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Effects of protein concentration and detergent on endotoxin reduction by ultrafiltration

Hyun Jang[1], Hyo-Seung Kim[1], Seung-Cheol Moon[1], Young-Rae Lee[2], Kang-Yeoul Yu[2,†], Byeong-Kil Lee[3], Hyun Zo Youn[3], Young-Ju Jeong[4], Byeong-Soo Kim[5], Sung-Ho Lee[5] & Jong-Suk Kim[2,]*

[1]R&D Center, Komipharm Co., Shihung, Departments of [2]Biochemistry and Institute for Medical Sciences, [3]Surgery, [4]Obstetrics & Gynecology, Chonbuk National University Medical School, Jeonju 561-180, [5]Department of Companion and Laboratory Animal Science, Kongju National University College of Medicine, Yesan 340-702, Korea

Lipopolysaccharide (LPS), found in the outer membrane of Gram negative bacteria, only exerts its toxic effects when in free form. LPS has three major parts, lipid A, the toxic component, along with a core polysaccharide and O-specific polysaccharide. LPS monomers are known to have molecular masses between 10 to 30 kDa. Under physiological conditions, LPS exists in equilibrium between monomer and vesicle forms. LPS removal by 100 kDa ultrafiltration was more efficient (99.6% of LPS removed) with a low concentration of protein (2.0 mg/ml) compared to a high concentration (20.1 mg/ml). In the presence of different detergents (0.5% Tween 20, 1.0% taurodeoxycholate and 1.0% Triton X-100), LPS removal was more efficient at low protein concentrations (2.0 mg/ml) compared to high protein concentrations (20.1 mg/ml). [BMB reports 2009; 42(7): 462-466]

INTRODUCTION

For more than 100 years, it has been known that gram-negative bacteria contain a heat-stable toxin called endotoxin (1, 2). Given their molecular characteristics, bacterial endotoxins are some of the most difficult compounds to understand. Even so, their molecular structure, chemical and physical diversity as well as their broad spectrum of biological activity have been revealed by research in this field. The chemical composition of LPS as well as its structure is well characterized, yet many questions remain to be answered about the role of endotoxin in human health, especially its pathophysiology. Endotoxin is an integral part of the outer cell membrane of gram-negative bacteria as it is responsible for organization and stability (3, 4). Approximately three-quarters of it remains firmly anchored within the bacterial cell wall, but it is also continuously liberated into the surrounding medium.

Chemically, endotoxins are lipopolysaccharides (LPSs) that consist of three biologically, chemically, genetically and serologically different parts. The O-antigen is comprised of a chain of repeating oligosaccharide units with strain specificity against anti-sera while the core oligosaccharide has a conserved structure with an inner KDO-heptose region and outer hexose region. The most conserved part of LPS is lipid A, as it shows very similar structures among different bacterial genera. It consists of a β-1,6-linked disaccharide of glucosamine covalently linked via amide and ester bonds to 3-hydroxy-acyl substituents comprised of 12-16 carbon atoms (2). The molar mass of the LPS monomer is about 10 kDa, although it can be as high as 15-20 kDa depending on the nature of the variable oligosaccharide chain (5). LPS molecules form aggregates with high stability and are therefore capable of existing in variable forms such as aggregates, micelles and even vesicles (4-7).

Gram-negative bacteria are widely used in the biotechnology industry to produce proteins or economically viable molecules. However, bacterial LPS has been recognized as a major cause of pyrogenic reactions encountered during the administration of biotherapeutics and vaccines. Methods for removing LPS from bioproducts although developed are dependent on the product being processed; none of them are applicable as a general method. Therefore, depending on the bacterial strain and nature of the target biomolecules, identifying and characterizing the basic properties of endotoxin are the first steps in developing an LPS removal process. For ultrafiltration removal, important considerations for LPS reduction include the size distribution in aqueous solution when mixed with other diverse biomolecules, interactions between different proteins (8-10), charge and the presence of detergents (9, 11, 12). In this paper, LPS removal from homogenized bacterial cells will be examined using ultrafiltration at different protein concentrations and in the presence of detergents. Analysis of the size distribution of LPS in aqueous solution using size exclusion chromatography will also be presented.

*Corresponding author. Tel: 82-63-270-3085; Fax: 82-63-274-9833; E-mail: jsukim@chonbuk.ac.kr
†Present address, Jeonju BioMaterials Institute, Jang-dong 452-74, Jeonju, Korea

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RESULTS

The molecular mass distribution of LPS in aqueous solutions consisting of homogenized cells

The LPS monomer has a molecular mass known to be between 10 to 30 kDa, its variability owing to the oligosaccharide chain. However, as shown in Fig. 1A, the LPS in aqueous solutions consisting of homogenized bacterial cells eluted mostly in the void volume or high molecular weight fraction. This is direct evidence that LPS exists not only in monomeric form but also in aggregate form, either as micelles or vesicles. The first major LPS peak was eluted at 8 ml while the second LPS peak was eluted at 15 through 20 ml. LPS from the first peak most likely was in the form of large micelles or vesicles, with molecular weights more than 200 kDa. The second LPS peak most likely corresponds to monomeric LPS, or small micelles of LPS. LPS molecules were critically separated into monomeric form when in aqueous solution. In summary, 40% of all LPS eluted in the void volume and more than 50% eluted between 15 and 20 ml.

The effect of protein concentration on LPS removal efficiency

Table 1 shows that the LPS removal efficiency depends on the total protein concentration. *S. flexneri* 2a bacterial cell homogenates were diluted serially with PBS (pH 7.4 up to 16-fold and all samples were diafiltered against PBS (pH 7.4) using cross-flow ultrafiltration with 100 kDa NMWCO membranes. LPS removal efficiency ranged from 37.9% through 99.6%, with more dilute samples demonstrating better efficiency. Samples obtained during diafiltration were tested for LPS concentrations by the LAL test, which found LPS reduction occurred in 1 of 16 diluted samples (Fig. 1B). Most of the LPS had been removed from the parental solution by 10 volume exchanges. LPS molecules of molecular size less than the 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. Final trace amounts of LPS remaining presumably combined with other biomolecules.

Effect of detergent concentration on LPS removal efficiency during ultrafiltration and microfiltration

Table 2 shows the effect of different detergents and detergent concentrations on LPS removal efficiency in serial dilutions of up to 10-fold. Diluted homogenized bacterial solution was mixed with detergent and incubated for 30 min at 4.0°C followed by filtration through a 100 kDa NMWCO ultrafiltration unit. For the solution diluted 1 to 10, the amount of LPS in the retentate was less than 100 EU per μg of total protein with a

Table 1. Effect of total protein concentrations on LPS removal efficiency

Dilute ratio	LPS concentration at start (EU/μg)	LPS concentration at UF retentate (EU/μg)	LPS removal efficiency (%)
Neat*	226.96	140.83	37.9
1 in 4	184.14	60.70	67.0
1 in 8	81.18	9.33	88.5
1 in 16	75.38	0.80	99.6

*Total protein concentrations of no dilute bacterial cell homogenates: 8.8 mg/ml.

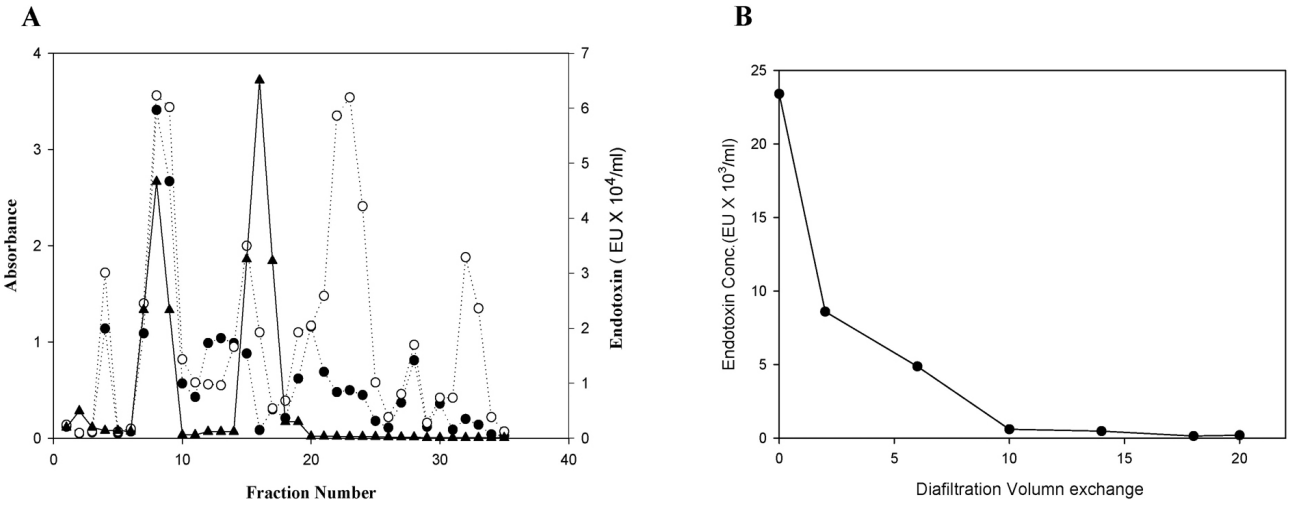

Fig. 1. LPS size distributions in aqueous solution of homogenized bacterial cell. Homogenized bacterial cell solution was fractionized by size exclusion chromatography (A). LPS removal during diafiltration (B). Absorbance at 280 nm (●), absorbance at 254 nm (○), and LPS concentration EU/ml (▲).

Table 2. Comparison of detergents effects on LPS removal efficiency between 1 in 10 dilute and neat of bacterial homogenate solution

Dilution ratio	Detergent	LPS of homogenate (EU/μg)	LPS remaining retentate (EU/μg)	Removal efficiency
1 : 10	0.5% Tween20	400.86	17.85	95.5%
1 : 10	1.0% Triton X-100	426.9	8.2	98.1%
1 : 10	1.0% TDC	385.0	1.75	99.5%
No	0.5% Tween20	410.6	179.6	56.3%
No	1.0% Triton X-100	405.6	104.5	74.3%
No	1.5% Triton X-100	390.5	240.38	38.5%

Table 3. Efficiency of detergent concentration on LPS micro-filtration using 0.2 μm nominal pore size filter

Detergent concentration tween 20%	LPS concentration in permeate (EU/ml)
0.0	5.04×10^4
0.5	3.66×10^5
1.0	4.66×10^5
2.0	7.13×10^5

Homogenate (1 : 10 dilute) Endo-toxin Concentration : 1.69×10^6 EU/ml.

removal efficiency greater than 95%. In undiluted solutions, LPS removal efficiency was less than 74%. We used three types of non-ionic detergents. The LPS removal efficiency for 1.0% TDC (taurodeoxycholate) was 99.5%, the best result compared with 95.5% for 0.5% Tween 20 (polyethylene glycol sorbitan monolaurate) or 98.1% for 1.0% Triton X-100. At a constant detergent concentration of 1.0% Triton X-100, the LPS removal efficiency for the solution diluted 1 to 10 was better than the undiluted condition. It was actually expected that the higher detergent concentration would result in a higher LPS removal efficiency. However, diafiltration using 1.5% Triton X-100 showed the opposite effect. The LPS removal efficiency of 74.3% using 1.0% Triton X-100 was reduced to 38.5% using 1.5% Triton X-100. Table 3 shows the effect of detergent concentration on LPS removal during microfiltration using a 0.2 μm hydrosart filter. It was found that the higher the concentration of Tween 20 (up to 2.0%), the greater the passage of LPS into the permeate and therefore the more efficient removal of LPS. When no detergent was present in the starting material, only 3% of the LPS passed through the membrane. However, adding more Tween 20 increased the passage of LPS into the permeate and, at 2.0% Tween 20, 42% of the LPS was removed.

DISCUSSION

The endotoxin of Gram-negative bacteria resides in the LPS, making up the bulk of the outer cell membrane along with phospholipid and protein. LPS is an amphipathic compound with a large hydrophilic polysaccharide chain and a tail containing a hydrophobic fatty acid. 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. In this study, size-exclusion chromatography of homogenized bacterial cell solutions (Fig. 1A) demonstrated that LPSs exist as large micelles and aggregates. The presence of divalent cations, such as Ca^{2+} and Mg^{2+}, favors formation of LPS aggregates, micelles and vesicles as opposed to monomeric LPS. Size-exclusion chromatography detailed the size relationship of LPS compared to target molecules, providing basic information for the development of purification methods. It was shown that 40% of the total LPS eluted in the void volume. Meanwhile, a high concentration of detergent shifted the equilibrium from aggregates and micelles to monomers. The presence of detergent most likely releases LPS monomers from aggregates, as shown by our ultrafiltration results. Bacterial cytoplasm contains divalent cations, which may cause LPS from the bacterial membrane to aggregate upon their release. LPS micelles and vesicles are typically very stable, however a detergent can release LPS monomers from aggregates (10, 13) thereby increasing the amount of LPS monomer passing through the membrane.

LPS shows a remarkable capability to interact with other substances, especially basic protein (pI $>$ 7) by electrostatic interaction (8). 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. Generally, hydrophobic interactions with proteins are thought to be the likely explanation since Ca^{2+} enhances the interaction between LPS and protein while also stabilizing their binding. Dilute solutions, with or without detergent, allow better removal efficiency compared to an undiluted solution. Two factors can be inferred as explanations (Table 2). Dilute samples by definition reduce the concentration of LPS and shift the equilibrium toward releasing LPS monomer from aggregates, therefore decreasing the chance of forming LPS micelles. In dilute solution, LPS in monomeric form was easily passed through the ultrafiltration membrane while LPS micelles and vesicles or even aggregates were dissociated to monomers by adding detergent. For high concentrations of detergent, more LPS existed in the

filtration permeate. Therefore, molecular filtration may be applicable to the removal of bacterial LPS from solution. It is necessary to assess the state of LPS aggregation in the particular solution to be purified and choose a filter of pore size suitable for achieving sufficient separation of LPS from the purified solute.

MATERIALS AND METHODS

Bacterial strain and culture media

The bacterial strain used was *Shigella flexneri* 2a BS 11 and was kept in a -70°C ultra-low temperature freezer. The initial culture was grown on solid LB agar media and bacterial cell number was expanded in the flask culture prior to seeding the bioreactor. The media composition for the flask and fermentation culture consisted of 10 g/L yeast extract (Oxoid Ltd.), 0.8 g/L citric acid (Sigma co.), 10 g/L glucose (Junsei co.), 0.35 g/L $MgSO_4$-$7H_2O$ (Junsei co.), 1.5 g/L $(NH_2)_2HPO_4$ (USB corporation), 11.0 g/L KH_2PO_4 (USB corporation) and 5.0 ml of trace mineral solution. The trace mineral solution was composed of 27.0 g/L $FeCl_3$-$6H_2O$ (Sigma co.), 2.0 g/L $ZnCl_2$-$4H_2O$, 2.0 g/L $CaCl_2$-$6H_2O$, 2.0 g/L Na_2MoO_4-$2H_2O$, 1.9 g/L $CuSO_4$-$5H_2O$ and 0.5 g/L H_3BO_3. Trace mineral dissolved in 5.0 M of HCl solution and was sterile filtered through a 0.2 μm pore size syringe filter whose membrane was made by polysulfone, acid resistant material. Media and trace mineral solution was sterile filtered through a 0.2 μm filter (Sartorius AG. Sartopore300) placed in situ on the bioreactor during sterilization.

Fermentation conditions

Initial culture was performed in a baffled shake flask containing 150 ml of media. Flasks were incubated in a shaker cabinet (Sartorius AG. CERTOMAT BS-T) set at 150 rpm and 32°C for 12 hours. The cell harvest from the flask culture was added aseptically to a 2.0 L Biostat B-DCU (Sartorius AG.) bioreactor containing 1.5 L of sterilized media. The pH was controlled at 7.8 by automatic addition of 10% NH_4OH or 10% HCl while the temperature was controlled at 37°C. Dissolved oxygen (DO) was set at 35% air saturation by automatic adjustment of the impeller speed, and air sparging flow rate was set at 1.5 L/min (1.0 VVM).

Bacterial cell harvest and homogenization

The bacterial cells were separated from the culture broth using a 0.2 μm hydrosart cassette (0.1 m^2, Sartorius AG), fitted into a cross-flow filtration apparatus (Sartorius AG. Sartoflow Alpha). The cells were concentrated from 5.0 L to 1.0 L. The concentrated bacterial cells were homogenized using a high-pressure homogenizer (Avestin C5) while cell breakage was determined by measuring viable cell count. More than 99.5% of the cells were broken as determined by loss of viable count.

Ultrafiltration for LPS reduction

1,000 ml of bacterial cell homogenate was concentrated to 500 ml using a 30 kDa nominal molecular weight cut-off (NMWCO), hydrosart ultrafiltration cassette (0.1 m^2, Sartorius AG.,) fitted into the cross-flow filtration system. Using constant volume diafiltration (CVD) (i.e. maintaining permeate flow equal to the rate of buffer addition to the retentate) the concentrate was then diafiltered against 20 volume changes of 10 mM potassium phosphate buffer pH 7.6.

LPS assay

LPS content was measured by *Limulus* amebocyte lysate (LAL) test using a kinetic turbidimetric assay kit (Cambrex Bio Science). Turbidity was measured at 340 nm using a microplate reader (ELX808 ultra microplate reader Bio-TEK instruments Inc.) and the result obtained was compared to that of standard LPS (*E. coli* O55 : B5 LPS Cambrex Bio Science). The LPS concentration was calculated using WinKQCL Version 3.00 software (Cambrex Bio Science). The protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

Size-exclusion chromatography (FPLC, AKTA explorer 100, Amersham bioscience) with a UV detector and a Superdex 200 (exclusion limit 100 kDa for dextrans), pre-packed column 10/300 GL (GE Healthcare) was used for the total protein, nucleic acid and LPS assays. Before column loading, the samples were prepared by filtering 0.2 ml of the bacterial cell homogenate through a 0.2 μm pore size, syringe filter unit (Sartorius AG.). The sample was injected and flowed onto the column by pumping PBS at a flow rate of 0.5 ml/min; 1.5 column volumes were collected. Elution was performed with the same buffer at a flow rate of 32 cm/h and was monitored by UV absorption at 280, 260 and 220 nm. Eluted 1.0 ml fractions were analyzed for protein, nucleic acid and LPS concentrations.

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