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Rong Ma[ab]; Dai-Di Fan[abc]; Wen-Jiao Xue[d]; Jian-Yu Xing[abe]; Chen-Hui Zhu[ab]; Xiao-Xuan Ma[ab]
[a] Shaanxi Key Laboratory of Degradable Biomedical Materials, School of Chemical Engineering, Northwest University, Xi'an, Shaanxi, China [b] Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi'an, Shaanxi, China [c] Xi'an Giant Biogene Technology Limited Company, China [d] Shaanxi Provincial Institute of Microbiology, Xi'an, China [e] School of Environmental Science and Engineering, Chang'an University, Xi'an, China

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Rong Ma,[1,2] Dai-Di Fan,[1,2,3] Wen-Jiao Xue,[4] Jian-Yu Xing,[1,2,5] Chen-Hui Zhu,[1,2] and Xiao-Xuan Ma[1,2]

[1]*Shaanxi Key Laboratory of Degradable Biomedical Materials, School of Chemical Engineering, Northwest University, Xi'an, Shaanxi, China*
[2]*Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi'an, Shaanxi, China*
[3]*Xi'an Giant Biogene Technology Limited Company, China*
[4]*Shaanxi Provincial Institute of Microbiology, Xi'an, China*
[5]*School of Environmental Science and Engineering, Chang'an University, Xi'an, China*

Interaction between human-like collagen (HLC) and endotoxin makes endotoxin removal from HLC more complicated. In this study, a simple, effective, and low-cost method was developed for endotoxin removal. A 0.4% (volume fraction) TritonX-100 solution was used to dissociate the HLC-endotoxin complexes and Q XL resin was used to remove endotoxin from the HLC solution. Product solution containing 1.2 mg · ml^{-1} HLC in Tris-HCl buffer (pH 7.5) and 50 mM NaCl was added to the Q XL resin; the endotoxin removal efficiency was more than 98% and HLC recovery of up to 95% could be obtained; HLC purity was also enhanced after this process. The residual TritonX-100 was removed by using a superdex-200 column. This method greatly reduced the cost of purification but provided high endotoxin removal efficiency and HLC recovery.

Keywords anion-exchange chromatography; endotoxin removal; endotoxin-protein complex; human-like collagen; TritonX-100

INTRODUCTION

Bacterial endotoxins are lipopolysaccharides (LPS) present at the outer cell surface of gram-negative bacteria, including *Escherichia coli* (1). They exhibit strong biological effects at very low concentrations in human beings (threshold level: 1 ng per kg body weight and hour) and in many animals when they enter the blood stream (2). In the biotechnology industry, gram-negative bacteria, especially *E. coli*, are widely used to produce recombinant products such as peptides and proteins; thus, endotoxin removal becomes the critical step before their therapeutic application.

Although many methods such as phase separation (3), anion-exchange chromatography, and affinity adsorption (4) have been reported for endotoxin removal from protein solutions, in situations where the endotoxin binds to the product proteins, the removal of endotoxins is almost always problematic (5). Owing to protein-endotoxin interactions, endotoxin removal from protein solutions requires techniques that result in strong interactions with endotoxin, and affinity chromatography has been proven to be one of the most effective methods (2,6). However, this method is not highly reproducible and may be accompanied by a significant loss of the product being purified. In addition, the adsorption capacity of adsorbents is generally low (7). A anion-exchange chromatography has also been widely used because it has low running cost when compared to affinity chromatography and it is easy to scale-up; however, significant ionic interactions may be present between the protein and the resin or the endotoxin and the protein as well. Therefore, binding of the endotoxin can often result in large losses in protein recovery, or the endotoxin will be able to flow through the column because it is tightly associated with the flow-through protein (8).

In this study, we developed a simple and low-cost method for endotoxin removal using human-like collagen (HLC). Our previous research showed that the HLC can interact with endotoxin, forming HLC-endotoxin complexes, and the nonionic surfactant TritonX-100 can dissociate these complexes; thus, the amount of TritonX-100 used for the dissociation of HLC-endotoxin complexes was investigated first. Then, an anion-exchange column with poor protein binding characteristics was chosen and

Received 23 November 2009; accepted 8 April 2010.
Address correspondence to Dai-Di Fan, College of Chemical Engineering, Northwest University, Xi'an, P. R. China. Tel.: +86-29-8830-5118; Fax: +86-29-8830-2348. E-mail: fandaidi@nwu.edu.cn

an appropriate buffer condition was determined to maintain high endotoxin removal efficiency and protein recovery. Finally, a simple method was adopted to remove the residual TritonX-100.

EXPERIMENTAL

Materials

Tris and TritonX-100 were purchased from Amresco Co. and Genview Co. respectively. All other reagents were of analytical grade. Glassware was heated in the oven at 210°C for 3 h. All solutions were prepared with endotoxin-free water.

The protein solution was loaded onto an ion-exchange column using an ÄKTA purifier (GE Healthcare). The bed volume was 1 ml. The column was packed with Q Sepharose (quaternary amino) XL resin. Before each use, the column was cleaned by perfusion with 0.5 M NaOH, allowed to stand for 2 h at 4°C, and washed with endotoxin-free water to obtain a neutral pH. Chromatography was performed at a constant flow rate of $0.5\,mg \cdot ml^{-1}$. All flow-through fractions during these steps were collected and analyzed.

Salt and TritonX-100 were removed with a Superdex-200 column (GE Healthcare) at a flow rate of $0.5\,mg \cdot ml^{-1}$ and the dimensions of the column were 10×300 mm.

Fermentation and Purification of Recombinant HLC

A recombinant *E. coli* plasmid containing a kanamycin resistance gene that allows temperature induction was constructed in our lab. Batch and fed-batch cultivations were carried out in an in situ autoclaved fermentor to achieve high-level expression of the target protein (9–11).

The cultured cells were harvested by centrifugation and were washed thrice with distilled water; the suspensions were disrupted twice in a high-pressure disrupter (Rannie 15.5, Denmark APV) at 700 bar. The homogenate was treated with sodium chloride (NaCl) for precipitation and then with hydrochloric acid until a pH of 2 was obtained for the removal of the non-target protein; the supernatant was then desalted by an ultrafiltration filter (CUF-100, 30 kDa cut-off, Millipore).

Endotoxin Assay

To determine the endotoxin concentration, chromogenic limulus amoebocyte lysate (LAL) test kit Pyrochrome® (Associate of Cape Cod, Inc. USA) was used. The reaction mixtures were measured at 405 nm by a Microplate Spectrophotometer (Powerwave, Biotech, USA) and endotoxin concentrations were determined by comparing the results to a standard curve.

The endotoxin removal efficiency (ERE) was calculated by the following formula (12):

$$ERE = \frac{m_{feed} - m_{flowthrough}}{m_{feed}} \quad (1)$$

where m_{feed} is the amount of endotoxin in the feed loaded on the column and $m_{flow\text{-}through}$ is the amount of endotoxin in the flow-through solution.

Protein Assay

The HLC concentration was determined by an immunodetection method according to the description in "QIA express Detection and Assay Handbook" (Qiagen).

The protein purity after purification was characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) performed using Mini-PROTEAN Tetra Cell (Bio-rad).

Effects of TritonX-100 Concentration/HLC Concentration

A $1\,mg \cdot ml^{-1}$HLC solution prepared with endotoxin-free Tris-HCl buffer (pH 7.5, 0.05 M) was mixed with TritonX-100, and the final concentrations of TritonX-100 were 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% volume fraction. These solutions were incubated at 4°C for 30 min. A sample of volume 2 ml was applied to the Q column, and the flow-through was collected for the endotoxin assay.

In the second experiment of this part of the study on the effects of protein concentration, HLC solutions of concentrations ranging from 0.2 to $2.0\,mg \cdot ml^{-1}$ were prepared with endotoxin-free Tris-HCl buffer (pH 7.5, 0.05 M). These solutions were mixed with 0.4% TritonX-100, and incubated at 4°C for 30 min; then a 2 ml sample was fed into the Q column. Fractions were collected for endotoxin assay.

Effects of pH

The effects of pH on the ERE and protein yield were studied in this part. HLC feeds were prepared with endotoxin-free Tris-HCl buffer (pH 7, 7.5, 8, 8.5, and 9); the buffer concentration was 0.05 M and the HLC concentration was $1.2\,mg \cdot ml^{-1}$. These solutions were mixed with 0.4% TritonX-100, and incubated at 4°C for 30 min. Feed of volumn 2 ml was applied to the Q column. Fractions were collected to detect the endotoxin and HLC.

Effects of NaCl Concentration

HLC solutions were prepared with endotoxin-free Tris-HCl buffer (pH 7.5, 0.05 M) containing 20, 30, 40, 50, 60, 70, or 80 mM NaCl; the HLC concentration was $1.2\,mg \cdot ml^{-1}$. All the solutions were mixed with TritonX-100 at a final concentration of 0.4%, and then, each solution was incubated at 4°C for 30 min. Feed of volumn 2 ml was applied to the Q column; fractions were collected for endotoxin and HLC assays.

Desalting and TritonX-100 Removal

HLC solution was prepared with endotoxin-free Tris-HCl buffer (pH 7.5, 0.05 M) containing 50 mM NaCl,

and the HLC concentration was 1.2 mg · ml^{-1}. This solution was mixed with TritonX-100, at a final concentration of 0.4%; then, the solution was incubated at 4°C for 30 min. Feed of volumn 2 ml was applied to the Q column; 500 µl flow-through fractions were collected to load into the Superdex-200 column, and the other fractions were collected for SDS-PAGE.

RESULTS AND DISCUSSION

Effects of TritonX-100 concentration/HLC Concentration

The existence of HLC-endotoxin complexes resulted in the masking of endotoxin molecules and consequently to a partial escape from removal procedures (8). As shown in Fig. 1, the ERE was very low without TritonX-100. When the TritonX-100 concentration was increased to 0.4% (Fig. 1), the ERE increased significantly from 47.3% to 97.3%. This change might have occurred because the nonionic surfactant TritonX-100 could dissociate these complexes and greatly enhance the ERE. On increasing the TritonX-100 concentration to higher than 0.4%, the ERE remained almost constant. This demonstrated that the TritonX-100 concentration of 0.4% volume fraction was enough to dissociate the HLC-endotoxin complexes existing in the 1 mg · ml^{-1} HLC solution.

The above discussion about the dissociation effects of TritonX-100 was also supported by the results in Fig. 2; when the HLC concentration was low, ERE was almost constant at approximately 98%. When the HLC concentration was increased from 1.2 mg · ml^{-1} to 2.0 mg · ml^{-1}, the ERE dropped drastically from 97.6% to 73.2%. The reason for this was that in a certain HLC concentration range, the HLC-endotoxin complexes could be dissociated by TritonX-100. Further increase in the TritonX-100 content is not suitable since it would increase the difficulty in the TritonX-100 removal step and it might affect protein stability. Therefore, we concluded that the optimal HLC concentration was 1.2 mg · ml^{-1} and the optimal TritonX-100 content was 0.4%. From the results in Fig. 2, we could also conclude that the concentration of the protein has a significant effect on the amount of protein-endotoxin complexes formed (13).

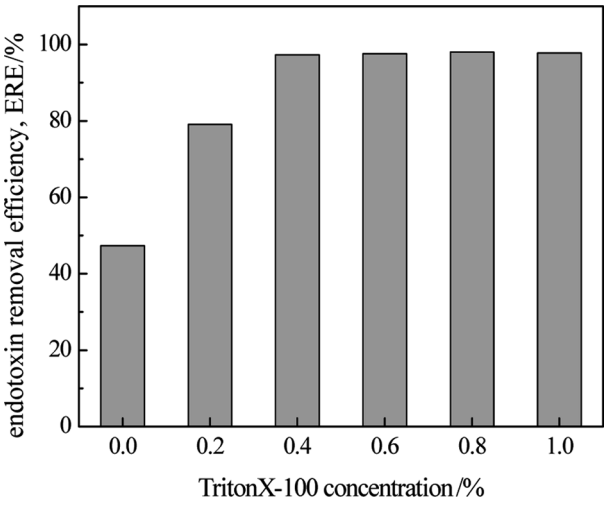

FIG. 1. Effect of TritonX-100 concentrations on endotoxin removal efficiency.

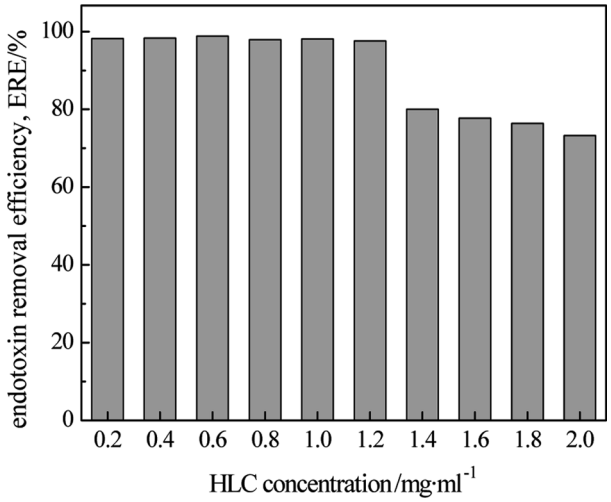

FIG. 2. Effect of HLC concentrations on the endotoxin removal efficiency.

The results of this part also provided evidence for the dominant role of the hydrophobic interaction between HLC and endotoxin. It has been reported that electrostatic interaction is the main mechanism of interaction between the endotoxin and protein (14). However, electrostatic interactions could not be the dominant factor in this research because the isoelectric points of HLC and endotoxin are approximately 6 and 2, respectively, (15) and HLC and endotoxin were both negatively charged under the experimental conditions (pH 7.5). Therefore, the mechanism of complex formation between HLC and endotoxin may involve hydrophobic interactions (16). The major hydrophobic part of the endotoxin is lipid A; it consists of a β-1,6-linked disaccharide of glucosamine, which is covalently linked to 3-hydroxy-acyl substituents with 12–16 carbon atoms via amide and ester bonds (Fig. 3) (8); further, proline and hydroxyproline in collagen are both hydrophobic amino acids. These hydrophobic groups might interact with each other and form complexes. The role of the surfactant TritonX-100 in this experiment was assumed to involve interaction with the endotoxin and alleviation of the hydrophobic interactions between endotoxins and proteins.

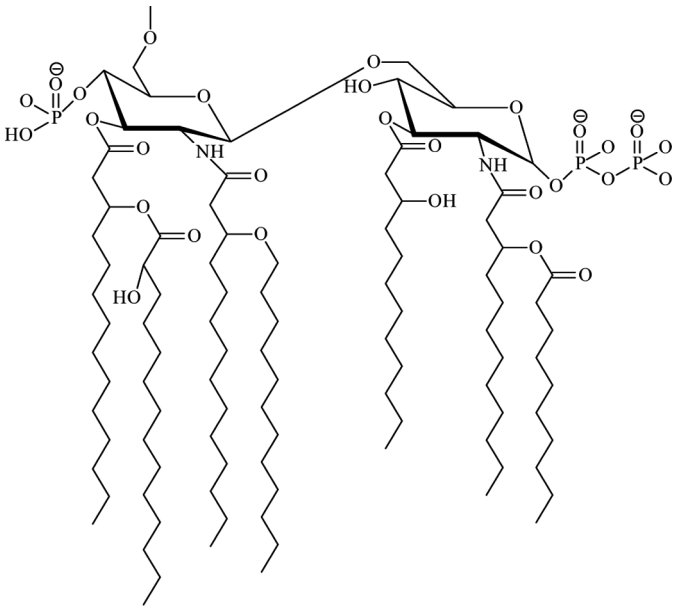

FIG. 3. Schematic view of the structure of lipid A.

pH Effects

It was important to maintain the pH of the protein solutions high enough to prevent the protein from acquiring a strong positive charge, because although positively charged proteins are more likely to pass through the Q resin, they also maintain strong ionic interactions with the endotoxin (17). On the other hand under a high pH condition, owing to the interaction between the protein and the resin, the high ERE must be accompanied by a decline in protein recovery. As shown in Fig. 4, the maximum HLC recovery was only 87.7% when the pH was increased from 7.0 to 9.0. Therefore, in the next study, NaCl was added to enhance the protein recovery.

Figure 4 also illustrates that the HLC recovery was decreased to 50.8%, when the pH increased to 9.0, because when the pH increased, more protein was bound to the column, and thus HLC recovery declined. Endotoxins were always negatively charged within the pH range used in this study; thus, the pH effects on the ERE should be very limited. However, when the pH was increased to 8.5 and 9.0, the ERE decreased; the maximum HLC recovery and ERE were obtained at pH 7.5. The binding of the protein to the column reduces the endotoxin binding sites; this may be responsible for the decrease in the ERE, and this can provide a good explanation for the question as to why the ERE and HLC recovery declined at the same time in this experiment.

Effects of NaCl Concentration

Figure 5 shows that HLC recovery was enhanced with an increase in the concentration of NaCl; however, when the NaCl concentration was increased from 50 mM to 80 mM, the HLC recovery did not change significantly. Figure 5 also shows that when the NaCl concentration was above 50 mM, the ERE dropped drastically. This result illustrates that ionic strength had a positive effect on protein recovery; it weakened the interactions between the protein and the column. Therefore, the HLC recovery was obviously enhanced. Figure 6 shows that not only did HLC bind to the column but also all the protein impurities were adsorbed to the column; the HLC purity was also enhanced after this process. On the other hand, increased ionic strength had a negative influence on endotoxin removal; the reason for this may be that NaCl weakened the interactions between the endotoxin and

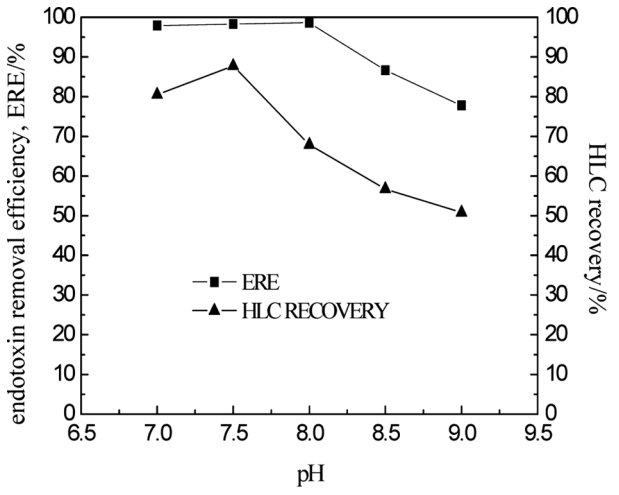

FIG. 4. pH effects on the endotoxin removal efficiency and protein recovery.

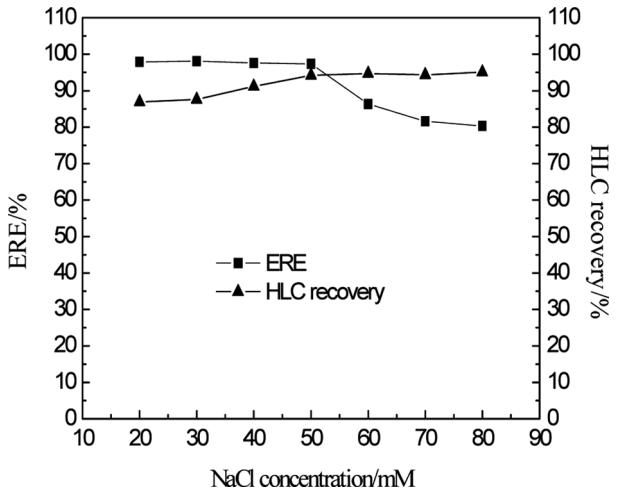

FIG. 5. Endotoxin removal efficiency and protein recovery at various NaCl concentrations.

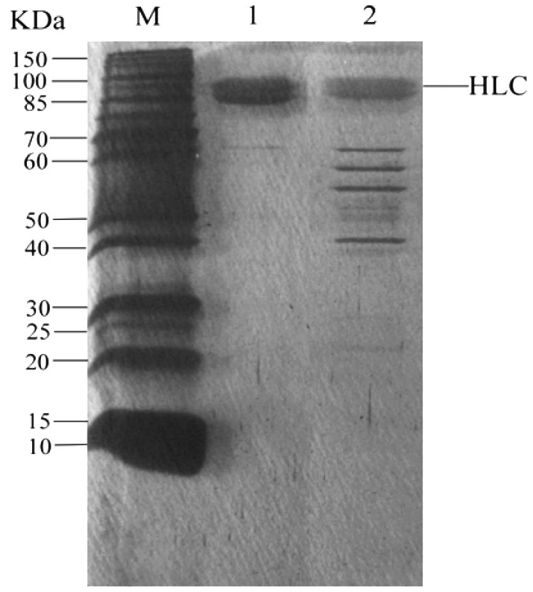

FIG. 6. The SDS-PAGE results. (Protein samples were treated by TCA/Acetone precipitation and the SDS-PAGE gel stained by silver. Lane M: molecular weight marker; Line 1: protein sample after the chromatography; line 2: the protein sample before chromatography process.)

purification resin (5). Therefore, when the NaCl concentration increased, the ERE decreased.

Results of Desalting Process

Peak 1 at 7.17 min in Fig. 7(a) is the peak for HLC (the molecular weight of HLC is 96 KDa). Chromatographic peak 3 in Fig. 7(a) at 28.87 min proved to be that of TritonX-100 by comparing with the TritonX-100 standard (peak 4 in Fig. 7b). The molecular weight of TritonX-100 is approximately 646.86 Da and it is larger than NaCl. According to the principles of gel chromatography, small molecules get trapped in the pores of the beads and slowed down but large molecules simply flow down the column; therefore, the larger molecules come out quickly. In the situation of this experiment, because of the molecular weight of TritonX-100 and NaCl were both below the separate range of the Superdex-200 (10,000∼600,000 D), they should be eluted together theoretically. However, Fig. 7 showed that the peak time of TritonX-100 was after that of NaCl. This may be explained as follows, there may be some forms of interactions between TritonX-100 and the gel column resin, and just this interaction led the peak time of TritonX-100 to be delayed. The results of Fig. 7(a) illustrated that the residual TritonX-100 could be successfully removed during the process of desalting.

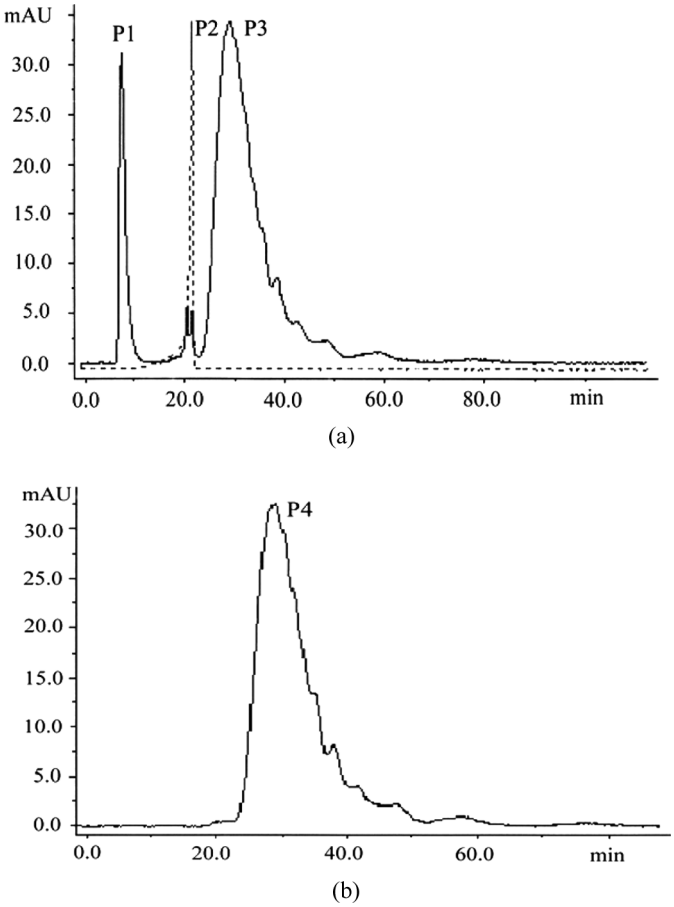

FIG. 7. Desalting and removal of TritonX-100. Solid line: UV280; dashed line: Conductivity. Chromatographic conditions; column: Superdex^{TM} 200 (10 mm × 300 mm), mobile phase: endotoxin free water, flow rate: 0.5 ml · min^{-1}, sample: 500 μl; Peaks; 1: HLC, 2: NaCl, 3: TritonX-100, 4: TritonX-100.

CONCLUSIONS

In this study, a simple and low-cost method was developed for endotoxin removal from HLC solutions. By using this method, the ERE and HLC recovery was enhanced significantly. The TritonX-100 concentration of 0.4% was found to be optimal for the dissociation of the endotoxin-HLC complexes in 1.2 mg · ml^{-1} HLC solution; Tris-HCl buffer (pH 7.5) with 50 mM NaCl was used to obtain an ERE higher than 98% and HLC recovery of up to 95%; the HLC purity was also enhanced after this process. The residual TritonX-100 could be removed effectively with gel filtration chromatography. This low-cost method successfully dissociated the endotoxin-HLC complexes and removed the endotoxins from the HLC solution; meanwhile, the problem of the residual TritonX-100 was also solved. Further research should focus on the scale-up of the chromatographic conditions and on the study of the hydrophobic interaction between HLC and the endotoxin.

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