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Endotoxin Removal from Recombinant Human-like Collagen Preparations by Triton X-114 Two-phase Extraction

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Abstract: Endotoxin removal from recombinant proteins is considered to be an indispensable step in bioprocesses before the application in biomaterials for pharmaceutical industry. This study was carried out using nonionic detergent Triton X-114 to eliminate endotoxin molecules from human-like collagen preparations. With the evaluation of four factors (Triton X-114 concentration, temperature, time and pH) which were mainly affecting the two-phase extraction behavior, the optimal experimental condition was determined. Under this condition, nearly 98~99% endotoxins were removed with high protein recovery (95.1%). The residual Triton X-114 was efficiently separated from HLC preparation via ultrafiltration procedure with microgram amounts of residues left. Through toxicity test *in vitro*, the processed HLC was proved to be non-toxic to BHK21 and acceptable as a novel biomaterial. Therefore, Triton X-114 two-phase extraction can be used as an effective way to remove endotoxins from HLC preparations.

Key words: Endotoxin, human-like collagen, triton X-114 extraction, protein purification

INTRODUCTION

Endotoxin is a basic constituent of outer membrane of gram-negative bacteria which consists of lipopolysaccharides (LPS) and trace amounts of proteins. When the outer membrane is impaired due to the cell dissolution or destruction, endotoxins are liberated from cells and become the frequent contaminations of the target products. It has been revealed that when endotoxins, even at a very low concentration, access to the blood stream of the mammalian host, strong biological effects may occur (Bito, 1977). Because of its high toxicity in vivo and in vitro, the removal of endotoxin is a vital and prerequisite process for products derived from bioprocesses, especially from parenterals (Liu *et al*., 1997). Human-like Collagen (HLC) is a recombinant protein expressed by recombinant *Escherichia coli* (*E. coli*) BL2 expression system through gene engineering (Fan *et al*., 2002). With the special characteristics of biocompatibility, processability, water solubility and little immunogenic reaction compared to animal-derived collagen, HLC is considered to be a good candidate as biomaterial (Yang *et al*., 2009; Zhu *et al*., 2009). Thus, the removal of endotoxins is a significant procedure for HLC derived from microbial bioprocesses before applied to material industry.

Modern biotechnology offers a lot of techniques to remove endotoxins from protein solutions, depending on specific properties and structures of different proteins themselves. The use of detergents in two-phase partition system has been used to remove hydrophobic contaminants from hydrophilic proteins. As a nonionic detergent, Triton X-114 is considered to be low-toxic and readily biodegradable. With the addition of Triton X-114 into protein solution, stronger nonpolar interactions are formed between alkyl chains of lipid A of LPS and the detergent to interfere the interactions of aggregates of LPS and HLC molecules (Petsch and Anspach, 2000). When temperature is raised, the solution separate into two phases. Solubilized protein tends to appear mainly in the upper phase while Triton X-114, together with LPS, concentrates in the lower phase.

Aqueous two-phase micellar system has been reported to be a simple and cost-effective strategy in removing LPS from protein preparations (Lopes *et al*., 2010). In this study, we employed Triton X-114 two-phase extraction procedure to eliminate LPS contaminants from HLC preparations. We aimed to (i) Evaluate the effects of four main factors on phase separation behavior in search for an effective condition, (ii) To eliminate residual Triton X-114 from proteins and determine the concentration of the residue and (iii) To estimate its toxicity through cell culture and viability analysis.

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MATERIALS AND METHODS

Materials: Triton X-114 was purchased from Amresco (OH, USA). Tachypleus Amebocyte Lysate (TAL) and TAL Reagent Water (TRW) were purchased from Chinese Horseshoe Crab Reagent Manufactory CO. Ltd., China. Human-like collagen (HLC, China patent number: ZL01106757.8, Mw = 97,000) was supplied by our laboratory. All the other reagents were of analytical grade. All solution transfers were performed using LPS-free devices. The used glassware was dried in an oven at 250°C for 1~2 h. Non-pyrogenic, sterile plastic ware was used at all times to prevent LPS contamination.

Triton X-114 two-phase extraction: This study aimed to study the effects of different concentrations of Triton X-114 (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0%, v/v), incubation temperature (25, 31, 37, 43, 49 and 55°C), incubation time (1, 2, 3, 4, 5 and 6 h) and pH of the solutions (6.0, 6.5, 7.0, 7.5 and 8.0) might have on two-phase extraction. These four factors were determined one by one in the order above. Triton X-114 at different concentrations was added to the protein solutions which were prepared with specific amount of HLC in PBS buffers (5 mg mL^{-1}) of different pH. After incubated at 4°C for 30 min with constantly stirring, the sample was placed in a thermo-regulated device, previously set at the desired temperature and maintained there for sometime to form two phases. Then the sample was centrifuged (9000 r min^{-1}, 10 min) at 25°C and the upper aqueous phase containing HLC was carefully separated from the sample. The concentration of endotoxin was determined in each sample, as well as protein recovery. All experiments were performed in triplicate.

The endotoxin concentration was quantitative determined approximately by TAL gel-clot assay according to Chinese Pharmacopoeia (2010) and the protein recovery was calculated in every sample after the concentration of HLC was determined by hydroproline colorimetry (Yin *et al*., 1994).

Triton X-114 removal and residue quantitation: The residual Triton X-114 in HLC solutions was eliminated through ultrafiltration. Membrane (Minimate TFF Capsule, Pall, USA) with 10 kDa nominal-molecular-weight cut-off was employed. LPS recontaminations were prevented all the time with careful operation.

Poly ethylene oxide groups of nonionic detergents can react with ammonium cobaltothiocyanate to form blue precipitates which are extracted into methylene dichloride phase. The concentration of Triton X-114 residue was determined through a spectrophotometric procedure (Weber *et al*., 1964). Standard solutions of Triton X-114 were prepared in order to get a standard equation which were performed in a parallel test to control the conditions of analysis.

Cell culture and viability analysis: The cytotoxicity of processed HLC was assessed using baby hamster kidney cells (BHK21) through Methylthiazolydiphenyl-tetrazolium bromide (MTT) assay which was performed as this reagent could produce dark-blue formazan crystals when incubated with viable cells. The cells were cultured at a density of 0.5×10^4 cells mL^{-1} on 96-well plates (200 μL $well^{-1}$) in a CO_2 (5%) incubator at 37°C. After incubation for 24 h, culture media with desired samples were added to 96-well plates (100 μL $well^{-1}$). After incubation for 24, 48 and 72 h in a CO_2 (5%) incubator at 37°C, 20 μL of MTT was added to each well, after which the cultures were incubated at 37°C for an additional 4 h. The cells were washed gently with PBS (pH = 7.5) to remove untransformed MTT and sample residues. DMSO (100 μL) was then added to each well to dissolve the MTT formazan purple crystals. Absorbency of the solution was measured at 490 nm using an Enzyme-linked Immunosorbent Assay (ELISA) Reader (MODEL550, Bio-Rad, USA). The mean value of six parallel samples was analyzed and the whole test was repeated twice.

RESULTS

Triton X-114 two-phase extraction: A series of experiments were performed to study the four factors (different concentrations of Triton X-114, incubation temperature, incubation time and pH of the solutions) in order. When the best condition of one factor was settled, the following experiment designs were based on the determination. The results were presented in Fig. 1. Fig.1a showed that with the increasing of Triton X-114 concentration, the endotoxin level in HLC decreased quickly at the beginning but it remained in the same level when came to 0.6%. The curve of protein recovery nearly changed in the same way. Temperature had a great influence on the behavior of two-phase separation as shown in Fig. 1b. When the temperature was low, the endotoxin concentration was higher and the HLC recovery exceeded 100%. This was probably because Triton X-114 did not completely react with LPS and was separated from the protein solution. The best result was obtained with the temperature of 43°C. And time did not affect the result so much as shown in Fig. 1c. Nevertheless, pH of the protein preparation at 7.5 was performed to have the higher HLC recovery and lower

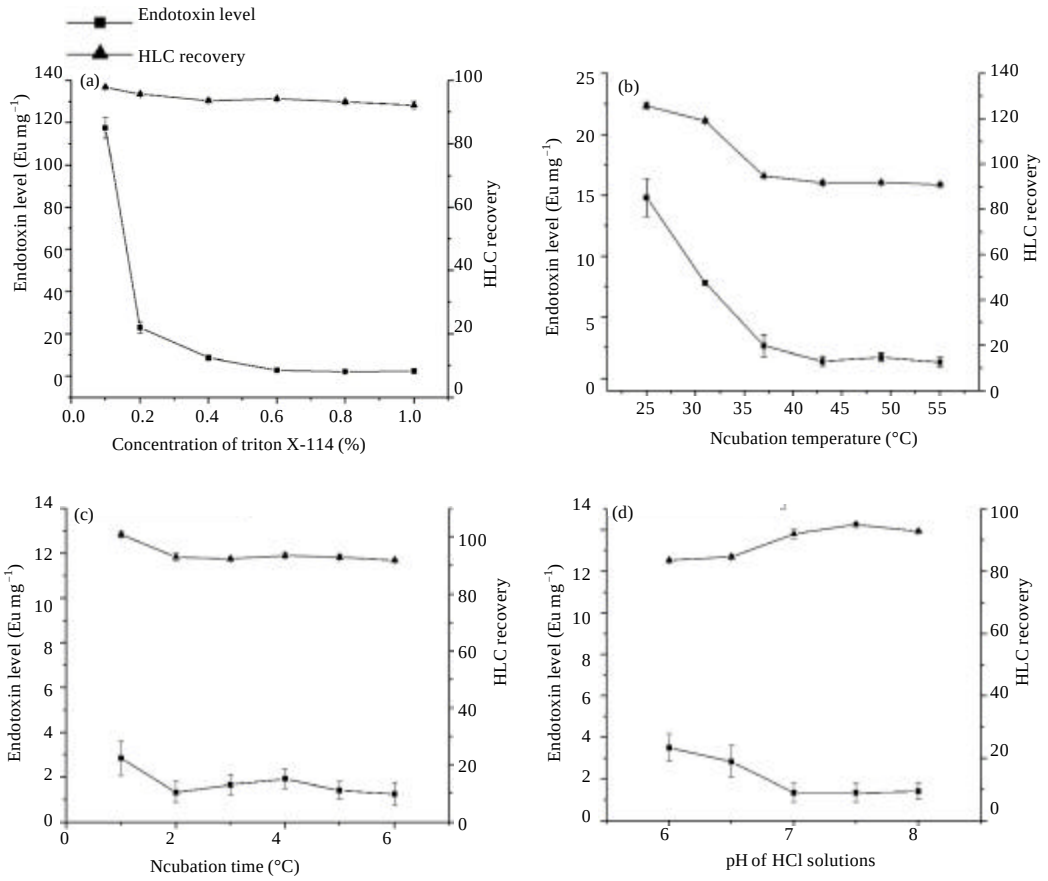

Fig. 1(a-d): Effects of different concentrations of (a) Triton X-114, (b) Temperature, (c) Time and (d) pH of the solutions on two-phase separation behavior, analyzed by endotoxin level and protein recovery

endotoxin content than other conditions (Fig. 1d). In conclusion, the best result was obtained under Triton X-114 concentration of 0.6%, the HLC preparations (pH = 7.5) incubated at 43°C for 2 h. Under this condition, the endotoxin level was reduced to 1.5~2.0 EU mg^{-1} with HLC recovery of 95.1% which was considered to be a great improvement in endotoxin removal.

Triton X-114 removal and residue quantitation: After Trition X-114 extraction procedure, the Triton X-114 residues in HLC preparations were efficiently removed by ultrafiltration and the residual concentration was determined using an ELISA Reader. The determination of absorption of the standard solutions of Triton X-114 (0, 50, 125, 250, 375 and 500 μg mL^{-1}) was performed in a parallel test to obtain a standard equation. The quality of fit of the linear relation was checked by the determination of R^2 which was high in the model. That indicated a good fit between the concentration of Triton X-114 and the absorbency at 620 nm, as presented in Fig. 2.

Then the Triton X-114 residue of HLC sample was determined and the final value was calculated through the standard equation (y = 0.0005x - 0.0023). The result showed that only 9.8 μg residues in 1 mg protein which was considered to be low and safe in pharmaceutical industry.

Cell culture and viability analysis: MTT assay was performed as a routine method to evaluate the toxicity of processed HLC as there were microgram amounts of Triton X-114 residues left. The proliferation of BHK21 was analyzed quantitatively by the optical density value at 490 nm (OD_{490}) which was shown in Fig. 3. Compared with the blanks, the other two groups were found to promote BHK21 proliferation which proved that HLC could stimulate the normal growth of cells.

With cell incubation time increasing from 24 to 72 h, OD_{490} of the two groups (the controls and samples) both increased and cell proliferation on these two groups showed no significant difference which implied the sample

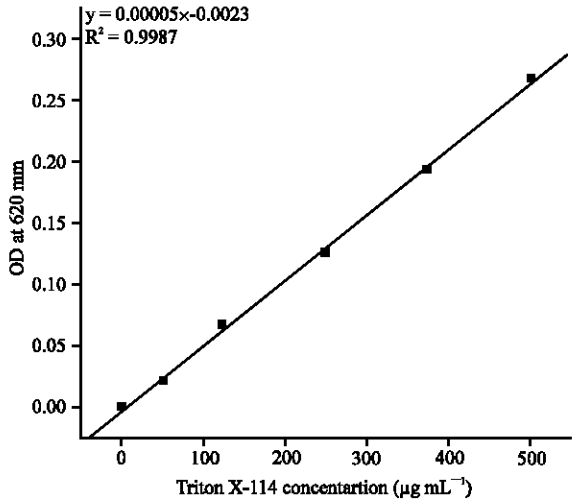

Fig. 2: The linear relation between the concentration of Triton X-114 and OD at 620 nm

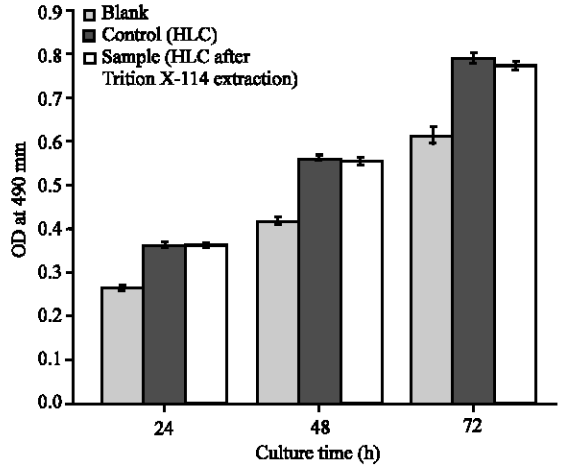

Fig. 3: Proliferation graph of BHK21 cultured for 24, 48 and 72 h, analyzed by MTT assay. Data are presented as the Mean±SD (n = 3)

with trace amount of Triton X-114 residues had little toxicity on viable cells. Therefore, HLC after processed through Triton X-114 two-phase extraction was tested to be a safe biomaterial for further utilization.

DISCUSSION

Endotoxin contamination and removal techniques have been studied for many years in pharmaceutical industry and different methods have been employed to different target products due to their specific properties. In this study, We demonstrated that by performing Triton X-114 phase extraction, endotoxin level in HLC preparations was reduced by 98~99% while protein recovery was still high (over 95%). The results showed that this detergent did not cause vital protein loss when it separated LPS from HLC molecules. Also, through the follow experiments of MTT assay it was proved that the residues in HLC did not have any significant influence on the bioactivity of HLC. These results clearly showed that large amounts of endotoxins were efficiently removed from highly contaminated medium to the detergent-rich phase. This process could be repeated several times to get further purification.

However, even 98~99% of endotoxins were removed; the residual endotoxin concentration was still high. To reduce more LPS molecules to a tolerable limit, this method should be integrated, for instance with an affinity chromatography process. Therefore, the two-phase extraction protocol can be used as a first step in endotoxin removal due to its efficient cost before affinity chromatography. In general, numerous deep researches should be investigated to obtain further achievements.

CONCLUSION

In this study, four factors (Triton X-114 concentration, temperature, time and pH) were evaluated through single-factor experimental design to obtain the optimized condition of Triton X-114 two-phase extraction. Under detergent concentration of 0.6%, the HLC preparations (pH = 7.5) incubated at 43°C for 2 h could get the best result which was below 2.0 EU mg^{-1} in endotoxin level with HLC recovery of 95.1%. The Triton X-114 residue was removed through ultrafiltration, then the residue quantitation was tested which showed only 9.8 μg mg^{-1} and considered acceptable after toxicity test. In conclusion, the use of Triton X-114 was proved to be simple and efficient as a first step to eliminate large amounts of endotoxins from recombinant HLC preparations.

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