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Lipopolysaccharide aggregates in native agarose gels detected by reversible negative staining with imidazole and zinc salts

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ABSTRACT

We investigated the use of imidazole and zinc salts for the detection of lipopolysaccharide (LPS) aggregates separated by native agarose gel electrophoresis (NAGE). As a result, a new staining procedure was established by which as little as 1.5 μg of *Escherichia coli* O55:B5 LPS aggregates was detected by means of inducing a clear, transparent pattern, contrasted against an opaque background. *E. coli* O55:B5 LPS preparations treated with nucleases and proteinase K proved that the reverse-stained LPS pattern is not related to any potential artifacts caused by unrelated biomolecules (e.g., nucleic acids, proteins). After this, we showed that the procedure is applicable to two-dimensional LPS separation using NAGE/SDS-PAGE, while at the same time confirming that real polydisperse LPS aggregates are represented by the stained profile. Also, we demonstrated the general applicability of this stain to the detection of different NAGE-separated LPS aggregates (e.g., from *E. coli* 026:B6, *E. coli* 0111:B4, *Salmonella minnesota* Re595). Finally, using lysozyme as a model protein, we found that imidazole–zinc may be combined with Coomassie brilliant blue R-250 into a double-staining process to enable the use of NAGE for investigating the interaction of cationic proteins and LPS aggregates and protein or LPS concentration effects on protein–LPS binding.

Lipopolysaccharides (LPSs)[1] are structurally related highly complex glycolipid molecules from the outer membrane of Gram-negative bacteria. Binding of LPS to Toll-like receptor 4/MD-2 (e.g., during infection) triggers proinflammatory signaling cascades that contribute to septic shock, target organ damage, and death [1].

The LPS molecules, which are amphiphilic, tend to form high-molecular-mass supramolecular aggregates (e.g., filaments, spherical-like particles, ribbon-like structures, micelles). The size and shape of these aggregates depend on LPS concentration and other physico-chemical parameters ([2] and citations therein]). Santos et al. proposed that the critical concentrations for the transitions between monomer and premicelle (preaggregate) oligomer and between premicelle oligomer and large aggregate are defined as CMC (critical micelle concentration) and CMCa (apparent CMC), respectively [3]. Aurell et al. found that even at very low concentrations (e.g., 10 pg/ml), lipopolysaccharides may be highly aggregated into doublet structures without any visible signs of the LPSs breaking up into individual LPS monomers [4]. The aggregation of lipopolysaccharides also depends on the conformation and hydrophobicity of the contributing glycoform-specific LPS. Other factors which influence LPS aggregation are temperature, pH, water content, and concentration of mono- and divalent cations [2]. The ratio of the cross sections of the hydrophilic and hydrophobic moieties determines whether the formed LPS aggregates are micellar, lamellar, or nonlamellar inverted in shape [5]. Given that only the aggregated and not the monomeric forms of a variety of LPS chemotypes are properly detected by LPS-binding proteins and immune cells [5], it has been suggested that at least for the initial steps of cell activation aggregates are the biologically active units of the endotoxin.

Today, LPSs are frequently separated by surfactant (e.g., sodium dodecyl sulfate [SDS], deoxycholate)–polyacrylamide gel electrophoresis (PAGE). Using this powerful analytical technique, multiple LPS samples or multiple components in a single sample are analyzed. A wealth of information can be obtained using slab–PAGE alone or in combination with other characterization techniques (e.g., mass spectrometry, bioassays). This includes purity of LPSs, glycoform-specific composition, molecular weight of components and primary (e.g., oligosaccharide) sequences, minor

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[1] *Abbreviations used*: LPS, lipopolysaccharide(s); NAGE, native agarose gel electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NF-κB, nuclear factor-κB; LZM, lysozyme; CBB, Coomassie brilliant blue R-250.

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structural changes due to mutations in genes responsible for LPS biosynthesis, and cell-based biological activities.

However, the surfactant–PAGE technique is not particularly suitable for separating, analyzing, and micropurifying native LPSs in aqueous dispersions. In theory, surfactant–PAGE can be replaced with native agarose gel electrophoresis (NAGE) for the separation of LPS aggregates. Purification based on NAGE might be particularly useful for complementing conventional purification methods, and allowing the effective removal of proteins, nucleic acids, polysaccharides, lipids, or small ionic compounds, which are common contaminants in LPS preparations. In practice, once the experimenter located the lipopolysaccharide aggregates of interest, it would then be relatively straightforward to directly recover LPS aggregates from agarose gels for further downstream applications, including structural characterization or activity-related studies. However, the successful use of NAGE as a reliable preparative purification method for identification and characterization of LPS aggregates will depend on the availability of appropriate gel detection stains. When developing a method to this end, the most important considerations should be that the staining method is reversible and does not compromise the chemical integrity and the biological properties of the target LPS aggregates. Other features that should combine the ideal detection method are: (i) ease of use (a few steps), (ii) fast to perform, (iii) low cost, (iv) nontoxic or as environmentally friendly as possible, and (v) compatibility with subsequent applications and experiments (e.g., mass spectrometry, bioassays, immunoblotting).

Maccari and Volpi reported the detection, down to the submicrogram level (0.05–0.5 μg), of various agarose-separated *Escherichia coli* LPS serotypes by staining with the cationic dye toluidine blue followed by the Stains-All method [6]. Here we report a different approach, which allows the reversible negative staining of LPS aggregates on agarose gels with imidazole and zinc salts. We have chosen to use these salts based on our extensive experience on them for visualizing glycoform-specific LPS species on polyacrylamide gels as well as such other biomolecules as proteins and nucleic acids [7]. Also, we show that imidazole–zinc may be combined with Coomassie brilliant blue R-250 (CBB) into a double-staining process to enable the use of NAGE for detecting and studying cationic protein (e.g., lysozyme)–LPS interactions. The new imidazole–zinc procedure increases the availability of staining methods while satisfying most of the aforementioned requirements for an ideal stain suitable for the reversible detection of NAGE-separated LPS aggregates and cationic protein–LPS complexes.

Materials and methods

Materials

Smooth-type LPSs from *E. coli* O55:B5, *E. coli* O26:B6, *E. coli* 0111:B4, and *Salmonella minnesota* Re595, as well as chicken egg white lysozyme, were obtained from Sigma-Aldrich (Germany). Diafiltration of LPSs recovered from agarose gels was performed using centrifugal filters (Millipore's Amicon Ultra-15, 10,000 or 30,000 MWCO [molecular weight cutoff] and Centricon Ultracel YM-10). Chemical reagents for electrophoresis, staining, and other purposes were of analytical grade and were purchased from different commercial suppliers (e.g., Merck, Sigma-Aldrich, Bio-Rad).

Native agarose gel electrophoresis

Lyophilized LPS was rehydrated into stock water solutions, at a concentration of 5 mg LPS/ml water per aliquot, and stored at either 4 or −20 °C until use. LPS samples in water, gel loading buffer (e.g., 0.025% bromophenol blue, 30% glycerol in 1X TAE) and distilled water were appropriately mixed, after which aliquots (e.g., 22 μl) were loaded in agarose submarine gels for electrophoresis. Of note, the volume (e.g., 22 μl) of sample loaded was sufficient for reaching the gel surface; this was required to enable better reverse staining of gels which is limited mostly to the gel surface. The gel and buffer electrophoresis systems was Tris–acetate–EDTA (TAE) made of 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0. The agarose gels were run at 80 V for approximately 2 h, until the dye front reached the bottom of the gel.

Detection of LPS aggregates in the agarose gel by staining with imidazole–zinc

Volumes of the solutions used at all stages were sufficient to ensure that gels were totally submerged, thus avoiding uneven staining and the gel surface drying out. Unless stated otherwise, 50 ml was used and the staining procedure was performed at room temperature.

Step 1: Following the run, the gel is washed (2 × 15 s) with distilled water to remove excess electrophoresis reagents, followed by incubation with 0.2 M imidazole for 20 min under gentle mechanical agitation.

Step 2: The imidazole solution is discarded, the gel washed twice with distilled water for a few (e.g., 15) s to remove excess imidazole, and placed over a dark background. A 0.2 M zinc sulfate solution is added while gently agitating (e.g., manually) for a few seconds. The LPS staining pattern is visualized rapidly as transparent and colorless bands over an opaque background.

Step 3: After a few seconds (e.g., 10–30 s) of incubation, the developer (zinc) solution is poured off, to avoid subsequent band overstaining, and the gel is rinsed with abundant distilled water for several times to remove excess zinc and ensure that the staining process stops. During this step, the staining contrast will become optimal.

Once reverse stained, the gel can be scanned, photographed, or stored in distilled water preferably at 4–8 °C until use. Specifically, we scanned all of the gels using a Syngene gel documentation system; the image of the gel placed in the darkroom was captured with the GeneSnap software (Ver. 6.00.21 from Synoptics Ltd). If the staining pattern fades during storage, the gel may then be restained without significant loss of sensitivity. It is worth noting that before restaining, the gel should be incubated in 100 mM EDTA for 15–30 min, to redissolve the zinc imidazolate precipitate that has deposited on the gel surface. After completely unstained, the gel is washed with distilled water (3 × for 15 min).

Three other procedures were tested for their ability to detect NAGE-separated LPS aggregates, as described below.

Zinc–imidazole. This procedure was essentially carried out as described for polyacrylamide gels [8,9], with a few modifications. Briefly, either the boiling or the washing step in 30% acetonitrile was replaced with a washing step in 100 ml distilled water at room temperature (3 × 15 min) before zinc–imidazole staining. This step is aimed at removing electrophoresis reagents which might otherwise interfere with the zinc staining process. After this, the gel was incubated in 15 mM zinc sulfate for 25 min. The solution was discarded and residues of zinc sulfate were removed by rapidly washing the gel with abundant water (30 s), before adding the next staining solution. Then, the gel was soaked and agitated in 0.2 M imidazole for 3–5 min to develop an opaque background on the gel surface while monitoring visually for the development of any reverse-staining pattern. After the development period was completed, the imidazole solution was replaced immediately by water to stop overstaining. The gel was washed briefly (three changes, 1 min) and subsequently scanned.

Zinc–imidazole–sodium carbonate. This procedure was performed as described elsewhere (e.g., [10]). In short, the