl, pH 9
B7	CF/2 M NaCl, pH 5.5
B8	CF/2 M NaCl/2 M urea, pH 5.5

*Tris – 50 mM Tris-HCl buffer, CF – 50 mM citrate-phosphate buffer.

The adsorbent was first equilibrated with 30 ml of the 50 mM citrate-phosphate buffer containing 300 mM NaCl at pH 5.5. The feed pH and composition were adjusted to pH 5.5 and NaCl concentration of 300 mM. The feed volume was either 9 ml (runs S1–S5) or 13 ml (run S6) and the feed flow rate was 0.5 ml/min. After the feed loading was finished, the bed was washed with 3.6 ml of the adsorption buffer. Several ways of gradient elution to recover rhEPO from the column were tested (Table 1). The eluent composition was adjusted using different buffers labelled B1–B8 (Table 2). The elution flow rate was 0.5 ml/min and the UV and conductivity signals were monitored during the whole experiment. Elution fractions were collected and the rhEPO/total protein concentration was determined.

Protein assays

BCA method

Protein concentration in batch equilibrium experiments was determined using the BCA method. Working solution was prepared by mixing bicinchoninic acid (BCA) from Sigma-Aldrich (St. Louis, USA) with a 4 % CuSO₄ solution in the ratio of 50 : 1. First, a 25 µl sample was pipetted into a microplate well. Using a multichannel pipette, 200 µl of the working solution was added to the sample. The microplate was incubated for 30 minutes at the temperature of 37 °C. The sample absorbance was measured at the wavelength of 562 nm. Bovine serum albumin (BSA) in the concentration range of 0.05–1 g/l was used for calibration.

Bradford method

Protein concentration in elution chromatography samples was determined using the Bradford method due to the interference of Tris-HCl buffer with the BCA reagent. The Bradford agent is a mixture of 0.01 % Coomassie Brilliant Blue G-250, 4.7 % ethanol, and 8.5 % phosphoric acid in deionized water. It was filtered before use. Then, 20 µl of the analysed sample and 1500 µl of the Bradford agent were pipetted into test tubes. After 10–15 minutes, absorbance was measured at two wavelengths, 590 and 450 nm. BSA solution in the concentration range of 0.05–2 g/l was used for calibration.

ELISA method

rhEPO concentration was determined using a commercial kit of sandwich ELISA method (Sigma-Aldrich). The sample volume of 100 µl was transferred into a microplate well which was incubated for 2.5 hours at the temperature of 25 °C. It was then washed four times with 300 µl of wash solution. After adding 100 µl of biotinylated detection antibody into

each well, the well plate was incubated for one hour at room temperature. The wells were again washed four times as before. A streptavidin-HRP solution (100 μ l) was added into each well and the well plate was incubated for 45 minutes at gentle shaking. The washing was repeated again. Finally, 100 μ l of ELISA colorimetric reagent TMB were added into each well and the well plate was incubated for 30 minutes in dark at gentle shaking. To stop the reaction, 50 μ l of 0.2 M sulphuric acid were added and the absorbance at 450 nm was measured.

In run S6, a recently developed indirect ELISA method was used (Molnár et al., 2019).

Results and discussion

Equilibrium experiments for the determination of optimal rhEPO adsorption conditions were done in the pH range of 5.5–7.5 and NaCl concentration range from 0 to 300 mM. Equilibrium adsorbed amounts of rhEPO, q_{EPO} , and total proteins, q_{Prot} , are shown in Figs. 2a and 2b. It is evident that higher pH has negative influence on the adsorption of both rhEPO and total proteins. On the other hand, higher salt concentration promotes rhEPO binding in spite of lower binding of total proteins. This effect is advantageous for selective separation already in the adsorption phase. Fig. 2a shows that the highest amount of rhEPO was adsorbed at pH 5.5 and 300 mM NaCl. Therefore, these conditions were chosen for chromatography feed.

Efficiency of rhEPO separation was examined in six elution chromatography runs (Fig. 3). For efficient elution, desorption by 1 M NaCl was first examined in runs S1–S3. Different pH was also applied in runs S1–S3. Each of these runs started with feeding a salt-free eluent. The NaCl concentration was later increased to 1 M in a shock-wave gradient. In the final elution phase, a shock-wave gradient increase of pH from 7 (run S1) or 8 (run S2) to the value of 9 was applied. Figs. 3a–3c show abrupt increase of outlet protein concentration demonstrated by the

increase of UV signal after the application of each gradient change. Numbered peaks denote each elution phase.

These results demonstrate a synergistic effect of pH and NaCl concentration on protein recovery. The existence of several elution peaks implies strong protein binding albeit not so strong as in case of multimodal anion exchanger applied to the separation of this product (Kurák et al., 2019). A significant fraction of adsorbed proteins was eluted using salt-free eluents (peak 1 in Figs. 3a–3c). The protein yield increased from 23 % at pH 7 to 50 % at pH 8 and finally to 74 % at pH 9. The application of 1 M NaCl in the second elution phase led then to almost complete protein recovery. Only in run S1, 6 % of proteins were recovered in the third phase. Unfortunately, sensitivity of the ELISA method allowed only semi-quantitative estimation of the eluted rhEPO. This was much higher in the second fraction than in the first one in runs S1 and S2. In run S3, approximately equal amounts of rhEPO were detected in both fractions.

Following these results, a linear pH gradient from 7 to 9 and salt-free eluent were applied in the first phase of run S4. Fig. 3d shows that no positive effect was achieved and the separation performance was very similar as in run S1. Considering the distribution of rhEPO and impurity proteins in individual fractions in runs S1–S4, the best selectivity of rhEPO separation was achieved in run S2 carried out at pH 8.

Fig. 3e presents the results of run S5 which was designed at the same pH in both the loading and the elution phases. An eluent with stronger desorption effect had to be applied from the beginning of the elution. It is clear that neither 2 M NaCl nor 2 M NaCl/2M urea were efficient enough. Complete protein desorption was achieved only when pH was increased to 9 in the final elution phase.

Fig. 3f presents the results of run S6 in which the same process conditions were used as in run S2 ex-

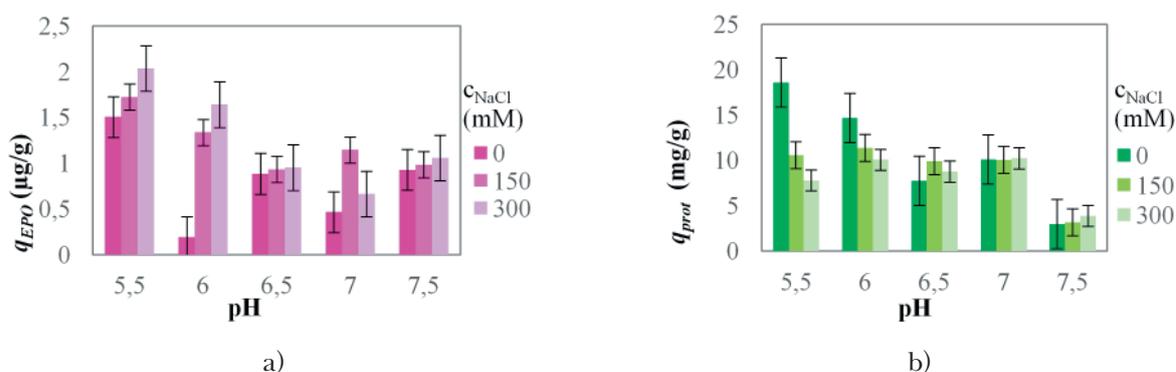


Fig. 2. pH and NaCl concentration influence on adsorbed amount of a) rhEPO and b) total proteins.

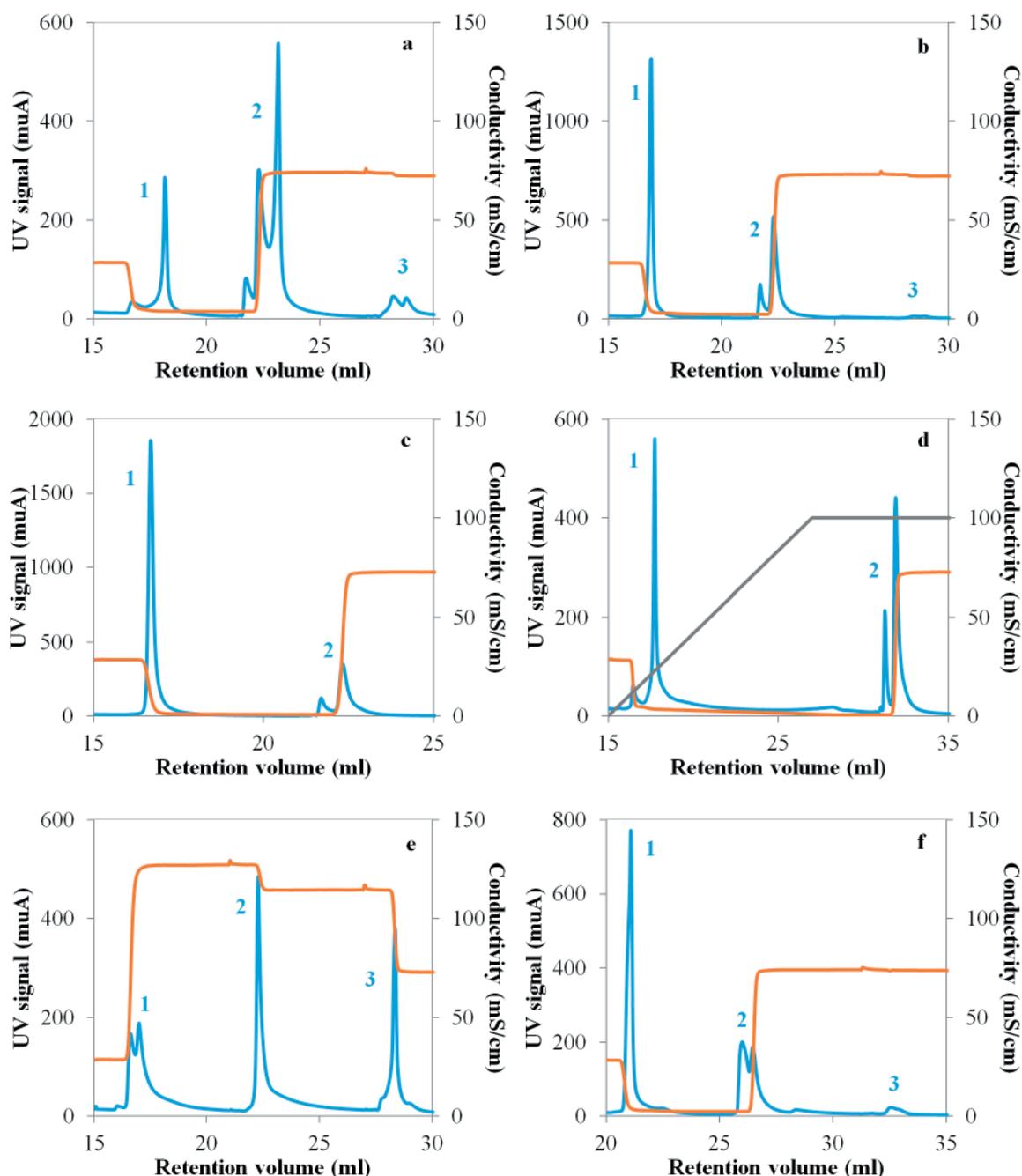


Fig. 3. Chromatograms of individual separation runs: a) S1, b) S2, c) S3, d) S4, e) S5, and f) S6. Blue and orange lines represent UV and conductivity signals, respectively. Peak numbers denote individual elution phases.

cept for a higher feed volume. Since a product from a different cultivation batch was used in this run, the rhEPO and protein loads were more than twice higher. Figs. 3b and 3f show that the chromatograms of runs S2 and S6 are very similar. The protein recovery yields in both phases were almost identical again. Due to a higher rhEPO feed concentration in run S6 and better reproducibility of a recently developed analytical method (Molnár et al., 2019), the rhEPO concentration in the elution fractions, the measurement reliability was higher. It was found that the rhEPO purity was around

90 % in the first elution fraction (peak 1) and 60 % in the second elution fraction (peak 2). The total rhEPO yield was however only slightly below 50 %, similarly as for a multimodal anion exchanger in our previous work (Kurák et al., 2019).

Conclusions

Adsorbent Capto MMC ImpRes has been proven to be a good candidate for rhEPO capture step in rhEPO separation. The most selective binding of rhEPO from a post-cultivation supernatant was

observed at pH 5.5 and NaCl concentration of 300 mM in batch equilibrium experiments. Chromatographic runs were used to optimize elution conditions. Two shock-wave gradients, in which pH was increased to 8 and the NaCl concentration to 1 M, provided the best separation performance. The rhEPO purity of about 80 % was reached when the yield of this target protein was 50 %.

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