Materials Science and Engineering C 42 (2014) 124–129

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Effect of Tris-acetate buffer on endotoxin removal from human-like collagen used biomaterials

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ARTICLE INFO

Article history:
Received 16 October 2013
Received in revised form 7 May 2014
Accepted 13 May 2014
Available online 20 May 2014

Keywords:
Endotoxin removal
Human-like collagen
Tris buffer
Biomaterials

ABSTRACT

Protein preparation, which has active ingredients designated for the use of biomaterials and therapeutical protein, is obtained by genetic engineering, but products of genetic engineering are often contaminated by endotoxins. Because endotoxin is a ubiquitous and potent proinflammatory agent, endotoxin removal or depletion from protein is essential for researching any biomaterials. In this study, we have used Tris-acetate (TA) buffer of neutral pH value to evaluate endotoxins absorbed on the Pierce high-capacity endotoxin removal resin. The effects of TA buffer on pH, ionic strength, incubation time as well as human-like collagen (HLC) concentration on eliminating endotoxins are investigated. In the present experiments, we design an optimal method for TA buffer to remove endotoxin from recombinant collagen and use a chromogenic tachypleus amebocyte lysate (TAL) test kit to measure the endotoxin level of HLC. The present results show that, the endotoxins of HLC is dropped to 8.3 EU/ml at 25 mM TA buffer (pH 7.8) with 150 mM NaCl when setting incubation time at 6 h, and HLC recovery is about 96%. Under this experimental condition, it is proved to exhibit high efficiencies of both endotoxin removal and collagen recovery. The structure of treated HLC was explored by Transmission Electron Microscopy (TEM), demonstrating that the property and structure of HLC treated by TA buffer are maintained. Compared to the most widely used endotoxin removal method, Triton X-114 extraction, using TA buffer can obtain the non-toxic HLC without extra treatment for removing the toxic substances in Triton X-114. In addition, the present study aims at establishing a foundation for further work in laboratory animal science and providing a foundation for medical grade biomaterials.

1. Introduction

Recombinant protein is mainly produced by gene engineering, but the recombinant products are always contaminated by bacterial endotoxins [1,2]. In biotechnology industry, endotoxin has been recognized as a main factor impeding the application of bioproducts in clinical research. Endotoxins, mainly a lipopolysaccharide (LPS) complex, which is an integral outer membrane component derived from the cell walls of the typical gram-negative bacteria [3,4] and is believed to be one of the principal mediators of the cardiovascular, pulmonary, and renal organ dysfunction observed in patients with sepsis [5]. Unfortunately, endotoxin can tolerate high temperatures (250 °C for more than 60 min) or non-neutral pH values compared to other compounds [6], namely, it is too stable to be eliminated easily. Besides, endotoxin has a certain negative effect on organism, and can adhere strongly to many materials and once present is difficult if not impossible to remove completely [7]. The major problem with endotoxin in humans is that it may easily lead to strong biological effects such as fever and shivering, and larger amounts of it even lead to irreversible septic shock or sepsis, and inflammatory cascade and death [8–10]. As a consequence, endotoxin removal from protein is required and makes bioprocessing fully challenging [6–9]. In many cases, gentle protein expressed in *Escherichia coli* expression systems has several endotoxins, which must be reduced to an acceptable level without changing their own properties.

Though many methods have been proposed for removing endotoxin, such as adsorption on anion exchanger or biological affinity and mixed mode materials [11], the performance of these solid phase adsorbents for endotoxin removal depends on pH, salt concentration or ionic interactions, and buffer conditions [12–16]. In recent years, affinity chromatography using modified polymyxin B has been confirmed to have a high affinity for endotoxins [17–19]. However, these methods are not highly reproducible and often accompanied by significant loss of the product being purified [20].

There are numerous methods to remove endotoxin from protein preparation, including ion exchange membrane [21], ion exchange filter [22], various non-ionic surfactants (such as Triton X-100, Triton X-114, NP40, Tween 20, Tween 80, and MOPS [23]) in amounts of 0.1–30% extraction. These non-ionic surfactants can partition endotoxins to the hydrophobic detergent-rich phase preferentially, whereas proteins

Abbreviations: HLC, human-like collagen; TAL, tachypleus amebocyte lysate; TA, Tris-acetate; LPS, lipopolysaccharides; PBS, Phosphate.

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http://dx.doi.org/10.1016/j.msec.2014.05.015
0928-4931/

prefer the aqueous phase [24]. Furthermore, anion exchange combined with Triton X-100 for endotoxin removal has been reported in the literature [25]. However, the binding capacity of anion exchanger is generally limited by the amount of endotoxins from recombinant protein. The removal of residual detergent needs an extra step and adds to cost. Meanwhile, endotoxins' residual in protein still affects the quality of recombinant products [25]. So, in order to use protein within the framework of clinical studies, European and American pharmacopeia demand that proteins should fall below specific boundary values for endotoxin level (e.g. immune serum globulin 0.91 EU/ml, corresponding to 5 EU/kg bodyweight and hour (dosage = EU/kg * h), [26]). The outcome of endotoxin assays has been addressed by many researches [27], achieving low endotoxin levels is particularly challenging for protein to meet regulatory or assay-specific threshold levels such as the US Food and Drug Administration guidelines for medical devices and parenteral drugs [13,28,29].

In this study, we considered a mild TA buffer to remove endotoxin from HLC solution, whose endotoxins could be absorbed on Pierce high-capacity endotoxin removal resin. The removal of endotoxin using TA buffer was based on the fact that Phosphate (PBS), Tris–HCl buffer and NaCl have a positive effect on the dissociation of collagen and endotoxins [12,30], but they could not be reduced to low-level endotoxin and applied to biomaterials and clinical test, the major problem was that relative non-ionic surfactants or $CaCl_2$ were usually added into PBS, Tris–HCl buffer for endotoxin removal [25,31,32]. The use of additives could affect protein recovery and stability [33]. In order to evaluate the character of HLC treated by TA buffer, TEM was used for detection of collagen properties to verify the reliability of TA buffer applied for endotoxin removal from HLC.

2. Materials and method

2.1. Materials

Tris, NaCl, and NaOH were purchased from Sigma. All other chemicals were of analytical grade. The collagen was produced in our laboratory as previously described [34] and was expressed by *E. coli* with a cloned and reversed partial cDNA derived from the mRNA coding for human collagen [35]. Pierce high-capacity endotoxin removal resin was purchased from Thermo Scientific. Endotoxin-free 15 and 50 ml conical collection tubes (made in Mexico) were purchased from Corning. Chromogenic tachypleus amebocyte lysate (TAL) test kit PYROTELL® was purchased from Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China.

2.2. Methods

2.2.1. Treatment with the high-capacity endotoxin removal resin

According to the instruction of the resin of Thermo Scientific, before the first use and after each subsequent use, 1 ml Pierce high-capacity endotoxin removal resin packed with the column was washed with five resin-bed volumes of 0.2 N NaOH in 95% ethanol for 2 h at room temperature. After 2 h, it was centrifuged at 650 ×*g* for 1 min to remove 0.2 N NaOH in 95% ethanol and 0.2 N NaOH in 95% ethanol would be discarded. Then, the column was washed three times with five resin-bed volumes of 2 M NaCl and five resin-bed volumes of endotoxin-free ultrapure water, after each wash, the solution was centrifuged at 650 ×*g* for 1 min to remove the NaCl or water. Finally, it was ready for the following use.

2.2.2. Preparation of TA buffer with different ionic strengths

For the adsorption study 10, 20, 25, or 40 mM of TA buffer with 100, 120,150, or 200 mM of NaCl was prepared with sterile pyrogen-free water. The buffer prepared was filtered through 0.45 μm filters and degassed to prevent air bubbles from clogging the column and reducing the flow. The level of endotoxins in the buffer prepared was equal or below 0.25 EU/ml. The buffers were ready for use. And the resin was washed three times with five resin-bed volumes of TA buffer and then the solution was centrifuged at 650 ×*g* for 1 min to remove it. Then the resin and buffers were ready for use.

2.2.3. pH

In addition, in order to study the effect of pH on the endotoxin removal efficiency and protein recovery, HLC concentration of 3 mg/ml was prepared with buffers at various pHs: 6.0, 7.0, 7.5, 7.8, and 8.0 respectively. All buffers were composed of 25 mM TA buffer containing 150 mM NaCl. Before adding the resin, HLC solutions were filtered through 0.45 μm filters and degassed.

2.2.4. Preparation of HLC solutions

HLC is a macromolecular water-soluble protein with molecular weight of 97 kDa [36]. The collagens lyophilized were dissolved in TA buffer (25 mM TA buffer containing 150 mM NaCl, and using acetate for regulating pH to 7.8) separately to prepare different collagen concentration levels, to which was added 8 ml to 1 ml of resin respectively.

2.2.5. Effect of incubation time

HLC solutions added to the resin were incubated with end-over-end mixing for different periods of time at room temperature. After incubating, the processed protein was collected by centrifugation (1100 ×*g*, 5 min) in the endotoxin-free corning tubes. And endotoxin adsorbed on the resin was desorbed by 2 M NaCl. Following, the high-capacity endotoxin removal resin was regenerated as described in Section 2.2.1.

2.2.6. Endotoxin detection

Lots of methods can be used to detect endotoxin. Using a lysate from horseshoe crab (*Limulus polyphemus*) amebocytes has been considered as a more quantitative in vitro assay in the 1970s [37]. Its principle is based on the clotting reaction of Limulus Amebocyte Lysate Reagent with endotoxin for judging whether endotoxin limit complies with the requirement, this is the standard assay used today and TAL gel clot assay is highly sensitive [37]. Besides, the rabbit pyrogen test was the first assay developed to test solutions destined for injection into humans. This test is based on the ability of endotoxin to cause fever in rabbits and is sensitive to 0.5 EU/ml.

In our study, TAL test kit PYROTELL® was used to determine endotoxin residual of HLC. The endotoxin content of different fractions is determined by TAL assay according to the manufacturer's protocol (Chinese Horseshoe Crab Reagent Manufactory, Xiamen, China). The TAL test was conducted by mixing 100 μl of TAL solution and 100 μl of sample processed in a TAL test tube, which was incubated at 37 °C for 60 min. At least three groups of tests were used for each sample. The test utilized a coagulation (gelation) process, in which the TAL solution reacted with LPS to form a gel during the incubation. Two kinds of results were obtained in our tests, including a positive reaction and a negative reaction. If the endotoxin level of the sample was beyond 0.25 EU/ml (the sensitivity of this test), the gel (positive) was formed and did not flow upside down. On the contrary, if gel-formation flowed upside down or the gel was not formed, the result was negative. All the steps must be operated in laminar flow cabinet.

2.2.7. HLC quantification

HLC concentration was determined with a 721E UV–Vis spectrophotometer (Shang Hai Spectrum Instrument Limited Company). HLC Recovery, R, of the adsorption process was determined by the following equation:

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

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