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Study on Endotoxin Removal from Human-like Collagen

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In this study endotoxin is removed from protein preparations using a Pierce® High-Capacity Endotoxin Removal Resin column. The effects of phosphate buffer and NaCl concentration on endotoxin removal and protein recovery have been investigated. Both PBS concentration and NaCl had significant effects on endotoxin removal from protein solutions. When PBS and NaCl were increased from 10 to 40mM and from 100 to 200mM respectively, the residual endotoxin was reduced from 8000 to 25 EU/ml in the PBS 25mM and NaCl 150mM. When protein concentration was varied from 1 to 5mg/ml, the residual endotoxin was reduced to about 20EU/ml in the PBS 25mM and NaCl 150mM. However, the incubation time had a critical effect on the endotoxin removal. Protein recovery was above 98% in all cases. This method greatly provided high endotoxin removal and HLC recovery.

Key words: Endotoxin removal, Human-like Collagen, Endotoxin-protein Complex, Lipopolysaccharides.

With the rapid development of biological engineering and life medicine, many important products have been developed. Multiple biological materials can be gained through the biological engineering. But expression engineering bacteria used often is *Escherichia coli* whose products are vulnerable to bacterial endotoxin pollution. Therefore, the effective means is needed urgently that it is high-performance to remove endotoxin. This basis is that it not only does not destroy the original effective ingredients, but can completely remove the endotoxin. And it is able to be producted largely using the development of the downstream products. At present, it is one of the general times for this happening in the biological technology products.

For the protein from the purification *Escherichia coli*, endotoxin pollution mainly comes from LPS. LPS, an integral component of the outer membrane of Gram negative[1] bacteria, is a potent stimulant of the immune system (Erridge *et al.*, 2002). If protein samples of the endotoxin pollution transfer to cells or inject to the animal body, it may cause a syndrome characterised by the massive release of proinflammatory cytokines, activation of clotting and complement cascades, and activation of leukocytes. For these adverse reactions, it is essential to remove endotoxins from all biological materials and products. Some commonly used methods for removing endotoxin contaminants have ultrafiltration (Li *et al.*, 1998, 1999), some special kits for small samples and ion exchange chromatography (Shibatani *et al.*, 1983), and so on. However, macromolecules such as protein, collagen, many ways have been tried for

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endotoxin removal. It has been reported that Triton X-114 phase separation, the use of polymyxin B(Aida *et al.*,1990; Matsumae *et al.*,1990; Morrison *et al.*,1976), anion-exchange chromatography, and affinity adsorption (Reichelt *et al.*, 2006)have been more successful in removing endotoxins from protein solutions. But protein and endotoxins have strong interaction common ways do not solve the binds of protein-endotoxin interactions to remove endotoxins of protein solutions. Though affinity chromatography has been proven to be one of the most effective methods(Anspach, 2001; Wei *et al.*, 2007), which does not deal with high products and leads to loss of the product being purified. In addition, the adsorption capacity of adsorbents is generally low(Lee *et al.*, 2003). A anion-exchange chromatography has also been widely used, mainly is easy to scale up, but HLC and endotoxin have the special interaction. So the nonionic surfactant TritonX-100 is chosen, which can dissociate from the interaction of HLC and endotoxin complexes(Rong *et al.*, 2010). But tritonX - 100 is a kind of low toxic substance, can not be easy to be cleared completely from HLC solutions. Thus HLC products will be produced into biological material, which is not safe.

In this study, Though buffer concentration, NaCl concentration and incubation time HLC-endotoxin are investigated. We improve the HLC concentration for large scale recombinant proteins and reduce the endotoxin to a level that experimental animals can tolerate.

MATERIALS AND METHODS

Materials

Sodium phosphate monobasic dihydrate, disodium hydrogen phosphate dodecahydrate and sodium chloride were purchased from DAMAO Reagent. All other chemicals were of analytical grade. The required HLC was expressed by E. coli with a cloned partial cDNA reversed from the mRNA coding for human collagen(Santos *et al.*, 1991) (Chinese patent number: ZL01106757.8). Glassware was heated in the oven at 210 °C for 3 h. All solutions were prepared with endotoxin-free water and degassed to prevent air bubbles from clogging the column and reducing the flow. Endotoxin-free 15 and 50ml conical collection tubes (Made in Mexico) were purchased from Corning.

Pretreatment of Pierce High-capacity Endotoxin Removal Column

The column was packed with 1ml Pierce High-capacity Endotoxin Removal Resin (Thermo Scientific). Before the first use and after each subsequent use, the column was washed with five resin-bed volumes of 0.2N NaOH overnight at room temperature or five resin-bed volumes of 0.2N NaOH in 95% ethanol for 1-2 hours at room temperature. The column was centrifuged at 650×g for 1 minute to remove 0.2N NaOH or0.2N NaOH in 95% ethanol and discard 0.2N NaOH or 0.2N NaOH in 95% ethanol. Then, it was washed three times with five resin-bed volumes of 2M NaCl and five resin-bed volumes of endotoxin-free, ultrapure water. The solution was centrifuged at 650×g for 1 minute to remove it. And it would be waited for the following use.

Preparation of buffers

Endotoxin binding to the resin occured at pH 6-8. The resin was equilibrated with an endotoxin-free buffer at neutral pH that includes 10-50mM sodium phosphate buffer containing 0.1-0.2M NaCl. The buffer pH was checked and adjusted to pH 6-8 with 0.1M NaOH or 0.1M HCl. The level of endotoxins in the buffer prepared was equal or below 0.5 EU/ml. The buffers prepared were filtered through 0.45 mm filters. Then, endotoxin-free buffer equilibrated with five resin-bed volumes.

Preparation of HLC endotoxin stock solution

HLC lyophilized was dissolved with PBS buffer to form different concentration. All the steps were finished in laminar flow cabinet as far as possible.

Incubation time

Different concentration HLC was added 8mL respectively and incubated at 4-22°C with end-over-end mixing for different times. The flow-through in the different times was collected. After the HLC solution was collected, adding endotoxin-free buffer eluted the collagen, one or two resin-bed volume elutions were sufficient.

Endotoxin assay

To determine endotoxins level a chromogenic TAL test kit PYROTELL® (Chinese Horseshoe Crab Reagent Manufactory CO., Ltd. XIAMEN CHINA) was used. The lyophilized lysate (PYROTELL®, Chinese Horseshoe Crab Reagent Manufactory CO., Ltd. XIAMEN CHINA) with

the sensitivity of 0.25 EU/ml was reconstituted using 0.1 ml of endotoxin free water(Chinese Horseshoe Crab Reagent Manufactory CO., Ltd. XIAMEN CHINA). A 0.1 ml sample was added into 1ml LAL glass tubes containing 0.1 ml of reconstituted LAL reagent and the mixture was incubated in water bath of 37 8C for 60 min. A positive reaction was indicated by the formation of a solid gel that did not collapse upon inversion of the tube. A negative result was characterized by the absence of such a gel. The endpoint of the assay is defined as the lowest concentration of endotoxin to yield a positive result(Lee *et al.*, 2003).

Protein assay

Absorption scanning from 325 to 1000 nm in this laboratory has indicated that the protein has an absorbance at around 540 nm. Therefore HLC concentration was measured with a 721E UV-Vis spectrophotometer (Shang Hai Spectrum Instrument Limited Company). Protein Recovery, R, of the adsorption process was determined by the following equation:

$$R=m^{frac}/m^{feed}$$

where m^{feed} is the amount of protein (mg) in the feed joined on the spin columns) and m^{frac} is the amount of protein (mg) in all fractions.

RESULTS AND DISCUSSION

Effect of PBS concentration and NaCl concentration on endotoxin removal of the HLC

In PBS buffer (pH 6.5), the concentration of endotoxins in the HLC collected of the phosphate buffer at 150 mM NaCl was 25 EU/ml and 200 EU/ml at 25 mM and 40 mM of phosphate, respectively (Fig. 1). The removal of endotoxins was successful at 25 mM phosphate containing 150mM NaCl. The results indicate that the concentration of phosphate had virtual effects on endotoxin removal(Lee *et al.*, 2003).

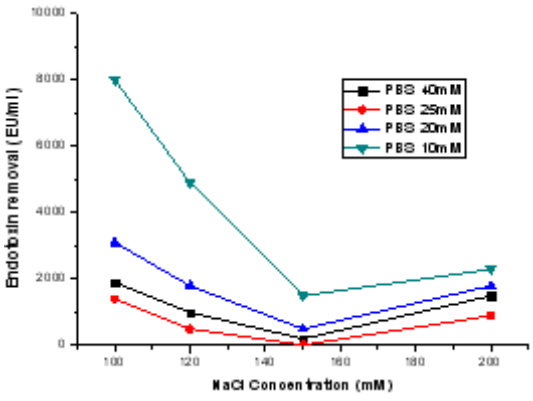

Fig. 1. Removal of endotoxin with PBS buffer. Each point represents the concentration of residual amount of endotoxin in HLC solution treated at different concentration of NaCl

From Fig. 1.seen, the concentration of residual amount of endotoxin in HLC solution treated had a general decreasing trend when the concentration of PBS was increased. But the optimal NaCl concentration, important, on the one hand, which could promote HLC - endotoxin complex untied, on the other hand, which could facilitate endotoxin for combining with the resin. However, NaCl concentration was too high, which could make HLC - endotoxin complex dissociated, but cannot make endotoxin combine to resin. Because high NaCl concentration had strong ionic strength to prevent the endotoxin combined to the resin. So Fig.1 also show that when the NaCl concentration and PBS concentration respectively was 15mM and 25mM, the residual endotoxin of HLC solution treated dropped drastically.

Effect of HLC concentration on on endotoxin removal of the HLC

In this part of the study (Section 3.1 and 3.2), HLC concentration prepared with buffer at various concentration was applied to the resin, as described in Section 2.4. Fig.1 shows how the change of PBS and NaCl effected the endotoxin removal. So the optimal concentration of PBS and NaCl was selected. And in such situation, HLC concentration should be considered. Therefore, Fig. 2 shows that the optimal HLC concentration was 4mg/ml. The residual endotoxin was quite low when HLC concentration was 1mg/ml, but the amount of HLC was low. With the increase of the amount of HLC, the residual endotoxin had a lower value when HLC was 4mg/ml. Because HLC concentration was above 4mg/ml, the resin could not adsorb too many endotoxins in HLC solution. From the results in Fig.2, we should conclude that the concentration of HLC has a significant effect on the amount of protein-endotoxin complexes formed(Li and Luo, 1997).

Effect of Incubation time on endotoxin removal of the HLC

Fig. 3 shows the endotoxin level under various incubation time. There was no difference

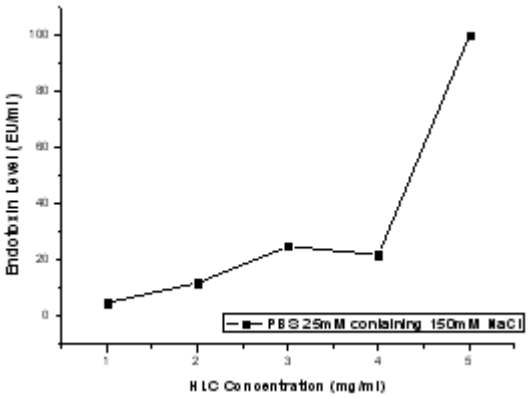

Fig. 2. Effect of HLC concentration on the removal of endotoxin. Each point represents the concentration of residual amount of endotoxin in HLC solution treated at PBS 25mM containing 150mMNaCl

in the endotoxin level from 2 h to 12 h incubation time. In addition, the residual of endotoxin had a lower value, which was about 20 EU/ml. These results demonstrate that the incubation time about 2 h had an important effect on endotoxin removal. The removal of endotoxin had no grand changes when the incubation time was increased to 4 or more. The appropriate incubation time could promote the endotoxin adsorbed to the resin adequately. If the incubation time was not sufficient, the endotoxin was not adsorbed to the resin fully. On the contrary, the long time had played no part in endotoxin removal. That's because resin had been adsorbed the endotoxin

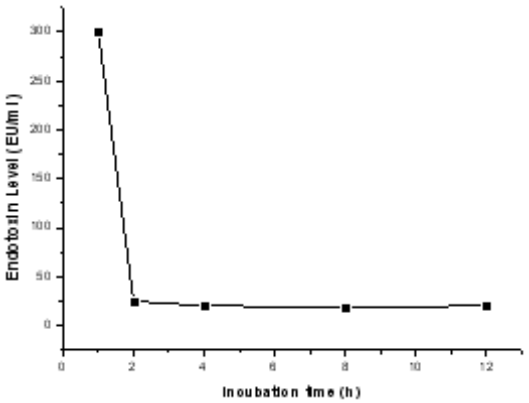

Fig. 3. Effect of incubation time on the removal of endotoxin. Each point represents the concentration of residual amount of endotoxin in HLC solution treated at PBS 25mM containing 150mM NaCl

and achieve saturation. Therefore, the incubation time is the most important parameter in determining the removal of endotoxin and the effects magnify with the concentration of HLC.

The recovery of HLC on the endotoxin removal recovery was a critical question. So this study not only removed the endotoxin, but also guaranteed the recovery.

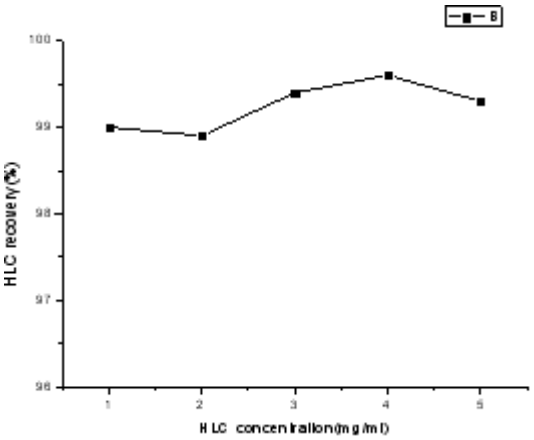

Fig. 4. HLC recovery on the endotoxin removal. Each point represents the HLC recovery in HLC solution treated

CONCLUSIONS

The optimal buffer conditions (buffer concentration, NaCl concentration) provided a 103 reduction in the endotoxin level using a Pierce® High-Capacity Endotoxin Removal Resin. The optimal concentration of PBS and NaCl in buffer was 25mM and 150mM, respectively. The right concentration of HLC in 25 mM PBS buffer containing 150 mM NaCl was 4mg/ml. The incubation time of 2 h was found to be optimal for the dissociation of HLC-endotoxin complexes in 4 mg/ml HLC solution. HLC recovery was up to 98%, meanwhile the endotoxin was efficiently removed to a level that experimental animals can tolerate. Further research should focus on the scale-up conditions for the endotoxin-free HLC producted and on the study of the removal mechanism between HLC and the endotoxin.

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