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Original Research

Characterization of Lipopolysaccharides: Gel Filtration, Ion-Exchange, Capillary Electrophoresis and Micro-purification of LPS

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Abstract:

Immune cells identify lipopolysaccharides, which are abundant on the surface of most Gram-negative bacteria, as a chemical linked with pathogens. They are known as endotoxins because of their ability to induce serious infections such as sepsis. Lipopolysaccharide is composed of O-antigen repeats, core oligosaccharide, and lipid A. One of endotoxin's primary bioactivities is lipid A. Purifying and analysing lipopolysaccharides is challenging due to their unique structure and amphipathic characteristic. Lipopolysaccharide extraction, purification, and analysis methods are reviewed in this chapter. Numerous investigations aimed at isolating and purifying LPS were undertaken after its very important causal involvement in diseases caused by gramme negative bacteria was recognised. So it should come as no surprise that numerous protocols and methods have been developed for the separation and purification of LPS from bacteria. Some of them include trichloroacetic acid extraction at 4ºC, aqueous butanol, triton/Mg+2, cold ethanol, and water extraction at 100ºC. Specific procedures for the purification of raw LPS using phenol, chloroform, petroleum-ether, and methanol have also been detailed. There has been recent success in purifying LPS from E. coli using a combination of sized exclusion chromatography and the Westphal method, which is based on the hot phenol extraction procedure. Due to its high yield, this technique is most often used for LPS extraction. One major drawback of certain suggested LPS purification techniques is the possibility of protein and nucleic acid contamination, which would make it impossible to trust the final product in sensitive assays like molecular and immunological tests. While ultracentrifugation can be used to remove contaminants' proteins, it typically results in decreased yields and a significant proportion of nucleic acids contaminating the sedimented LPS.

Keywords: Lipopolysaccharide, LPS, Lipid A, Extraction, Purification, Analysis

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Introduction:

The cell surface of the majority of Gram-negative bacteria contains lipopolysaccharide (LPS). It is composed of O-antigen repeats, core oligosaccharide, and lipid A. The specific structure of LPS varies among bacteria, with the lipid A component showing greater conservation than the core oligosaccharide and O-antigen repeats. Smooth type LPS (S-LPS) has O-antigen repetitions of varying sizes; rough type LPS (R-LPS) comprises core oligosaccharides of varying sizes but no O-antigens; and free lipid A make up the other two types of LPS. The structural diversity of LPS has prompted the development of specialised methods for its extraction, purification, and analysis. There isn't a one-size-fits-all solution for LPS extraction since the structures and amphipathic characteristics of LPS molecules in various bacteria vary. There are a number of approaches, and each one works better with a different set of LPS. Take the phenol-water extraction method as an example; it works well for S-LPS but not R-LPS [1, 2]. On the other hand, the ether extraction approach works better for R-LPS. There are now options for both large-scale and micro-scale extractions. Agents utilised by various. Several techniques for LPS extraction have been developed since its discovery. The former is better at extracting S-LPS, whereas the latter is better at extracting R-LPS. We have also devised a technique that efficiently extracts both S-LPS and R-LPS.

Phenol–Water Extraction:

To remove LPS, you can use a 45:50 (volume/volume) mixture of phenol and water. Above 65 ◦ C, this combination remains in a single phase. However, below 65 ◦ C, it splits into two distinct phases. At temperatures above 65 ◦ C, this combination can remove LPS and proteins from bacteria. Separation of phases happens when substances are cooled. The protein-containing phenol phase is separated from the polysaccharide- and nucleic acid-containing aqueous phase by LPS. For phenol-water extraction of LPS, the most common approach is as follows.

1. In a 25 mL solution of 50 mM sodium phosphate (pH 7.0) with 5 mM EDTA, dissolve 5 g of dry bacteria. Overnight at 4 ◦ C, stir in 100 mg of egg lysozyme to the suspension.

2. While the mixture is at 37 ◦ C, stir it for 20 minutes. Fill up to 100 mL with 50 mM sodium phosphate (pH 7.0) that contains 20 mM MgCl 2. To achieve final concentrations of 1 μg/mL, incorporate RNase A and DNase I. After one hour of incubation at 37 ◦ C, heat the mixture to 60 ◦ C for one more hour [3-6].

3. Bring the mixture to a temperature of 70 ◦ C in a water bath. Thoroughly combine with an equivalent amount of 90% (w/v) phenol that has been heated to 70 ◦ C. By whisking the mixture in an ice water bath, the resultant slurry is cooled down quickly.

4. Spin the mixture in a centrifuge set at 18,000 ×g for 15 minutes at 4 ◦ C. Separation of phases happens. Gather the liquid phase that includes the LPS.

5. Rinse the liquid phase with distilled water until the phenol smell is gone. Spin the dialyzate for 5 minutes at 1,100×g. After removing the pellets, spin the mixture at 105,000×g for 2 hours to separate the supernatant fractions. The polysaccharides in the gel-like pellets are called LPS. The LPS pellets should be resuspended in distilled water and then frozen.

Because proteins, especially lipoproteins, contaminate the extracted LPS, the phenol-water extraction methodology now incorporates additional purification steps, such as hydrolysis by proteinase.

Proteinase degradation is performed before phenol extraction according to the following protocol:

1) In a water bath set at 65 ◦ C, dissolve 500 mg of dry bacterial cells in 15 mL of a 10 mM Tris-HCl buffer (pH 8.0), which also contains 2% SDS, 4% 2-mercaptoethanol, and 2 mM MgCl2. To the cell mixture, add 1 millilitre of proteinase K solution (100 μg/mL), and then, for 1 hour, maintain the

sample at 65 <C. The next step is to soak the sample in water at 37°C for the night.

2 mL of 3 M sodium acetate should be added to the cell suspension and mixed well. Before allowing a precipitate to develop at -20 ◦ C, mix 40 mL of cold 100% ethanol with the cell suspension.

3. For 15 minutes, spin the mixture at 4,000×g in a centrifuge, and then remove the liquid on top.

4. Swirl the precipitate around in 9 millilitres of distilled water. Before vortexing, add 1 millilitre of 3 M sodium acetate. Return to vortexing after adding 20 mL of cooled absolute ethanol. In a temperature of -20 ◦ C, the suspension is let to precipitate.

5. Mix 9 mL of 10 mM Tris-HCl (pH 7.4) with the LPS that has precipitated, and then add 0.5 mL of DNase I (100 μg/mL) and 0.5 mL of RNase (25 μg/mL). 10. Wait 4 hours at 37 degrees Celsius.

After 30 minutes in a water bath heated to 65 ◦ C, add an equal volume of 90% phenol that has been preheated to 65 ◦ C, and continue incubating at 65 ◦ C for another 15 minutes. Cool the mixture to 4 ◦ C by placing it in an ice bath.

7. For 15 minutes, spin the cooled mixture in a centrifuge set at 6,000×g. After removing the watery layer, repeat the process of extracting the phenolic layer using the same amount of distilled water. Dialyze the combined liquids using several changes of distilled water over the course of two days. Lyophilization of the LPS is possible following dialysis.

Elimination of Ether:

Because R-LPS sometimes splits into the phenol phase, the phenol-water extraction method has a number of significant drawbacks. Consequently, R-LPS extraction using ether was created. The three components of the monophasic solution used in this procedure are petroleum ether (PCP), liquid phenol, and chloroform [7-10]. The extracts can be used without R-LPS, S-LPS, proteins, nucleic acids, or polysaccharides because they are insoluble in this solution. The standard proportions of phenol, chloroform, and petroleum ether in PCP are 2:5:8 (v/v/v), although 5:5:8 (v/v/v) also yields the desired result. A quick procedure for LPS ether extraction is as follows:

• After harvesting, rinse the cells with distilled water. A low LPS yield can be the consequence of using saline or some other salt solution. After washing, the cells are vacuum-dried until their weight remains consistent.

50 grammes of dry bacteria should be mixed with 200 millilitres of PCP, which is made up of aqueous phenol (90 grammes of dry phenol and 11 millilitres of water), chloroform, and petroleum ether (b.p. 40 ◦ C ∼ 60 ◦ C) in a volume ratio of 2:5:8. Get the mixture to mix well by heating it to a temperature between 5 and 20 degrees Celsius for 2 minutes.

After 15 minutes of centrifugation at 5,000×g, the liquid portion containing the LPS is poured into a circular flask through a filter. Just like before, the pellet is agitated and centrifuged after being re-extracted with the same quantity of PCP. One extract is made by combining the supernatant with the rest [11-14].

Using a rotary evaporator, strip the extracts of chloroform and petroleum ether. Put the leftover extracts in a centrifuge tube and dilute them with water until LPS forms a precipitate.

Retrieve the LPS that has precipitated by spinning the mixture at 3,000×g for a duration of 10 minutes. After three washes with 80% phenol and three washes with ether, the precipitates should be dried in vacuum.

The LPS precipitates should be dissolved in 50 mL of distilled water. The mixture should then be heated to 45 ◦ C and shaken until it becomes viscous. Purge the mixture by spinning it at 100,000 times the force of gravity for four hours. The LPS pellets should be dissolved again in water and then freeze-dried.

EDTA-Enhanced Extraction Method:

A technique that can be used for both S-LPS and R-LPS extractions was devised (Darveau and Hancock, 1983) due to the fact that LPS molecules can vary in size even within the same

organism. This technique involves the use of SDS and EDTA to remove impurities [15, 16], like proteins and peptidoglycan, via precipitation. The method's brief protocol is as follows:

Prepare a solution by dissolving 500 mg of dry bacterial cells in 15 mL of 10 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 100 µg/mL pancreatic DNase I, and 25 µg/mL pancreatic RNase A. Use a French press to break the cells. Bring the final concentrations of DNase I and RNase A to 200 and 50 µg/mL, respectively, by adding more of each. Proceed to incubate the mixture for 2 hours at 37 ᠆ C.

Twenty-five millilitres (mL) of a solution containing 0.1 M EDTA and 2% SDS at a pH of 9.5 is combined with 10 millimolar Tris-HCl (pH 8.0) after digestion. To remove the peptidoglycan, mix the solution and spin it at 50,000×g for 30 minutes at 20 ◦ C.

• Bring the concentration of pronase in the supernatant up to 200 µg/mL. Keep shaking constantly while incubating at 37 ◦ C overnight. Use centrifugation to separate the precipitates.

• Dissolve 0.375 M $MgCl_2$ in 95% ethanol and add two volumes, then mix. After bringing the mixture to room temperature, spin it in a centrifuge at 12,000×g for 15 minutes at 4◦C. Then, dissolve the solids in 25 mL of 10 mM Tris-HCl (pH 8.0) with 2% SDS and 0.1 M EDTA, and sonicate briefly. By adding 4 M HCl, the pH of the solution can be brought down to 7.0. To denature outer membrane proteins that are resistant to SDS [17, 18], incubate the mixture at 85 ◦ C for 30 minutes. Lower the temperature and add 4 M NaOH to bring the pH up to 9.5. Combine 25 µg/mL of pronase with the mixture and leave it to incubate overnight at 37 ◦ C with constant stirring.

Before centrifugation at 12,000×g for 15 minutes at 4 ◦ C, dissolve 2 volumes of 0.375 M $MgCl_2$ in 95% ethanol and use it to precipitate LPS at 0 ◦ C. To eliminate any leftover Mg^{2+} -EDTA crystals, dissolve the pellet in 15 mL of 10 mM Tris-HCl (pH 8.0), sonicate it, and then spin it at 1,000×g for 5 minutes. Gather the LPS pellets by centrifuging the supernatant at 200,000×g for 2 hours at 15 □ C with 25 mM $MgCl_2$.

Miniaturised Lipopolysaccharide Extraction:

Because there are situations where only a little quantity of bacteria is available or when only a tiny amount of LPS is required, various techniques for small-scale LPS synthesis have been devised. While the LPS extracted using these procedures may not have the highest purity, it is sufficient for analytical purposes [19-23]. The most basic protocol is as follows: The cells will be resuspended in 50 µL of lysing solution, which contains 2% SDS, 4% 2-mercaptoethanol, 10% glycerol, and 1 M Tris-HCl (pH 6.8) after being harvested from 1.5 ml of culture. The mixture will then be heated at 100 ◦ C for 10 minutes. After that, 25 µg of proteinase K is mixed with 10 µL of lysing buffer and left to incubate at 60 ◦ C for 1 hour. This LPS preparation is detectable by western blotting or acrylamide gel electrophoresis.

Micro-Extraction of Phenol from Water:

When comparing LPS from numerous clinical isolates, the traditional phenol-water extraction method is impractical due to its length of time required. Hence, the following is a fast micro-extraction method that has been devised. To summarise, 2×10^9 CFU of bacteria are rinsed in phosphate-buffered saline (pH 7.2) with 0.15 mM $CaC1_2$ and 0.5 mM $MgCl_2$ and then resuspended in 300 µL of distilled water. Then, at 68 ◦ C, add an equal volume of hot phenol (90%, stirring constantly). After cooling the mixture, the phenol-water phases can be isolated by centrifugation, and the watery phase can be discarded. The extraction is carried out again after adding 300 µL of distilled water to the phenol phase. A final concentration of 0.5 M sodium acetate is achieved by pooling the aqueous phases. 10 litres of 95% ethanol is coupled with it. Once incubated at -20 ◦ C for one night, the insoluble crude LPS can be retrieved by centrifugation. The precipitation process is repeated after re-dissolving the crude LPS in 100 µL of distilled water [24-31]. You can dissolve the remaining LPS precipitates in 50 µL of distilled water and then store them at -20 ◦ C. In order to study the

spread of invasive H. influenzae type b strains during an outbreak, this approach has been employed to extract LPS from several clinical isolates at the same time.

The Micro-extraction of Phenol-Chloroform:

In under half an hour, ethidium bromide (EtBr) can show the presence of LPS that has been isolated using this method (Kido et al., 1990). Centrifuge a 1.5 mL bacterial cell culture that has been incubated overnight. The bacterial pellet is combined with 200 µL of an alkaline solution that includes 3 g of SDS, 0.6 g of Trizma base, and 6.4 mL of 2 M NaOH in 100 mL of H 2 O. The mixture is then suspended in 100 µL of triethylamine (TAE) buffer. Following 70 minutes of heating at 60 ◦ C, the mixture is combined with phenol-chloroform at a ratio of 1:1 (by volume/volume). After a 10-minute centrifugation run at 16,000×g, remove the liquid at the top and combine it with 200 µL of H_2O and 50 µL of 3 M sodium acetate (pH 5.2). Two litres of ethanol are added to precipitate LPS [32-36]. The 20 µL solution of 50 mM Tris hydrochloride (pH 8.0) and 100 mM sodium acetate dissolves the precipitate, which is then precipitated with 2 volumes of ethanol. The LPS solution has a final concentration of approximately 2 mg/mL and is dissolved in 50 µL of H_2O.

Lipide A Extraction:

Lipid A Extraction from Leaf Stem Cells:

The acido-labile ketosidic link between lipid A and the ketodeoxyoctanoate (Kdo) in LPS is exploited to extract lipid A from LPS. The bond can be broken by applying heat or acid. Because it does not dissolve in water, lipid A is easily extracted by centrifugation. The lipid A moiety from LPS molecules has been released using harsh hydrolysis, such as 0.1 M hydrochloric acid at 100 ◦ C, and softer hydrolysis, using 1% acetic acid. Lipid A may undergo partial dephosphorylation and O-deacylation due to the severe hydrolytic conditions. This is helpful for removing monophosphoryl lipid A, but it may alter the lipid A's biological functions [37-41]. Claiming to be effective in cleaving the lipid A-polysaccharide link, milder hydrolysis conditions such sodium acetate at pH 4.5 have been demonstrated. You can add 1% SDS to the mixture when hydrolysis doesn't work. Using a solvent mixture of chloroform and methanol (2:1, v/v), the lipid A can be isolated from the hydrolytic process mixture. 34 It was X. Wang and colleagues.

Bacterial Lipid A Extraction:

Getting lipid A out of purified LPS is a very tedious and time-consuming process. As a result, a technique for obtaining bacterial lipid A was devised. This extraction process calls for the use of two different solutions. A solution comprising CHCl3/MeOH/H 2 O (1:2:0.8, v/v/v) is one example; glycerophospholipids and free lipid A are soluble in this solution (Bligh and Dyer, 1959). Lipid A is soluble in the hydrophobic phase of a two-phase solution of CHCl3/MeOH/H 2 O (2:2:1.8, v/v/v), while proteins and nucleic acids are soluble in the hydrophilic phase. The yield of lipid A can be increased by adding 1% SDS and subjecting the mixture to sonic irradiation. Lipid A extraction from bacteria often follows this approach.

Starting with an initial A 600 of 0.02, inoculate 400 mL of cultures with cells that have been grown overnight. After being grown to an A 600 of 1.2, the cells are harvested and treated with 100 mL of 50 mM HEPES buffer (pH 7.5) for two washes [42-46]. Next, 24 millilitres of phosphate-buffered saline (pH 7.4) is added to the washed cell pellets and mixed again.

A single-phase Bligh-Dyer mixture containing chloroform, methanol, and water (1:2:0.8, v/v/v) should be formed by adding 30 mL of chloroform and 60 mL of methanol to the cell suspension. The mixture should then be stirred constantly while incubated at room temperature for 60 minutes. Whirl in a centrifuge set to 1,000×g for precisely 20 minutes. Fifty millilitres of a one-phase Bligh-Dyer combination is used for a single washing of the recovered debris. Using centrifugation at 1,000×g for 20 minutes, the insoluble pellets are collected.

• If necessary, add 1% SDS to the 27 mL of 12.5 mM sodium acetate (pH 4.5) before heating the pellets containing LPS for 30 minutes at 100 ◦ C. The pH is then adjusted to 4.5 with the addition of glacial acetic acid following a short session of sonic irradiation.

• Once the mixture has cooled, add 30 mL each of chloroform and methanol to make it a two-phase Bligh-Dyer system.

• Stir until well combined, about 30 minutes. Whirl in a centrifuge set to 1,000×g for precisely 20 minutes. Gather the bottom layer that includes the free lipid A. By adding 30 mL of pre-equilibrated lower phase, the remaining upper phases are extracted a second time. The first lower phase is mixed with the lower phase that is collected by centrifugation. The combined lower phases are filtered to eliminate insoluble cell debris by passing them through a funnel that is clogged with glass wool.

• Use rotary evaporation to dry the combined lower stages and obtain crude lipid A.

Nano-Lipid A Extraction:

Lipid A, a characteristic molecule in Gram-negative bacteria, can be utilised for bacterial detection. Due to the urgency of the requirement for sensitive and quick detection methods, several strategies for micro-extraction of lipid A from entire bacteria have been devised. Two detection procedures, with a detection limit of 10-100 µg, can be executed in a single working day.

(1) A technique for hydrolysis that is promoted by SDS (Zhou et al., 1999): 500 micrograms of cells are mixed with 200 microliters of 1% SDS in 10 millimolar sodium acetate (pH 4.5) and left to incubate at 100°C for one hour [47-51]. Dried is the reaction mixture. Centrifugation at 10,000×g for 5 minutes is used to remove SDS after washing the mixture with 50 µL of distilled water and 250 µL of acidified ethanol, which is made by adding 100 µL of 4 M HCl to 20 mL of 95% ethanol. Afterwards, 400 µL of 95% ethanol is used to wash the samples twice. After centrifugation at 8,000×g for 5 minutes, the lipid A is removed from the pellets using 200 µL of a solvent mixture of chloroform and methanol (1:1, v/v). The nitrogen stream dries the lipid A-containing supernatant.

(2) Micro-extraction supported by isobutyric acid and ammonium hydroxide (El Hamidi et al., 2005; Tirsoaga et al., 2007a): In a 400 µL solution with a 5:3 v/v ratio of isobutyric acid to 1 M ammonium hydroxide, dissolve 100 mg of the cell sample, and then incubate at 100 ◦ C for 2 hours. Centrifuge the mixture at 2,000×g for 15 minutes at 4 ◦ C after cooling it with ice water. The next step is to lyophilize the supernatant after diluting it with water (1:1, v/v). Proceed to centrifuge the sample at 10,000×g for 5 minutes after washing it with 400 µL of methanol. Centrifugation at 8,000×g for 5 minutes follows dissolving and extracting the insoluble lipid A once with 200 µL of a chloroform and methanol (1:1, v/v) solvent. Use a nitrogen stream to dry the liquid that contained lipid A. Not every bacterial colony or cell produces the same lipid A species structure because, as a result of phase change, the expression levels of specific enzymes in bacteria fluctuate. Lipid A can be extracted from a single colony using a micro-extraction approach (Zhou et al., 2009). Lipid A can be examined from cell samples or even a single colony with this technique, which employs microwave-assisted enzymatic digestion and sodium acetate hydrolysis. Instead of using SDS to destroy the cells [52-58], proteinase K is utilised because both the cleanup process and the potential interference from contaminated SDS would significantly impact the mass spectrometry analysis. The whole procedure for preparing lipid A using this approach takes around 2 hours and has a detection limit of 1 µg.

(1) Cellular lipid A isolation. The cells that have been frozen (5 mg) are put into a 1.5 mL Eppendorf tube and mixed with 200 µL of a sodium acetate buffer (50 mM, pH 4.5) that includes proteinase K (60 µg/mL). The enzymatic digestion is conducted for 5 minutes at 58 ◦ C while subjected to microwave irritation at 50 W. Afterward, the mixture is allowed to sit at 100 ◦ C

for one hour. For five minutes, the reaction mixture was spun in a centrifuge at 10,000×g. Prior to centrifugation at 10,000×g for 5 minutes, the pellets are rinsed twice with 400 μL of methanol. Centrifugation at 8,000×g for 5 minutes follows the dissolution and extraction of lipid A with 200 μL of a solvent mixture of chloroform and methanol (1:1, v/v). The nitrogen stream dries the lipid A-containing supernatant.

2. Isolation of lipid A from a single colony. With great care, we remove the bacteria from each colony's growth plate and transfer them to a 1.5-milliliter eppendorf tube that contains 1% phenol in PBS. Swirl and tumble downward. Before suspending the cell pellets, wash them with deionized water. Then, add proteinase K (60 μg/mL) to 100 μL of 50 mM sodium acetate buffer (pH 4.5). The suspension is heated to 100 ◦ C for 1 hour after being irritated with a microwave. After 5 minutes of centrifugation at 10,000×g, the mixture is re-washed with methanol to remove any remaining pellets. A 100 μL solvent mixture of chloroform and methanol (1:1, v/v) is used to extract Lipid A from the pellets. After 5 minutes of centrifugation at 8,000×g, the liquid remaining after removing the lipid A is dried using a nitrogen stream.

Liberated Lipid A Extraction:

According to Reynolds and Raetz (2009) and Wang et al. (2006b), certain mutant or wild-type Gram-negative bacteria are able to produce free lipid A. Along with glycerophospholipids, free lipid A can be extracted using the single Bligh-Dyer technique outlined above. A 400 mL sample of bacterial cells is collected and washed twice with phosphate-buffer saline, to summarise. In 190 mL of a one-phase Bligh-Dyer mixture, the cell pellets are suspended. They are left to incubate at room temperature for 1 hour, stirring every so often. By adding 50 mL of chloroform and 50 mL of water, the supernatant that has been centrifuged at 4,000×g for 20 minutes and contains glycerophospholipids and free lipid A can be transformed into a two-phase Bligh-Dyer system. An increase in the recovery yield of lipids may be achieved by adjusting the pH of the upper phase to 1.5 using concentrated HCl (Nishijima and Raetz, 1979; Raetz et al., 1985). Centrifuge the mixture at 4,000×g for 20 minutes after vortexing it. We collect the lower phase and then re-extract the upper water-methanol phase by adding the pre-equilibrated lower phase. The last step is to mix the lower phases that contain lipid A and then dry them using rotary evaporation. Using DEAE chromatography, isolate lipid A from glycerophospholipids is necessary for this sample.

Lipopolysaccharide Purification:

Phospholipids, nucleic acids, lipoproteins, capsular polysaccharides, and peptidoglycan are among the additional macro-molecules found in bacterial cells that might contaminate LPS when it is recovered directly from the bacteria. Potentially mixed cellular reactions to the infected LPS have been reported [58-61]. Examples of similar host responses to bacterial infection can be induced by peptidoglycan and LPS, respectively, through Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4). According to Hirschfeld et al. (2000), cells that overexpress TLR2 become very reactive to even trace amounts of peptidoglycan in LPS. Hence, it is necessary to purge LPS preparations of any impurities. Various techniques have been devised to separate aggregates of LPS and contaminants and to purify LPS. When it comes to getting rid of the big bad guys, these techniques usually work. The physicochemical interactions between LPS and pollutants determine the efficiency of a specific technique.

Eliminating Impurities:

Since phospholipids are ubiquitous in biological membranes, they pose a significant threat to the purity of the LPS that is initially extracted. Mass spectrometry and thin-layer chromatography (TLC) can determine the extent to which phospholipids have been polluted. A chloroform-methanol mixture (1:2, v/v) could be used to wash the LPS samples in order to eliminate the contaminated phospholipids. Although LPS is insoluble in this solvent, phospholipids are. An further significant impurity in the LPS samples is lipoproteins. Ultraviolet absorption at specific wavelengths can be used to measure the level of

lipoprotein contamination. The LPS samples contain lipoproteins that can be broken down by proteinase K. It is imperative that all peptididoglycans be extracted from the LPS sample since these contaminants can trigger the same host response as LPS. Researchers have employed several dissociation agents, like SDS and mineral acid, to separate and extract peptidoglycan (Hirschfeld et al., 2000). According to Tirsoaga et al. (2007b), the effectiveness of these cells was measured by transfecting HEK293 cells with an NF-κB-dependent luciferase reporter gene and overexpressing TLR2. The acid treatment resulted in the most pure LPS and the lowest TLR2 activation, however all treatments can reduce TLR2 activation relative to the beginning LPS sample.

Tirsoaga et al., 2007b outlines the following steps for acid-promoted LPS purification:

The first step is to create a 20 mg/mL solution of LPS by dissolving 10 mg in 0.5 mL of 1 M hydrochloric acid. To achieve a final volume ratio of 3:2:0.25 (v/v/v) of chloroform, methanol, and 1 M hydrochloric acid, the suspension is first sonicated in an ultrasonic bath for 2 minutes. Subsequently, 6 mL of chloroform and 4 mL of methanol are added.

(2) After being subjected to an ultrasonic bath for 2 minutes, the mixture is subsequently spun at 2,000×g for 10 minutes. The particle and the supernatant are isolated.

(3) Vacuum evaporation is used to remove the supernatant. After drying the pellet under a nitrogen stream, the extraction process is performed twice, each time with water instead of acid. The remaining LPS is mixed with water at a concentration of 5-10 mg/mL and then spun at 300,000×g for 45 minutes at 4 ◦ C in an ultracentrifuge. The pellets can be lyophilized to retrieve the pure LPS. The following is an example of a combination technique that removes multiple contaminants at once: phospholipids, nucleic acids, lipoproteins, and peptidoglycan.

Put the LPS preparation in a 10 mM Tris-HCl solution with a pH of 8.0. Bring the concentration of RNase to 25 μg/mL and that of DNase I to 100 μg/mL. Hold the mixture at 37 degrees Celsius for two hours.

(2) Incorporate Proteinase K until the final concentration reaches 100 μg/mL, and then let the mixture to incubate at 37 ◦ C for two hours.

Thirdly, incorporate 5 millilitres of phenol that is saturated with water. Before centrifuging the mixtures at 3,000×g for 30 minutes at room temperature, make sure to vortex them thoroughly.

(4) In a water bath set at 4◦ C, dilute the liquid fraction with distilled water for 12 hours. Remove any insoluble particles by centrifuging the aqueous solution at 17,000×g for 20 minutes at room temperature after dialysis.

(5) Extract pure LPS by lyophilizing the liquid above the solid.

Separation of LPS by Gel Filtration Chromatography:

Isolating truncated LPS devoid of the lipid A moiety, as well as other heterogeneous LPS recovered from bacterial cells, is a primary goal of gel filtration chromatography. Chester and Meadow (1975) found that the latter method removed ester-linked fatty acids from the lipid A, reducing non-polar interactions and facilitating the separation of LPS components. The presence of detergents such deoxycholate (DOC), SDS, and Triton X-100 in the elution buffer ensures that the LPS molecule is disaggregated. There are two types of elution buffer that can be used with Sephadex G chromatography. A 10 mM Tris buffer with a pH of 8.0, 0.25% DOC, 1 mM EDTA, and 0.02% NaN 3 is one example. One alternative is a 0.12 M Tris-HCl buffer with a pH of 8.1, which includes SDS or Triton X-100, and 1 mM β-mercaptoethanol. Even Sepharose is eluted with the later buffer [62, 63]. Extensive dialyzing of the pooled fractions with the DOC-free elution buffer can remove DOC. Column and dialysis buffers with EDTA and high concentrations of monovalent cations are designed to extract samples free of multivalent cations.

Analysis of LPS by Ion-Exchange Chromatography:

Liposomal polysaccharide (LPS) can be separated using anion-exchange chromatography because of the negative charges in the core oligosaccharide and lipid A. For the removal of less hydrophobic LPS, this technique works well. Hydrolysis of LPS can eliminate some acyl chains of lipid A, which reduces its hydrophobicity. You can mildly hydrolyze LPS at 37 ◦ C for 30 minutes to O-deaylate it, or you can N-deacylate it in 4 M KOH at 120 ◦ C for 16 hours.

Latent Period Salivary Electrophoresis:

A high-resolution separation method, capillary electrophoresis (CE) is frequently employed for the examination of intricate mixtures of low-mass molecules, such as peptides, saccha- rides, and nucleotides. Since LPS contains polar groups such phosphates, phosphoethanolamins, carboxylates, and amino groups, it can be utilised for LPS purification. All attest that the coupling of CE with electrospray mass spectrometry (CE-ES-MS) has allowed for the identification and resolution of glycoform populations and substituted groups in LPS like never before. In order to detect species in heterogeneous native R-LPS, researchers have also utilised the Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR MS) coupled with CE. This method provides accurate mass measurements as well as high resolution.

Lipidomic A Lipid Extraction:

One common method for isolating lipid A is DEAE-cellulose ion-exchange chromatography. This technique relies on the phosphate, pyrophosphate, or phosphoethanolamine groups found on the lipid A molecule. Similarly, lipid A isolation can be accomplished via TLC, which is a method for detecting lipids. Furthermore, molecular polarity discrepancy-based chromatographic methods have been created. As an example, Que et al. (2000) reported that the lipid A components from Rhizobium etli could be separated using a Bio-Sil column. The components were eluted step-by-step with increasingly polar solvent volumes, such as CHCl 3 /MeOH (95:5 v/v), CHCl 3 /MeOH (90:10 v/v), CHCl 3 /MeOH (85:15 v/v), and CHCl 3 /MeOH (2:1 v/v). Rumour has it that a C 18 reverse column was used to isolate Rhizobium trifolii ANU843's crude lipid A from elution mixtures of water, methanol/water (2:1, v/v), methanol/water (4:1, v/v), pure chloroform, and water/methanol (2:1, v/v). Also, monophosphoryl lipid A can be effectively isolated using a number of different separation techniques. The process begins with the conversion of monophosphoryl lipid A to its free acid form using Chelex 100 (Na +) and Dowex 50 (H +) columns. Following this, diazomethane is used to methylate the lipid. It is possible to isolate monophosphoryl lipid A using the HPLC method. Purifying lipid A from 400 mL of bacterial cultures can be accomplished using the following approach.

(1) The solvent CHCl3/MeOH/H 2 O is mixed with 5 mL of DEAE-cellulose column (2.5×13 cm) in acetate form at a ratio of 2:3:1, v/v/v.

(2) Soak the sample of crude lipid A that was extracted in 8 mL of a mixture of CHCl3, MeOH, and H 2 O (2:3:1, v/v/v) before loading it onto the column. Gather the repeat as one fraction.

(3) Collect as a single fraction and wash the column with 25 mL of CHCl3/MeOH/H 2 O (2:3:1, v/v/v). Gradually increasing the NH 4 Ac concentration (30 mM, 60 mM, 120 mM, 240 mM, and 480 mM) in the aqueous part using the four column contents of CHCl 3, MeOH, and NH 4 Ac (2:3:1, v/v/v) elutes the different lipid A components. Fill a tube with 2 millilitres of elute.

The lipid A elution profile is monitored by directly dotting 20 µL of each fraction onto Silica Gel 60 TLC plates. By adding the right proportions of chloroform and water, the fractions that contain selective lipid A components are transformed into a two-phase Bligh-Dyer system. Once mixed, the components are extracted by spinning them at 4,000×g for 20 minutes at ambient temperature. Rotary evaporation is used to dry the pooled lower stages that contain lipid A.

Using preparative TLC to eliminate the minimal phospholipids contamination further purifies the

resultant lipid A. Here is a concise outline of the process.

(1) Apply the dissolved lipid A samples to a Silica Gel 60 analytical TLC plate that was created in a solvent system. Then, add the required volume of chloroform and methanol solution (4:1, v/v).

(2) The white stripes that are evident when the plates are drying at room temperature are actually lipid A. First, use a pencil to mark the bands. Then, using a clean razor blade, scrape them off.

3–Next, dry the debris and dissolve it in 3.8 mL of the Bligh–Dyer mixture of the single phase.

Fourth, add 2 millilitres of chloroform, 1 millilitre of methanol, and 1.9 millilitres of water to the suspension and mix it well to create a two-phase Bligh-Dyer system.

(5) Spin in a centrifuge for 10 minutes after vortexing. After collecting the lower phase that contains lipid A, it is dried using a nitrogen stream. After the sample has been thoroughly cleaned, it can be run through a DEAE-cellulose column to eliminate any remaining silica chips or other potential impurities.

Method for Micro-Purification:

Elution of LPS from polyacylamide gels by zinc-imidazole staining is the basis of the micro-purification process. It is a byproduct of the SDS-polyacrylamide gel micro-purification of proteins and nucleic acids using negatively stained zinc-imidazole. The LPS samples can be easily purified and recovered by passive elution from SDS-polyacylamide gels since the structural and biological characterisation have not been affected by zinc-imidazation detection. The recovery of rough and semi-smooth type LPS is around 70-80% after three hours of elution for gel micro-particles with an average size of 32 μM in water (Hardy et al., 1998). Smooth LPS with a high molecular mass typically has a recovery rate of 5 to 10% due to the strong aggregation in the gel, low water solubility, and diffusion rate from the gel to the water. To improve the yield and repeatability of smooth LPS, you can replace the distilled water with 1% SDS, DOC, or 5% TEA eluted for 3 hours or 2 minutes, respectively. Using 5% TEA as the elution buffer is more convenient than 1% SDS and DOC since it can be easily removed by simple evaporation.

Lipopolysaccharide Analysis:

Strains containing mutations in genes involved for LPS production have mainly contributed to our understanding of LPS's involvement in bacterial pathogenesis. Because of this, we need a screening method that can detect even subtle changes in LPS structure or composition quickly and accurately. After silver staining, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the analytical method most frequently utilised. It has been created to conduct SDS-PAGE on LPS that is 3-H, 14-C, or 33-P-labeled. LPS gels have been stained with EB, a fluorescent dye typically used to stain nucleic acids in gels. Sensitive reverse staining with zinc-imidazole salts was created as a solution to the problems associated with silver staining, such as the LPS's irreversible fixation and chemical alteration.

Living protein separation via electrophoresis:

Most commonly, changes in electrophoretic band profiles using SDS-PAGE are used to evaluate structural heterogeneity or modification of LPS. In place of glycine, tricine has been used for the gel and electrophoresis buffer in order to achieve a higher resolution of LPS. Substituting sodium DOC for SDS in electrophoresis can prevent LPS aggregation (Komuro and Galanos, 1988). The development of a bilayer stacking gel allowed for the detection of subtle variations in the low molecular weight bands of LPS in polyacrylamide gels. Below, you can see the essential components of this SDS-PAGE.

• Making gel: Cover the plates with distilled water containing 0.1% SDS and pour the 14% acylamide preparation gel on top, leaving a 4-centimeter overhang. Remove the water layer and rinse the gel top after 2 hours of polymerization. Next, layer the separating gel with a 3% acylamide solution of stacking gel A, making sure to reach a level slightly higher than the well comb's length.

Finally, cover the layer with 0.1% SDS. When the two hours have passed, wash the surface of stacking gel A with distilled water. Then, apply a layer of stacking gel B's 3% acylamide solution. With the exception of substituting 0.025 M Tris-0.192 M glycine buffer for the 0.125 M Tris-HCl (pH 6.8) in stacking gel A's solution, the two gels' compositions are indistinguishable. Spread the stacking gel A out so that the well comb's bottom touches the gel's top. Let it sit at room temperature for the night.

• Dye buffer: Mix together 0.59 mL of storage buffer (0.06 M Tris-HCl (pH 6.8), 1 mM EDTA, and 2% SDS), 0.4 mL of glycerol, 0.08 mL of 2-mercaptoethanol, and 0.04 mL of saturated bromophenol blue to make 2×solubilization buffer.

1. The upper reservoir should be filled with 0.25 M Tris-1.92 M glycine (pH 8.0), while the lower reservoir should be filled with the remaining buffer.

Prepare 10 µg of LPS samples by dissolving them in 10 µL of distilled water. Then, combine them with 10 µL of freshly made 2×solubilization buffer. Put the sample in a boiling bath and let it sit for 5 minutes. Transfer the samples to the gel's wells.

• Electrophoresis: The stacking gels move samples at 9 mA, whereas the separating gel uses 12 mM. Pulse the electrophoresis, remove the gel from the plate, and stain it when the dye reaches the bottom of the separating gel. Liner gradient gel electrophoresis was used to analyse tiny molecular LPS because big PAGE consumed a lot of time and samples.

Separation of high molecular weight LPS into uniform bands was also achieved using this electrophoresis technique. Following is a visual representation of the essential components of linear gradient gel electrophoresis for LPS.

• Making gel: For the stacking gel, use a 1.25 M Tris-HCl (pH 6.8) gel buffer, and for the linear gradient gel, use a 1.88 M Trsi-HCl (pH 8.88) resolving gel buffer. The ingredients for the linear gradient gel include a peristaltic pump, a small gel casting device, a gradient former (10 mL chamber), and water. Before making the linear gradient gel, degas and cool the monomer solutions. A linear gradient can be created in the casting unit by storing in the gradient former a 30% stock acylamide solution diluted with 50% glycerol, which allows for solutions of higher concentrations of acylamide. The water used to make the solutions is in a different container. The pump and gradient former are connected by a polyethylene tube that is filled with butanol that is saturated with water. Once the polymerization process has begun, transfer the contents of the chambers into the apparatus through a 0.4 mL/min pumping rate.

Mix 0.025 M Tris with 0.192 M glycine and 2% SDS to make the reservoir buffer.

• Preparing the samples: Fill a 60 mM Tris-HCl (pH 6.8) buffer with 2% SDS and 1 mM EDTA; dissolve the LPS samples until they reach a final concentration of 0.1% (w/v). Apply the solutions to the prepared gel after diluting them with 34 mM Tris-HCl (pH 6.8) buffer that contains 39% glycerol and 3.9% saturated BPB in water.

A constant voltage of 200 V is used during the electrophoresis technique, which is carried out at room temperature. The separating gel is stopped from being electrophoresed when the BPB reaches a point three millimetres below its base. Take the gel from the plate and colour it.

Ways to Apply Stain:

Metallic Silver:

Due to the availability of aldehyde groups made possible by the oxidation of the existing hexoses, the reactive residue in the silver stain is the polysaccharide component of the LPS molecule. However, during the first fixing and oxidisation processes, the retention of LPS fractions in the SDS-PAGE may be caused by the quantity of fatty acids in lipid A of LPS. Using the conventional silver stain method to identify specific LPS fractions containing partially deacylated S-LPS is inefficient. Thus, skipping the fixing step and increasing the staining procedure's oxidation time improve a modified

silver stain method. To visualise LPS in polyacrylamide gels using silver staining, less than 1 μg of LPS is enough.

An alternative method for staining silver is as follows:

No fixation is done before oxidising the LPS in the SDS-PAGE gel for 20 minutes at 22 ◦ C in a solution that contains 0.7% periodic acid, 40% ethanol, and 5% acetic acid. To remove the oxidation buffer, wash the gel three times with 5 minutes of distilled water.

- Use a freshly made staining solution, following the steps below, to leave the gel stained for 10 minutes: Combine 56 millilitres of 0.1 M sodium hydroxide with 4 millilitres of concentrated ammonium hydroxide. Mix 10 millilitres of 20% (w/v) silver nitrate with 200 millilitres of water while swirling constantly. The capacity is finally brought down to 300 mL by adding water.

- Use distilled water to wash the gel three times for a total of five minutes.

- Ten milligrammes of citric acid and one millilitre of 37 percent formaldehyde are combined in a 200 millilitre solution to produce the colour. Expose the sample to 10% acetic acid for 1 minute, then wash it several times with distilled water to stop the colour reaction.

Stain using Ethidium Bromide:

When staining LPS with acidic O-specific polysaccharides, which were not well visible with silver staining, the EB-staining approach came in handy. The staining effect of EB is more pronounced on high-molecular-weight LPS, which contains long O-specific polysaccharide chains, than on low-molecular-weight LPS. Here is the stain protocol as described by Kido et al. (1990): Afterwards, take the gel out of the glass plate and submerge it in a 30 μg/mL EtBr solution for 10 seconds. After 30 minutes of gentle shaking in distilled water, the gel will be stain-free. With the help of a transilluminator operating at 302 nm, LPS bands can be seen.

The most significant disadvantage of zinc-imidazole stain silver staining is the irreversible fixation and chemical modification of the LPS, which results in lower sensitivity due to excessive background. The procedure is also time-consuming, hazardous, and expensive. For this reason, a sensitive reverse stain method based on zinc and imidazole salts was created to extract LPS from gel slices, which could then be used in structural and biological investigations. By adding imidazole, a staining backdrop can be created while the zinc-LPS complex remains in the form of translucent, colourless bands. What follows is the staining protocol.

(1) To eliminate SDS and other compounds associated with electrophoresis, incubate the gel in boiling distilled water for 15 minutes three times after electrophoresis.

To proceed, soak the gel for 15 minutes in a solution of 10 mM zinc sulphate.

(3) A soak of three minutes in 0.2 M imidazole with agitation is required for the gel. The LPS-containing zones, however, stay clear and colourless while a homogeneous white backdrop precipitates down the gel's surface. By positioning the gel a few centimetres above a black backdrop, one may observe the negative staining patterns.

(4) Once you've achieved a sufficient image contrast, rinse the gel three times with distilled water for one minute each to halt the reaction.

Immuoblotting Procedure:

Since the O-antigen in LPS can bind certain antibodies, the immunoblotting technique has been used to explore LPS's immuno-chemistry. Standard practice dictates using the antiserum as the primary antibody and then peroxidase-conjugated anti-rabbit IgG as the secondary antibody. Monoclonal antibodies can attach to the particular O-antigen; these antibodies serve as the primary antibody, while a secondary antibody conjugated with alkaline phosphatase allows for visualisation. Following electrophoresis, the gel containing LPS is transferred to a nitrocellulose membrane by soaking it in transfer buffer. The nitrocellulose blots are visualised by using the first antibody followed by the secondary antibody in a sequential manner. The last step is to use

agents that generate colour to detect the attached antibody.

Separating lipid A species is a breeze with the use of thin-layer chromatography (TLC). Applying a chromogenic chemical spray after chromatography allows for the coloration of such lipid A molecules. Solvents such as CHCl3/MeOH/H2O/NH4 OH (40:25:4:2, v/v/v/v) or chloroform/pyridine/88% formic acid/MeOH/H2O (60:35:10:5:2, v/v/v/v/v) are used to develop the lipids after they have been spotted onto a Silica Gel 60 TLC plate. Charring at 145 ◦ C with a spray of 10% sulfuric acid in ethanol can be used to identify the lipids once they have dried. Additionally, autoradiography methods for lipid A measurement were developed, which can significantly improve detection sensitivity. Additionally, ESI MS may examine the isolated lipid A.

Conclusion:

Researchers mostly rely on LPS and lipid A to comprehend the infection process of bacterial pathogens because these molecules are essential for Gram-negative bacteria and can stimulate the immune response. Increasing the productivity and purity of LPS and lipid A extraction and purification procedures, as well as LPS analysis methods, still requires further modification.

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