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A two-step method for extraction of lipopolysaccharide from *Shigella dysenteriae serotype 1* and *Salmonella typhimurium*: An improved method for enhanced yield and purity

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ABSTRACT

Bacterial lipopolysaccharide (LPS) has been widely used as an antigen and adjuvant in immunological applications. Amongst the methods developed for extraction of LPS, hot phenol extraction (HPE) method is the gold standard. However, the HPE method provides poor yield of LPS (~4.5% by weight), is associated with relatively higher impurities of proteins and nucleic acids, and the acidic hot phenol can cause a degradative effect on LPS. In this work a two-step extraction (TSE) method was developed using a non-capsulated, [*Shigella dysenteriae serotype-1*] (Sd1) and capsulated [*Salmonella typhimurium type B* (StB)] species as model pathogens. The TSE method takes advantage of growth kinetics of bacteria wherein a two-step sequential approach for LPS extraction was employed. In step-1, culture supplemented with $CaCl_2$ during early log phase of growth was induced to release LPS by the effect of EDTA at their late exponential phase of growth. In step-II, cells with left over LPS were subjected to modified HPE method that reduced both the degradative effect of acidic hot phenol and associated impurities. The LPS produced using TSE method enabled not only enhanced yield (*~2.78* and *~2.91 fold* higher for Sd1 and StB respectively) requiring nearly similar duration of extraction, but also was structurally and functionally comparable with LPS produced using HPE method and commercially procured LPS. Overall, the developed TSE method is relatively more efficient (enhanced yield), clean (healthy extraction with reduced impurities), safe (reduced handling of larger pathogenic culture) and cost-effective for LPS extraction with potential for scale up.

1. Introduction

Bacterial lipopolysaccharides (LPS) are abundantly available on the surface of Gram negative bacteria and act as a penetration barrier for large foreign molecules thereby protecting the bacteria from external shock. Structurally, LPS is a tripartite glycolipid molecule composed of Lipid A- the inner most region (buried in the outer membrane) followed by the intermediate *core polysaccharide* region, and the outer most O-antigen region (Morrison and Ryan, 1987). LPS has diverse role in immunological applications. The LPS molecule or its less toxic monophosphoryl lipid A (MPL®) variant serves as a natural adjuvant by triggering TLR4 mediated immune responses which have been shown to have a profound effect on clonal expansion of T and B lymphocytes (De Smedt et al., 1996; Mata-Haro et al., 2007). Having its role in determining the serotyping of bacteria, the LPS is responsible for causing fever and other immunological complications (Erridge et al., 2002). LPS has also been widely explored as a protective antigen for developing vaccines against a wide array of Gram negative bacteria including *Shigella spp.*, *Salmonella* spp., *Hemophilus influenza*, *Neisseria gonorrhoea* and various *Escherichia coli* species (Alexander and Rietschel 2001; Eisenstein and Angerman 1978; Lindberg et al., 1991; Zhong, 1999). The LPS from *Shigella dysenteriae* and *Salmonella typhimurium* is known to play a crucial role as a virulence factor during pathogenesis, wherein, post infection, majority of the generated protective antibodies have been found to be against its O-antigen (Dmitriev et al., 1976; Liu et al., 2010; Sandlin et al., 1996; Zhong, 1999).

Since the late 1950s, a large number of methods have been developed for extraction of LPS from bacteria (Mirzaei et al., 2011; Shnyra et al., 2000) with the intent to extract intact LPS with its O-antigen (*smooth-LPS*). Among the various methods reported for extraction of LPS, conventional aqueous hot phenol extraction (HPE) method by Westphal et al. (Westphal and Jann, 1965) is probably the most popular method for extraction of smooth-LPS. The extracted LPS has been widely used for applications ranging from adjuvant to protective antigen, thereby, making the HPE method a gold standard for extraction of smooth-LPS from bacteria. However, in spite of having multiple advantages, the HPE method is associated with the limitation of poor yield of LPS (~4.5% by weight). Moreover, the LPS produced using conventional HPE method has been associated with large impurities (protein and

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nucleic acids) (Muck et al., 1999). Also, the use of hot acidic phenol (90% phenol at 65–68 °C having pH ~ 5.0,) for extraction of LPS in conventional HPE method has been reported to have a degradative effect on the structure of LPS (Tsang et al., 1974; Yi and Hackett, 2000). These limitations have prompted modifications to the extraction process that helped overcome the shortcomings of the HPE method. Consequently, there have been multiple reports that involved modifications (Lindsay et al., 2009; Muck et al., 1999; Pupo, 2011; Rezania et al., 2011; Venter and Lues, 2003; Yi and Hackett, 2000) in order to reduce the degradative effect of acidic hot phenol (Tsang et al., 1974; Yi and Hackett, 2000) or to reduce the impurities of proteins and nucleic acids (Micoli et al. 2013; Muck et al., 1999; Rezania et al., 2011) or to improve the yield of LPS (Darveau and Hancock, 1983; Ridley et al., 2000). While there have been modifications that have attempted to overcome the limitations of the conventional HPE method, a method that provides both higher yield and purity of LPS remains a challenge.

Hence the goal of the present study was to develop a method for extraction of LPS from pathogenic bacteria that provides both higher yield and purity. For this *Shigella dysenteriae serotype 1* (Sd1) and *Salmonella typhimurium type B* (StB) were chosen as model pathogens in order to demonstrate the suitability of proposed method for both non-capsulated (Sd1) and capsulated (StB) bacteria. Both Sd1and StB are Gram negative food borne pathogens present worldwide. Sd1 causes severe bacillary dysentery or bloody diarrhea (Shigellosis) which is responsible for >164.7 million cases globally (Kotloff et al., 1999). Similarly, StB is another ubiquitous mucosal pathogen which has global impact for causing Salmonellosis in around 1.3 billion cases annually with almost similar symptoms as Shigellosis (Coburn et al., 2007; Jansen et al., 2011). LPS purified from both *Shigella* and *Salmonella* species has been used as protective antigen.

Since it is known that Gram negative bacteria tend to lose their LPS in culture medium (shedding) as they enter their late exponential phase of growth (Hoekstra et al., 1976), a sequential approach for extraction of LPS was employed. This approach [two step extraction (TSE) method] initially (step-I) took advantage of the growth kinetics of bacteria while minimizing the shedding of LPS which led to a partial improvement in yield. Step-I combined with a modification to the conventional HPE method (step-II) resulted in a significant improvement in yield while keeping the duration of extraction nearly unchanged. Further, the milder conditions used for extraction helped reduce the degradative effect of phenol (compared to conventional HPE method) and resulted in improved quality of LPS while further enhancing the yield of LPS. Lastly, the use of enzymes as a final polishing step led to improvement in purity of extracted LPS. The cumulative effect of step I, step II and enzymatic polishing allowed for significant improvement in yield and purity of the extracted LPS.

2. Materials and methods

Shigella dysenteriae serotype 1 (Sd1, BCH-518) was received from National Institute of Cholera and Enteric Disease (NICED) Kolkata, India and *Salmonella typhimurium type B* (StB, MTCC No. 3224) was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Ampicillin salt, dialysis membrane, and thiobarbituric acid (TBA) were procured from HiMedia, Mumbai, India. Lipopolysaccharide (LPS) from *Shigella flexneri 1A*, *E*-Toxate™ endotoxin detection kit, E-Toxate™ pyrogen free sterile water, and 2-keto-3-deoxyoctanate (KDO) ammonium salt were procured from Sigma Aldrich, Missouri, USA. Reagents including calcium chloride, ethylenediaminetetraacetic acid (EDTA), deuterium oxide, magnesium chloride, tris-buffer, phenol, dextrose anhydrous, sulphuric acid, pancreatic RNase A, pancreatic DNase I, proteinase K and bicinchoninic acid (BCA) assay kit were procured from Merck, Mumbai, India. N-Cetyl–N,N,N,trimethylammonium bromide (CTAB) and sodium arsenite were procured from Loba Chemie, Mumbai, India, malachite green dye from Qualigens, Mumbai, India, and sodium meta-periodate from Sisco laboratory, Mumbai, India. Ultrapure type-1 water at resistivity below 18.2 MΩ · cm (purification system, Millipore, Billerica, Massachusetts, USA) was used for all experiments.

2.1. Pathogenic culture

Both the species (Sd1 and StB) were sub-cultured in nutrient broth using a class II type A2 clean bench bio-safety facility (Biosafety cabinet, Equitron, India) as per the protocol approved by institutional biosafety committee (IBSC). Cultures were maintained at 37 °C in an aerated incubator shaker at 125 rpm and growth was monitored by recording optical density (O.D.) of culture at 600 nm using a spectrophotometer (UV1,Thermo Scientific, Pittsburgh PA, USA).

2.2. Extraction and purification of LPS using HPE method

A flask containing nutrient broth was inoculated with 1% v/v of overnight culture ($O.D._{600nm}$ > 1.0) of respective species (Sd1 or StB) incubated as mentioned above. At the late exponential phase (Sd1 at $O.D_{600nm}$ ~ 1.3 and StB at $O.D._{600nm}$ ~ 1.6), growth was stopped by transferring the culture onto ice followed by harvesting of cells by centrifugation at 5000 rpm for 20 min at 4 °C (Z383K, Hermle, Germany). Pellets were then lyophilized for approximately 36 h (Alfa 1–2 LD, Christ, Germany) to calculate dry bacterial cell mass and subjected further for extraction of LPS using conventional HPE method as reported by Westphal et al. (Westphal and Jann, 1965). In this method, the extraction of LPS from phenol phase was performed three times (with 20 ml, 15 ml and 10 ml water maintained at 65–68 °C respectively). Crude LPS was recovered from the pooled aqueous phase and subjected to further purification as reported in the conventional method. Purified LPS samples were lyophilized for ~36 h to yield a white-fluffy powder (purified LPS) that was used for further characterization.

2.3. Extraction and purification of LPS using TSE method

In the TSE method (Fig. 1), a flask containing nutrient broth was inoculated with overnight culture of Sd1or StB and growth was monitored till it reached early log phase ($O.D._{600nm}$ ~ 0.6). At this stage, the culture was supplemented with $CaCl_2$ (200 μM) and was allowed to grow further till late log phase ($O.D._{600nm}$ ~ 1.5) where growth was stopped by transferring the culture onto ice. Cells were harvested as stated under conventional method and the pellets were dispersed in small volume (15 ml) of 1× PBS (pH ~ 7.4). Cell suspension was lyophilized (~36 h) to calculate dry mass of cells. Dry cells were further re-suspended in 0.1 M tris-HCl buffer (pH ~ 8.0) in an incubator shaker maintained at 37 °C, 100 rpm for 30 min. Cells suspension was supplemented with EDTA (50 mM) and after 5 min of incubation under similar conditions EDTA effect was stopped by adding approximately double concentration of $MgCl_2$ (100 mM). At this stage cells were re-centrifuged to obtain crude-LPS released in supernatant by EDTA with the pellet having bacterial cells with leftover LPS on their surface. Both the supernatant and the pellet were lyophilized independently to calculate the dry mass of both the fractions. Fraction consisting of crude-LPS released by EDTA was designated as *step-I* of extraction. The second fraction containing cells with leftover LPS was used for further LPS extraction that was designated as *step-II* of extraction. Briefly, cells were suspended in tris-HCl buffer and allowed to grow for 15 min at 37 °C. Turbid cell suspension was treated with ampicillin (50 μg/ml) followed by incubation for another 1 h. Following incubation, cell suspension was used for LPS extraction using modified hot phenol extraction method. Briefly, hot phenol saturated with tris-HCl (pH 8.2) and supplemented with 1% isoamyl alcohol [referred to as *PTI* (Phenol tris-isoamyl) mixture] was used for LPS extraction. The pH and temperature of PTI mixture were maintained at