Factors affecting endotoxin removal from aqueous solutions by ultrafiltration process

Abdel-Nasser Abbass El-Moghazy*

Department of Microbiology and Immunology, Biotechnology Center, EIPICO, Faculty of Pharmacy, Al Azhar University, Nasr city, Cairo, Egypt

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This study presents factors affecting endotoxin removal from bacterial cells homogenates with ultrafiltration process. Endotoxin removal was more efficient at low protein concentrations (3 mg/ml) compared to high protein concentrations (30 mg/ml). Efficiency increased from 28.9% in undiluted sample to 99.8% in diluted samples (1:256). Highest removal efficiency of endotoxin occurred at pH 7-8. In presence of different detergents (0.5% Tween 20, 1% Triton X-100, 1% Triton X-114, and 1% taurodeoxycholate), endotoxin removal was more efficient at diluted samples compared to undiluted samples. Polysulfone membrane represent an efficient method in reducing endotoxin from solutions containing biomolecules.

Keywords: Detergent, Endotoxin, Lipopolysaccharide, Size-exclusion chromatography, Ultrafiltration

Introduction

Bacterial endotoxins are lipopolysaccharides (LPS), which are typically derived from outer cell walls of Gram negative bacteria1. Such LPS are composed of a hydrophilic polysaccharide moiety, which is covalently linked to a hydrophobic lipid moiety (lipid A)2-3, which is most conserved part of endotoxin and is responsible for biological toxicity4. Endotoxins possess potential biological toxicity to human and cause pyrogenic and shock reactions in mammalians5. Exposure to endotoxins affects structure and function of cells and organs, changes metabolism, triggers a coagulation cascade, and modifies haemodynamics6. Serious endotoxin infection can cause septic shock leading to severe hypertension, cardiovascular collapse, tissue injury, multiple organ failure and death7. Gram-negative bacteria are widely used to produce recombinant DNA products (peptides and proteins), which are always contaminated with endotoxins8. Removal of endotoxins from final bioproducts has always been a challenge, especially in situations where endotoxins bind proteins9,10. Many methods11-13 have been developed for removal of LPS form proteins.

This study presents LPS removal from bacterial cell homogenate (BCH) using ultrafiltration and size exclusion chromatography (SEC).

Experimental Section

Materials

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO). E. coli BL21 was used as bacterial strain and kept in a -70°C ultra-low temperature freezer. BL21 cells were inoculated in Erlenmeyer flask containing 100 ml of LB medium and grown overnight at 37°C with shaking (200 rpm).

Fermentation Conditions

Initial culture was performed in shake flask containing 100 ml of LB media. Flasks were incubated in a shaker cabinet (Sartorius AG.) set at 200 rpm and 37°C for 24 h. Cell harvest from flask culture was added aseptically to a 20.0 l Applikon (Applikon biotechnology-The Netherland) fermentor containing sterilized LB media (10 l). pH was set at 7.0 by automatic addition of 10% NH4OH or 10% HCl while temperature was set at 37°C. Dissolved oxygen (DO) was set at 30% air saturation by automatic adjustment of impeller speed, and air sparging flow rate was set at 1.2 vvm (air volume/medium volume/min).
**Cells Disruption & Homogenization and Cell Viability Determination**

Cell pellet was recovered from culture broth by centrifuging culture solution (10 l) at 5000 rpm in SLA 5000 rotor (Sorvall) for 15 min, and then suspended in 10 mM potassium phosphate buffer (PPB) (pH 7.2). Suspension was passed into high-pressure homogenizer (Stansted Fluid Power Ltd-UK) three times at 1000 bar and 90 ml/min and viable cell count was measured. Cell viability was determined by plating serial dilutions of cell suspension in sterilized physiological saline onto LB agar plate before and after passage through cell disruptor and survival cells were estimated. A negative control of medium without cells was used. Viable cells were counted in heart infusion agar after cultivation at 37°C for 48 h.

**Ultrafiltration**

BCH was concentrated using Sartocon ultrafiltration cassettes (Polyethersulfone) with nominal molecular weight cutoffs 30 kDa (Sartorius AG) under increased pressure (1 bar), membrane was fitted into cross-flow filtration system (Sartorius AG), and concentrate was then diafiltered against 30 volume changes of 10 mM PPB (pH 7.2). Diluted BCH solution was mixed with detergent and incubated for 30 min at 4.0°C followed by filtration through a 100 kDa NMWCO ultrafiltration unit.

**LPS Assay**

Glassware used for assay was depyrogenated at 250°C for 2 h and all solutions were prepared using ultrapure deionized non-pyrogenic water. LPS assay was tested by Limulus amebocytes lysate (LAL) assay, performed using Pyrogent Plus (Cambrex Bio Science), at manufacturer’s instruction. Samples (100 µl), LPS standards (0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 EU/ml), or endotoxin-free water (negative control) was mixed with LAL (100 µl) in a microcentrifuge tube. Mixture was incubated at 37°C for 1 h. A positive test is the formation of hard gel that permits complete inversion of tube or vial without disruption of gel. All other results (soft gels, turbidity, increase in viscosity, or clear liquid) are considered negative.

**Estimation of Protein and Bradford Assay**

Protein concentration was estimated with a spectrophotometer (SmartSpec 3000-Biorad-Labs) at 280 nm. Protein concentration in supernatant fractions was determined by Bradford assay using bovine serum albumin (BSA) as standard. Standard BSA was diluted to 0.1 mg/ml and serially twofold dilution was prepared. Colour was developed by adding Biorad reagent. Absorbance was measured at 595 nm using a spectrometer (SmartSpec 3000-Biorad-Labs). Standard curve was generated and sample concentration was calculated.

**Gel Filtration Chromatography**

All chromatography was performed on AKTA explorer 100 systems (Amersham Biosciences) with a gradient pump (P-900), a variable wavelength detector (UV-900), and a pH and conductivity monitor (pH/C-900) and equipped with a Superdex 75 10/300 GL column (GE Healthcare). Column was equilibrated with pyrogen-free PBS buffer, followed by loading samples (flow rate, 0.25 ml/min) after filtration through 0.2 µm syringes filter (Sartorius AG.). Then, 1-5 ml fractions were tested for protein, nucleic acid and LPS.

**Results**

**LPS Removal during Diafiltration**

Removal efficiency (RE) of endotoxin from BCHs by diafiltration is shown (Fig. 1). Separation was carried out at room temperature, with membrane area 0.7 m² and using 10 mM PPB (pH 7.2). LPS molecules of molecular size less than UF membrane cut-off were filtered first, followed by an equilibrium shift from aggregate and micelle form to monomeric form. Repeated diafiltration continuously processed monomeric LPS through filtration.

**Effect of pH, Flow Rate and Detergent**

Highest RE was at pH 7-8 and RE decreased with deviation from neutral pH (Fig. 2). Ultrifiltration was used to remove endotoxin from BCH using polysulfone membrane (area, 0.7 m²). RE of cassette decreased not too much with increase of flow rate (Fig. 3).
Under effect of using detergent in RE of endotoxin (Fig. 4), RE was found very high of diluted samples (99.5%) compared to that of undiluted samples (55%). LPS RE for 1.0% TDC (taurodeoxycholate) was 99.8%, and it was the best compared with other detergents.

Effect of Protein Concentration
Serial dilutions of BCHs were diafiltered against PBS using cross-flow ultrafiltration with 100 kDa membranes. RE of LPS increased by decrease of protein concentration (Fig. 5).

Removal Efficiency of LPS
BCHs were diluted serially with PBS (pH 7.2) up to 256-fold and all samples were diafiltered against PBS (pH 7.2) using cross-flow ultrafiltration with 100 kDa NMWCO membranes. LPS RE ranged from 28.9% to 99.8% (Fig. 6).

Chromatographic Profile of LPS Preparation
Molecular mass distribution and size of LPS in aqueous solutions of cell lysates was analyzed by gel filtration chromatography using Superdex 75 10/300 GL.
Of two peaks obtained (Fig. 7), first peak represented LPS (35%; mol wt, > 300 kDa) was eluted at 5-10 fractions and mostly from higher-molecular weight fractions or large micelles or vesicles. Second peak represented lower molecular-weight micelles or monomeric (10–20 kDa) LPS (65%) and passed through column between fractions 16-20.

Discussion
As LPS is physically larger than other biomolecules, when isolated it forms aggregates and micelles in aqueous solution as one would expect for a major constituent of a biological membrane. This study demonstrated that, in aqueous solutions, endotoxins can self assemble in a variety of structures (monomers, micelles and aggregates). LPS monomer has a molecular mass between 10-30 kDa. However, LPS in aqueous solutions consisting of BCHs eluted mostly in void volume or high molecular weight fraction, indicating that LPS exists not only in monomeric form but also in aggregate form, either as micelles or vesicles. First major LPS peak was eluted at 5 ml while second LPS peak was eluted at 16-20 ml. LPS from first peak most likely was in the form of large micelles or vesicles (mol wt, > 300 kDa). Second LPS peak most likely corresponds to monomeric LPS, or small micelles of LPS. Of total LPS eluted, 35% was in void volume and 65% eluted between 16 and 20 ml.

In aqueous solution (physiological solutions), molecules form spheres or ribbons consisting of bilayers and also endotoxin molecules stick each other or aggregate to form supermolecule structure. These results were similar to reported values. Formation of aggregation may also be due to interaction between endotoxin and some other proteins, to form protein-endotoxin aggregation complexes and also may be due to presence of divalent cations in bacterial cytoplasm, which causes aggregation of LPS and promote formation of large structures like vesicles (>1000 kDa) LPS shows a remarkable capability to interact with other molecules, especially basic protein (pI > 7) by electrostatic interaction. Although interactions with neutral and even acidic proteins (pI < 7) are known at low ionic strength, it is not clear how these interactions take place. Dilute solutions, with or without detergent, allow better RE compared to an undiluted solution. Dilute samples reduce LPS concentration and shift equilibrium toward releasing LPS monomer from aggregates, therefore decreasing chance of forming LPS micelles. In dilute solution, LPS in monomeric form was easily passed through ultrafiltration membrane, while LPS micelles and vesicles or even aggregates were dissociated to monomers by adding detergent. Therefore, molecular filtration and size-exclusion chromatography may be applied for removal of bacterial LPS from solution.

Conclusions
Using ultrafiltration and SEC for endotoxin removal, there was significant effect of protein concentration on efficiency of high-flux polysulphone membranes in removing of pyrogenic substances. Detergents were found very effective for reducing endotoxin levels.

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References


