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Chromatographic removal of endotoxin from hemoglobin preparations
Effects of solution conditions on endotoxin removal efficiency and protein recovery

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Abstract

In this work, affinity chromatography was used to remove endotoxin from human hemoglobin preparations with a Sterogene Acticlean Etox column. The effects of solution conditions on endotoxin removal efficiency and protein recovery have been investigated. It has been found that cations Na^+ or Ca^{2+} reduced endotoxin removal efficiency from 73% (sample prepared with endotoxin-free water) to 31% (sample prepared with 0.15 *M* NaCl, ionic strength, $I=0.15$ *M*), and from 73% (sample prepared with endotoxin-free water) to 9% (sample prepared with 0.05 *M* $CaCl_2$, ionic strength, $I=0.15$ *M*). It has also been found that the protein recovery was increased from 90% to 96%, and from 90% to 99%, respectively.

Keywords: Endotoxins; Haemoglobin; Proteins; Toxins; Lipopolysaccharides; Polysaccharides; Protein-lipopolysaccharide complex

1. Introduction

Bacterial endotoxins are lipopolysaccharides (LPSs) derived from the outer cell membranes of Gram-negative bacteria [1]. Endotoxins are known to have potent biological effects in human and in many animal species when administered systemically [2]. In biotechnology industry, Gram-negative bacteria are widely used to produce recombinant DNA products, such as peptides and proteins. Bacterial endotoxins have been recognized by the industry as a major cause of the pyrogenic reactions that can be encountered during the administration of biotherapeutics. The removal of these physiologically active agents from final bioproducts has always been a challenge. It is known that LPS subunits (monomers) have a molecular mass of approximately 10 000 to 20 000 [3,4]. Normally, these subunits aggregate into vesicles with a molecular mass around 1 000 000 [5]. The aggregation is believed to be facilitated by cations such as Na^+, K^+, Ca^{2+}, Mg^{2+}. Because LPSs have negatively charged phosphate groups, cations, especially divalent cations such as Ca^{2+} and Mg^{2+} can act as ''bridges'' between LPS subunits, resulting in LPS bilayer sheets or vesicles with a diameter of the order of 0.1 μm in water [6].

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The stability of the large endotoxin vesicles maintained by the ''bridging effect'' of cations can be disturbed by introducing chelating agents such as ethylenediaminetetraacetic acid (EDTA). As a result, the endotoxin aggregates are broken down into LPS subunits [6]. It is also known that some proteins can disaggregate endotoxins and form complexes with LPSs in biological solutions, which makes the removal of endotoxin from protein-based biopharmaceutical products more complicated. Some of such proteins have been identified, such as polyclonal [7] and monoclonal antibodies [8], lipopolysaccharide binding protein (LBP) [9], bovine serum albumin (BSA) [10] and lipoproteins [11]. It is reported that hemoglobin not only can bind but also disaggregate LPSs, and enhance LPS activation of limulus amebocyte lysate (LAL) in a concentration dependent manner [12,13]. In previous studies, researchers in our research group investigated the concentration effects of hemoglobin and albumin on protein–LPS binding and endotoxin removal [14]. It was found that the proteins interacted with LPSs and formed protein–LPS complexes, resulting in the disaggregation of LPS vesicles. It is believed that the interaction of protein and LPSs causes the breakdown of cation bridges between LPS subunits and the subsequent disaggregation of LPS vesicles.

Numerous methods have been applied to remove endotoxin from protein solutions, including ion-exchange [15,16] and affinity chromatography [17–19], ion-exchange membrane [20], adsorption with functional particles [21], extraction [22], etc. However, in the situations where endotoxins bind product proteins, removal of endotoxins is almost always problematic. Significant product loss and low product yield can result from the separation steps employed to remove endotoxins. Chromatographic methods are widely used to remove endotoxin from biological solutions. However, few studies have addressed how the protein–LPS binding affects the removal of endotoxin from protein solutions in a chromatographic process. Moreover, it is unknown how the solutions containing cations in the feed affect endotoxin removal and protein recovery during a chromatographic removal of endotoxin from a protein–LPS complex.

Hirayama et al. [15] studied the use of cross-linked *N*,*N*-dimethylaminopropylacrylamide (DMAPAA) spherical particles for selective removal of endotoxin from protein solutions. They found that ionic strength has less effect on the endotoxin removal efficiency with DMAPAA than with immobilized histidine. In another study, investigators from the above group [16] studied the application of polyethyleneimine (PEI)-immobilized cellulose fibers for endotoxin removal from protein solutions. Matsumae et al. [18] applied immobilized histidine to remove endotoxin from protein solutions. The effects of pH and ionic strength on the removal of endotoxin were investigated. Although the influences of ionic strength on endotoxin removal were reported in the above articles, the effects of divalent cations on endotoxin removal and protein recovery were not studied. At the same ionic strength, divalent cations have much stronger influences on endotoxin aggregation state due to the ''bridging effect'' [23].

In this study, an affinity chromatography column was used to remove endotoxin from the hemoglobin preparations where the protein formed complex with LPS subunits. The effects of various solutions such as endotoxin-free water, NaCl solution and $CaCl_2$ solution on the endotoxin removal efficiency and protein recovery in the chromatographic process were investigated. The effects of protein–LPS binding on protein yield in a chromatography column have also been studied.

2. Experimental

2.1. Materials

2.1.1. Reagents

Endotoxin from *Escherichia coli* 026:B6 (Sigma, St. Louis, MO, USA) was used in all studies. All solutions were prepared with endotoxin-free water (LAL reagent water, Biowhittaker, Walkersville, MD, USA). Hemoglobin Ao (HbAo, ferrous), NaCl (>99%) and $CaCl_2$ (>99%) were purchased from Sigma.

2.1.2. Column

The experiments were conducted on a Acticlean Etox column (Product No. 4AA04), which was kindly provided by Sterogene Bioseparations (Carlsbad, CA, USA). The column dimensions were

3.5×0.7 cm. The column was packed with an Acticlean Etox affinity resin, which had an Actigel ALD support matrix (4% agarose) linked to a proprietary ligand through a secondary amine. Before each use the column was cleaned by perfusion with 30 ml of 1.0 *M* NaOH, allowed to stand at 4°C overnight, and washed with endotoxin-free water until neutrality. Samples were applied to the column at an approximate flow-rate of 0.3 ml/min. Elution was carried out at above flow-rate with endotoxin-free water as the mobile phase.

2.1.3. Labware

All glassware used was autoclaved at 15 p.s.i.g. (1 p.s.i.=6894.76 Pa) for 30 min with an electric pressure steam sterilizer (Model No. 25X, Wisconsin Aluminum Foundry, WI, USA) and followed with heating in an oven (Model 16, Precision Scientific, IL, USA) at 210°C for 3 h. All solution transfers were performed by endotoxin-free devices. Sterile, disposable plasticware was used at all times to prevent endotoxin contamination.

2.2. Protein assay

The absorption scanning from 250 nm to 700 nm in our laboratory shows that the endotoxin sample only has the absorbance peak at around 280 nm (the absorption spectrum is not shown in this paper). Therefore HbAo concentration was measured with a spectrophotometer (U-2000, Hitachi, Japan) at 415 nm to avoid the effect of endotoxin absorbance interference.

2.3. Endotoxin assay

To determine endotoxin concentration, a chromogenic LAL test kit QCL-1000 (modified procedure) [24] from Biowhittaker was used. The sensitivity of this test is 0.01 EU/ml. The reaction mixtures were measured at 405 nm by the spectrophotometer (Hitachi) and the results were compared to a standard curve to obtain endotoxin concentrations. However, HbAo also has a strong absorbance at 405 nm and deduction of HbAo absorbance may be necessary. The correction was done by deducting the HbAo absorbance from total absorbance of the reaction mixture.

2.4. Endotoxin removal from HbAo–LPS solution prepared with endotoxin-free water

Sterile, 98.1 μg/ml HbAo solution was spiked with endotoxin to 2.60 EU/ml and incubated for 30 min at 37°C in water bath in order to form HbAo–LPS complex [14]. Ten ml of this HbAo solution were perfused through the column at 0.3 ml/min. Six fractions were collected at 2 ml, 12 ml, 14 ml, 16 ml, 18 ml and 20 ml. Proper dilutions of samples taken from the fractions were needed in order to obtain the maximum endotoxin assay sensitivity.

2.5. Endotoxin removal from HbAo–LPS solution prepared with 0.15 M NaCl solution

HbAo prepared with 0.15 *M* NaCl solution was mixed with endotoxin to reach 2.04 EU/ml final endotoxin concentration and 93.3 μg/ml HbAo concentration. This solution was incubated for 30 min at 37°C. The ionic strength of the solution was 0.15 *M*, which was calculated by Debye–Huckel theory [25]. A 10-ml sample was applied to the column. Six fractions were collected at 2 ml, 12 ml, 14 ml, 16 ml, 18 ml and 20 ml.

2.6. Endotoxin removal from HbAo–LPS solution prepared with 0.05 M $CaCl_2$ solution

HbAo prepared with 0.05 *M* $CaCl_2$ solution was mixed with endotoxin to reach 1.82 EU/ml final endotoxin concentration and 90.7 μg/ml HbAo concentration. This solution was incubated for 30 min at 37°C. The ionic strength of the solution was also 0.15 *M*, the same as the ionic strength of NaCl solution mentioned in Section 2.5. A 10-ml sample was applied to the column. Six fractions were collected at 2 ml increments except fraction II, which was 10 ml.

2.7. Elution of pure HbAo solutions

Pure HbAo solutions were prepared with endotoxin-free water, 0.15 *M* NaCl and 0.05 *M* $CaCl_2$, respectively. The solutions were incubated for 30 min at 37°C. Ten ml of these solutions were applied to the column and 2-ml fractions were collected except fraction II, which was 10 ml. HbAo con-

centration in each fraction was measured by spectrophotometry at 415 nm.

3. Results and discussion

3.1. Endotoxin elution profiles of various feeds

Fig. 1 (case 1) shows the endotoxin elution profile when the feed was HbAo–LPS prepared with endotoxin-free water. We collected six fractions. The volume of each fraction was 2 ml except fraction II. Fraction II was the main fraction or the purified protein product. Its volume was 10 ml. The first 2 ml collection had trace amount of endotoxin due to the retention volume of the column was less than 2 ml.

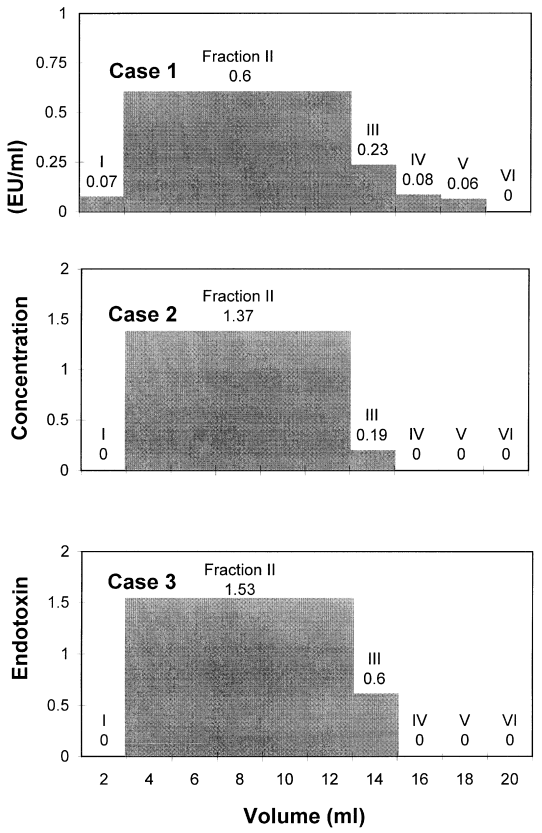

Fig. 1. Endotoxin elution profiles on Acticlean Etox column. Case 1, feed: 10 ml HbAo–LPS solution prepared with endotoxin-free water. Case 2, feed: 10 ml HbAo–LPS solution prepared with 0.15 *M* NaCl solution. Case 3, feed: 10 ml HbAo–LPS solution prepared with 0.05 *M* $CaCl_2$ solution.

Endotoxin concentration in the main fraction (fraction II) was the highest (0.6 EU/ml) among all the fractions. Starting from fraction III the endotoxin concentration in the subsequent fractions decreased as the elution process continued. The endotoxin content in fraction VI was zero, which means the endotoxin concentration is below 0.01 EU/ml, the test limit of the chromogenic LAL test. Fig. 1 (case 2) shows the endotoxin elution profile when the feed was prepared with 0.15 *M* NaCl solution. Fig. 1 (case 3) shows the endotoxin elution profile when the feed was prepared with 0.05 *M* $CaCl_2$ solution. By comparing the above three cases, it can be seen that the endotoxin peaks in case 2 and case 3 are narrower than those in case 1. This indicates the effects of Na^+ or Ca^{2+} in the solution on endotoxin profile in the eluted protein fractions.

3.2. Endotoxin removal efficiency with Acticlean Etox column for various feeds

The endotoxin concentrations in the protein–LPS mixtures before and after column elution are shown in Fig. 2 for cases 1, 2 and 3. Endotoxin removal

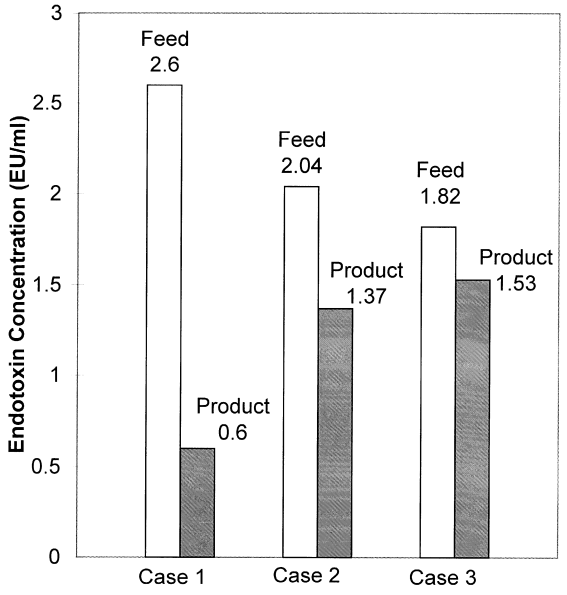

Fig. 2. Endotoxin removal from HbAo–LPS solution. Case 1, feed prepared with endotoxin-free water. Case 2, feed prepared with 0.15 *M* NaCl solution. Case 3, feed prepared with 0.05 *M* $CaCl_2$ solution.

Table 1
Endotoxin removal efficiency and HbAo recovery by Acticlean Etox column

Feeds	Endotoxin removed (%)	HbAo yield (%)	HbAo recovery (%)
HbAo–LPS prepared with endotoxin-free water	73	70	90
HbAo–LPS prepared with 0.15 *M* NaCl solution	31	83	96
HbAo–LPS prepared with 0.05 *M* $CaCl_2$ solution	9	85	99
HbAo prepared with endotoxin-free water	–[a]	71	94
HbAo prepared with 0.15 *M* NaCl solution	–[a]	86	100
HbAo prepared with 0.05 *M* $CaCl_2$ solution	–[a]	88	100

[a] Endotoxin removed (%) is not applicable.

efficiency of the column was summarized in Table 1 in term of removal percentage for all three cases. In case 1 where the feed was HbAo–LPS mixture prepared with endotoxin-free water, 27% of the total endotoxin in the feed was eluted with the product. In other words, 73% of total endotoxin was adsorbed by the column. In case 2 where the feed was HbAo–LPS mixture prepared with 0.15 *M* NaCl solution, 31% of the total endotoxin in the feed was adsorbed by the column. In case 3 where the feed was HbAo–LPS mixture prepared with 0.05 *M* $CaCl_2$ solution, 91% of the total endotoxin in the feed was eluted out with the product. In other words, only 9% of the total endotoxin was removed from the protein–LPS mixture by the column.

3.3. Protein elution profiles and recovery: pure HbAo solutions as the feeds to the column

From the engineering point of view we define the yield as the total amount of HbAo in the main fraction divided by the total amount of HbAo in the feed, and the recovery as the total amount of HbAo in the eluent divided by the total amount of HbAo in the feed. In this part of the study, pure HbAo solutions were employed as feeds to the column, as described in Section 2.7. The results were used to compare with the results obtained from experimental runs where HbAo–LPS mixtures were used as feeds to the column. The latter will be discussed in Section 3.4. The data of pure HbAo feed solutions served as a baseline for investigating the effects of endotoxin in HbAo solutions on protein recovery.

There were three cases in the pure HbAo elution study. To distinguish them from the previous three cases (cases 1, 2 and 3 mentioned in Section 3.1 Section 3.2), we designated them as case P-1, case P-2 and case P-3. In case P-1 pure HbAo solution was prepared with endotoxin-free water to serve as the feed to the column. In case P-2 and case P-3 pure HbAo solutions were, respectively, prepared with 0.15 *M* NaCl and 0.05 *M* $CaCl_2$ to serve as the feeds to the column. The HbAo elution profile of all three cases are shown in Fig. 3. It can be seen that the HbAo solutions prepared with NaCl or $CaCl_2$ resulted in narrower protein peaks than that of HbAo solution prepared with endotoxin-free water. The protein yields and recoveries for all the three cases were calculated based on initial feeds and the elution profiles in Fig. 3. The results are summarized in Table 1. When the HbAo feeds were prepared with salt solutions, the elution times were short and the protein recoveries were 100% for both case P-2 and case P-3. This means that there was no protein binding to the column. However, when the HbAo feed was prepared with endotoxin-free water, the elution took a longer time and the protein recovery was 94% for case P-1. This indicates slight protein

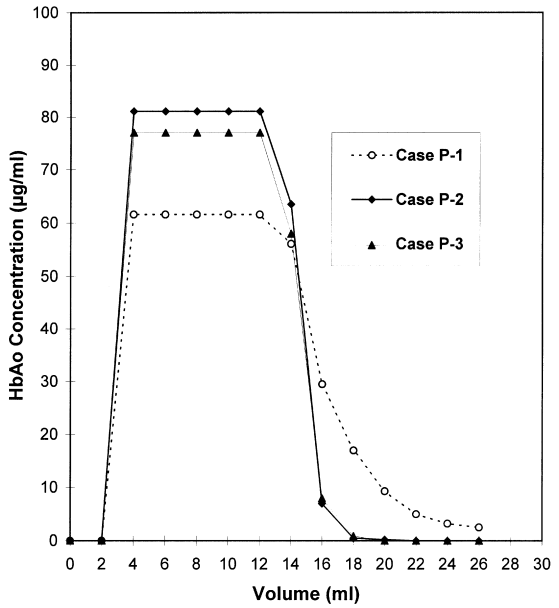

Fig. 3. HbAo elution profiles on Acticlean Etox column. Case P-1, feed: 10 ml HbAo solution prepared with endotoxin-free water. Case P-2, feed: 10 ml HbAo solution prepared with 0.15 *M* NaCl solution. Case P-3, feed: 10 ml HbAo solution prepared with 0.05 *M* $CaCl_2$ solution.

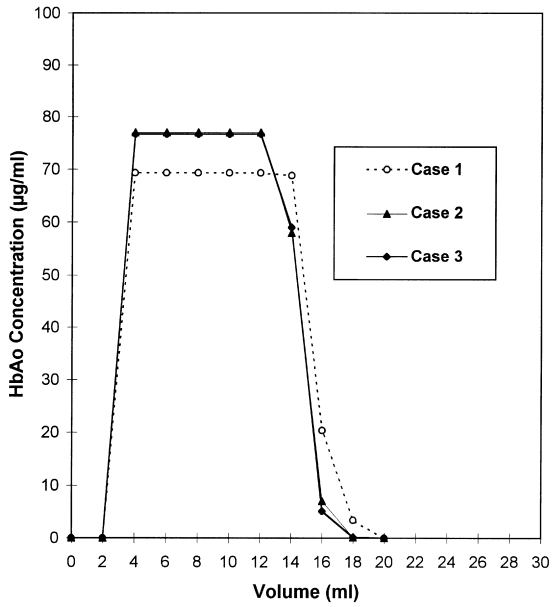

Fig. 4. HbAo elution profiles on Acticlean Etox column. Case 1, feed: 10 ml HbAo–LPS solution prepared with endotoxin-free water. Case 2, feed: 10 ml HbAo–LPS solution prepared with 0.15 *M* NaCl solution. Case 3, feed: 10 ml HbAo–LPS solution prepared with 0.05 *M* $CaCl_2$ solution.

binding to the column in this situation. In case P-1, when the elution mobile phase was switched from endotoxin-free water to 0.15 *M* NaCl, 6% of HbAo adsorbed in the column was eluted out and the total recovery was 100% (chromatogram not shown). Overall, there was almost no HbAo binding to the column when pure HbAo solutions were fed to the column.

3.4. Protein elution profile and recovery: HbAo–LPS solutions as the feeds to the column

In this part of the study, HbAo–LPS mixtures were employed as the feeds to the column, as described in Sections 2.4–2.6. The fractions for all three cases were collected for endotoxin and protein analyses. Endotoxin elution profiles and endotoxin removal efficiency have been discussed in Sections 3.1 and 3.2. Here we will report the investigation on protein elution profiles and protein recovery. We will also compare the results to those of pure HbAo elution presented in Section 3.3. There were three cases in the HbAo–LPS mixture elution study: case 1, case 2 and case 3, as described in Section 3.1. The HbAo elution profiles of all three cases are shown in Fig. 4. It can be seen that the HbAo solutions prepared with NaCl and $CaCl_2$ resulted in narrower protein peaks than that of HbAo solution prepared with endotoxin-free water. The protein yields and recoveries for all three cases were calculated based on initial feeds and the elution profiles in Fig. 4. The results are summarized in Table 1. The recovery increased from 90%, 96% to 99% when the feeds were prepared with endotoxin-free water, 0.15 *M* NaCl and 0.05 *M* $CaCl_2$, respectively.

3.5. Discussion

The results in Sections 3.1 and 3.2 show that cations in the feeds have significant influences on endotoxin removal efficiency of the column. Further, 0.05 *M* $CaCl_2$ affected endotoxin binding to the column much more than 0.15 *M* NaCl, despite that both solutions have the same ionic strength: 0.15 *M*. Cations in the feeds also affect the protein recovery under both situations of pure HbAo elution and

HbAo–LPS elution, which are presented in Sections 3.3 and 3.4, respectively. The results indicate that the column almost does not have affinity to HbAo, especially when the feeds were prepared with cation solutions. Further, the results show the effects of protein-LPS interaction on the protein recovery. By comparing the HbAo recovery in Table 1 we can see that the presence of endotoxin in protein solutions decreased the protein recovery. For example, when HbAo prepared with endotoxin-free water was fed to the column and eluted by endotoxin-free water as the mobile phase, the HbAo recovery was 94%. However, when HbAo–LPS mixture prepared with endotoxin-free water was fed to the column and eluted by endotoxin-free water as the mobile phase, the HbAo recovery was 90%. Similar changes in HbAo recovery also happened in the situations where the feeds were prepared with NaCl and $CaCl_2$ solutions. The decrease of the HbAo recovery is apparently due to the complexation between HbAo and LPS. Since LPS bound the adsorbent resin in the column, the HbAo molecules in the HbAo–LPS complex were kept in the column as well.

The adsorption mechanism in this process may be explained as follows. There are three types of interactions during the chromatographic process: resin and endotoxin, resin and HbAo, and HbAo and endotoxin. Endotoxin interacts with the resin via strong ionic and hydrophobic interactions, while HbAo interacts with the resin via weak ionic interaction. It is reported previously that the complexes formed between hemoglobin and LPS subunits are stable with a calculated dissociation constant, K_d, of $3.1 \cdot 10^{-8}$ M [12]. The forming of these complexes affects both the removal of endotoxin and recovery of the protein.

For an endotoxin removing step in downstream bioprocessing, it is ideal to have both high endotoxin removal efficiency and high protein recovery. We have summarized the endotoxin removal and protein recovery data of all three cases in Table 1. When HbAo–LPS mixture was prepared with endotoxin-free water, the column had the highest endotoxin removal efficiency, 73%, but the lowest HbAo recovery, 90%. When HbAo–LPS mixture was prepared with 0.05 M $CaCl_2$ solution, the column had the lowest endotoxin removal efficiency, 9%, but the highest HbAo recovery, 99%.

4. Conclusions

In this work, an affinity chromatographic column Acticlean Etox was used to remove endotoxin from HbAo preparations. It was found that the cations in the feeds had significantly decreased the endotoxin removal efficiency. It was also found that 0.05 M $CaCl_2$ in the feed reduced the endotoxin removal efficiency more than 0.15 M NaCl, although both solutions had the same ionic strength. Protein recovery of the endotoxin removal process was also investigated. It was found that the HbAo recoveries from the HbAo–LPS mixtures were lower than the HbAo recoveries from pure HbAo solutions in all cases, due to the complexation between HbAo and LPS. Finally, we noticed that when the feed was prepared with endotoxin free water, the column had the highest endotoxin removal efficiency but the lowest HbAo recovery; when the feed was prepared with $CaCl_2$ solution the column had the lowest endotoxin removal efficiency but the highest HbAo recovery. Therefore, a compromise may need to be made between high endotoxin removal efficiency and high protein recovery when carrying out optimization studies on a chromatographic endotoxin removal process.

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