Separation of bacterial capsular and lipopolysaccharides by preparative electrophoresis

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Introduction

Gram-negative bacteria produce a variety of polysaccharides that are important in determining plant- and animal-microbe interactions. These polysaccharides include extracellular, capsular and the cell wall lipopolysaccharides (EPSs, CPSs, and LPSs, respectively). In order to determine the role of one or more of these polysaccharides in a particular interaction it is necessary to purify the polysaccharide. While numerous procedures exist for the purification of these various polysaccharides, often a particular polysaccharide preparation can be significantly contaminated by another polysaccharide; for example, a CPS preparation is often contaminated by LPS. In addition, a purified polysaccharide is often heterogeneous. For example, CPSs and EPSs all consist of polymerized oligosaccharide repeating units in which the degree of polymerization is highly variable. The CPS can also be present as both a lipid bound molecule, and as a lipid-free polysaccharide (Jann and Jann, 1990, 1991; Whitfield and Valvano, 1993). The LPS is even more complex in that it has three structural regions: a lipid known as the lipid A, an oligosaccharide, known as the core, which is attached to the lipid A via Kdo, and a polysaccharide known as the O-chain that is attached to the core oligosaccharide. Furthermore, the O-chain polysaccharide consists of a repeating oligosaccharide with a varying degree of polymerization. Most pure LPS preparations exist as a mixture of LPS molecules, that is, a mixture in which some molecules do not have the O-chain polysaccharide (known as ‘rough’ or 

R-type LPS) together with those molecules containing various sizes of the O-chain polysaccharide (known as ‘smooth’ or S-type LPS), or truncated O-chains (known as ‘semi-smooth’ LPS). In order to assess the role of LPS in plant- or animal-microbe interactions, a procedure which readily separates the various LPS molecules on a preparative scale would be extremely valuable. Similarly, a procedure which allows the preparative separation of the CPS or EPS into molecular species of discreet sizes would also be very useful. It is known, for example, that only a particular size of EPS is biologically active in determining the ability of certain Rhizobium meliloti strains to invade the root nodule cells of its legume host, alfalfa (Battisti et al., 1992).

As mentioned above, current methods of extraction and purification of cell-associated polysaccharides often result in preparations which still contain significant amounts of contaminating polysaccharides. In most cases, separating CPS or EPS from LPS can only be achieved by the application of repetitive column chromatography using a combination of several different column matrices and eluants (Petrovics et al., 1993; Reuhs et al., 1993). The LPSs and CPSs have been characterized by polyaclrylamide gel electrophoresis (PAGE) on an analytical scale which allows one to visualize the various size ranges of these polysaccharides in a particular preparation (Pelkonen and Finne, 1989; Corzo et al., 1991; Petrovics et al., 1993; Reuhs et al., 1993). The LPS can be visualized by silver-staining, while the CPS (or EPS) can be visualized by fixing the gel in the presence of Alcian blue prior to silver-staining (Corzo et al., 1991; Petrovics et al., 1993; Reuhs et al., 1993). The PAGE analysis is usually done using deoxycholate (DOC) as the detergent since DOC facilitates the deaggregation of the various LPS molecules on a preparative scale from a crude extract, as well as fractionation of various polysaccharides into specific size ranges. This method employs preparative PAGE, as well as the ability of DOC to de-aggregate LPS thereby allowing its migration through the preparative polyacrylamide gel.

Results

Separation of polysaccharides from Rhizobium

Figure 1 shows the preparative PAGE results for the crude acidic polysaccharide extract from R. fredii USDA257. Similar results were obtained with other strains of R. fredii, R. leguminosarum, and R. meliloti (data not shown). The lanes labeled ‘C’ contain the crude polysaccharide mixture prior to preparative gel electrophoresis. Lanes 2–13 contain aliquots from every 10th fraction from the first stage of the preparative PAGE which was run in the absence of DOC. These bands are visualized only if Alcian blue is added to the fixing solution prior to silver-staining, a result characteristic of acidic polysaccharides (Petrovics et al., 1993; Reuhs et al., 1993). Each fraction
clearly contains a narrow size range of the polysaccharide. Lanes 14–25 contain aliquots from every 10th fraction from the second stage of separation which was done in the presence of DOC. The polysaccharides in these fractions can be visualized by silver-staining whether or not the fixing solution contained Alcian blue, a characteristic of LPSs (Petrovics et al., 1993; Reuhs et al., 1993). These fractions clearly show the separation of S-type from R-type LPS.

The fractions from preparative gel electrophoresis were dialedyzed and lyophilized, and their compositions were determined. The fractions represented by lane 2 contained, in the case of *R. fredii*, predominantly glucose and phosphate (determined by colorimetric analysis), and therefore most likely contain the phosphoglycerol substituted cyclic β-2-linked glucans commonly found in rhizobia (Breedveld and Miller, 1994). Fractions represented by lanes 3–13 contained glycosyl components consistent with that of rhizobial CPS. For example, in the case of *R. fredii* USDA205, these fractions contained its CPS which consists of a polymerized →3-α-D-Galp-1→5-β-D-Kdop-2→ disaccharide repeat unit (Reuhs et al., 1993). The CPS fractions, early to late eluting, contained low to higher molecular weight forms of this polysaccharide. Fatty acids and glycosyl residues indicative of LPS, were not found in these fractions. Fractions represented by lanes 14–25 contained glycosyl components and β-hydroxy fatty acids consistent with those previously published for *R. fredii* LPS (Reuhs et al., 1994).

Rhizobial EPSs can also be separated into discreet sizes and from a contaminating LPS by preparative PAGE. Figure 2 shows the results of a preparative PAGE purification of a crude preparation of *R. meliloti* EPS II, which consists of a →3-α-D-Glcp-1→3-β-D-Galp-1→ disaccharide repeat unit pyruvylated at C4 and C6 of the galactosyl residue and acetylated at C6 of the glucosyl residue (Levery et al., 1991). The analytical PAGE of the fractions eluting before the addition of DOC to the running buffer is shown in Figure 2. Analysis of these fractions show that they contain only EPS II and not LPS. The *R. meliloti* LPS (marked by the arrows in lane 1 of Figure 2) elutes only after the addition of DOC to the running buffer (data not shown).

**Separation of polysaccharides from enteric bacteria**

Figure 3 shows the preparative PAGE purification of the crude LPS preparation from a strain of *Salmonella enteritidis SE6-E21*. This strain does not produce a CPS. Lane ‘C’ contains a sample of the crude material. Lanes 2–5 contain every tenth fraction eluting from the preparative PAGE before the addition of DOC to the running buffer. Lanes 6–26 contain a sample from every tenth fraction eluting after the addition of DOC to the running buffer. Composition analysis of the material eluting in fractions 2–5 showed that it contains primarily ribose indicating that these fractions consist of RNA fragments that had contaminated the crude extract. Fraction 6–9 are enriched in glycosyl residues that are common to the core region of *Salmonella* LPS, and also contain small amounts of rhamnose and tyvelose indicating that these fractions also contain truncated versions of the O-chain polysaccharide; that is, rhamnose and tyvelose are found only in the O-chain of *Salmonella enteritidis* LPS (Hellerqvist et al., 1969). Thus, fractions represented by lanes 6–9 contain low molecular weight forms of the LPS which have truncated O-chain polysaccharides. Fractions represented by 10–26 contain greatly increased levels of O-chain compared to core oligosaccharide glycosyl residues indicating that these fractions contain S-type forms of the LPS which have various sizes of the highly polymerized O-chain polysaccharide.

As stated above, the fractions represented in lanes 6–9 contained LPS molecules which have truncated O-chains, that is,
Polysaccharide purification

Fig. 3. PAGE analysis of fractions obtained from the preparative PAGE purification of the crude polysaccharide extract from *S. enteritidis* SE6-E21. Well 'C' contains the crude polysaccharide prior to preparative PAGE. Samples from fractions prior to (wells 2—5), and after (wells 6—26) the addition of DOC to the running buffer were analyzed. The inset shows a second preparative PAGE separation of the low molecular weight LPSs present in the fractions shown in lanes 6—9.

'semi-smooth' LPS. As can be seen in Figure 3, the various forms of the 'semi-smooth' LPS were not separated during the initial preparative PAGE isolation. This is thought to be due to the fact that LPS molecules are quite hydrophobic and highly aggregated, and therefore during the initial electrophoresis with DOC, they migrate partially through the gel with the 'DOC front' resulting in the incomplete separation of the various sizes 'semi-smooth' LPS. Therefore, fractions containing the 'semi-smooth' LPS were combined, dissolved in DOC-containing sample buffer, and run a second time by preparative PAGE in the presence of DOC. The results (Figure 3, inset) show that the second preparative PAGE did separate the various forms of the 'semi-smooth' LPS from one another.

The polysaccharides in the crude extract from another enteric pathogen, *Proteus mirabilis*, were also purified by preparative gel electrophoresis. Unlike the *S. enteritidis* strain above, the LPS from this *P. mirabilis* strain is actually an R-type LPS since it does not contain an O-chain polysaccharide (Gygi et al., 1995). In addition, this bacterium produces an acidic CPS composed of mannose, galacturonic acid, glucose, N-acetylgalactosamine, and N-acetylglucosamine (Gygi et al., 1995). This CPS eluted from the preparative gel prior to the addition of DOC. After the addition of DOC, a polysaccharide with a high electrophoretic mobility eluted, followed by a polysaccharide with a low mobility. Figure 4 shows the results of analytical PAGE analysis of the combined CPS-containing fractions eluting prior to DOC addition, and fractions containing polysaccharides with high and low electrophoretic mobility eluting after DOC addition. The polysaccharide of low mobility can be visualized by silver-staining only when Alcian blue is added to the gel fixing solution; this is also true for the CPS that eluted prior to addition of DOC. As previously stated, the visualization of these polysaccharides only when Alcian blue is added to the fixing solution indicates that they are CPS. The material with high electrophoretic mobility silver stains with or without the addition of Alcian blue to the fixing solution, suggesting that this material is LPS.

Composition analysis of these isolated polysaccharides showed that the high mobility polysaccharide is LOS in that it has typical glycosyl and fatty acyl residues of an LPS core oligosaccharide and lipid A, for example, heptose, glucosamine, β-hydroxymyristate, etc. (Gygi et al., 1995). Composition analysis also verified that the material eluting prior to the addition of DOC was CPS since it contains all of the glycosyl residues which characterize this CPS (Gygi et al., 1995). The low mobility polysaccharide, eluting only after the addition of DOC, had a glycosyl composition identical to that of the CPS, except that it contained a stearic acid acyl residue (Gygi et al., 1995). Therefore, the low mobility polysaccharide may be lipid-linked CPS, for example, possibly to phosphatidic acid as is the case with some other CPSs (Jann and Jann, 1990, 1991; Whitfield and Valvano 1993). Thus, in the case of this strain of *P. mirabilis* polysaccharide. Lane 'C' contains the crude polysaccharide prior to preparative PAGE. Those fractions eluting prior to the addition of DOC to the running buffer (lane 2), and of the low (lane 3) and high (lane 4) molecular weight polysaccharides eluting after the addition of DOC to the running buffer were analyzed.
Discussion

Bacterial surface polysaccharides are important virulence factors in both animal- and plant-microbe interactions (Leigh and Coplin, 1992; Noel, 1992; Jann, 1983). The CPSs and EPSs can act by masking the invading bacterium thereby protecting it from the defense mechanism of the host cell (Jann, 1983). Some of this 'masking' effect is thought to be due to the structural similarity of a particular polysaccharide to the carbohydrate of the host cell (Jann, 1983). The presence of a large proportion of S-type LPS on the surface of a bacterium has also been correlated with its virulence (Guard-Petter et al., 1995), for example, resistance to the complement system (Vukalovich, 1992). In the case of rhizobia, it is known that LPS structural alterations occur when bacteria are grown under conditions that induce expression of nodulation (nod) genes (Tao et al., 1992; Noel 1993; Reuhs et al., 1994), and from rhizobia isolated from root nodules, or from bacteria grown under physiological conditions that are thought to mimic the nodule environment (Kannenberg and Brewin, 1989, 1994; Vandenv-Bosch et al., 1989; Wood et al., 1989; Sindhu et al., 1990; Tao et al., 1992; Kannenberg et al., 1994; Noel, 1993; Reuhs et al., 1994). Furthermore, it has also been demonstrated that only a certain size range of \( R.meliloti \) EPS has the ability to complement the infection defect of an EPS defective mutant (Battisti et al., 1992). Thus, to better understand the structure-function relationships of these polysaccharides in plant- and animal-microbe interactions, it is important to be able to preparatively separate the various size classes of EPSS, CPSs, S-type, 'semi-smooth', and R-type LPSs.

In this report it has been shown that preparative PAGE can be used to (1) separate CPS from LPS, (2) separate EPS from contaminating CPS, (3) separate CPS from lipid-bound EPS, and (4) separate discrete sizes of CPS, EPS, and LPS. This method takes advantage of the ability of PAGE to separate these molecules with a high degree of resolution, and of the ability of DOC to deaggregate LPS. The preparative PAGE procedure is based on reports which show that analytical PAGE in the presence of DOC (Krauss et al., 1988) or gel filtration chromatography using a DOC-containing eluant separates various molecular forms of LPS from a variety of bacteria (Peterson and McGroarty, 1985). In addition, analytical PAGE has been used to characterize a variety of acidic polysaccharides (Pelkonen and Finne, 1989; Corzo et al., 1991; Petrovics et al., 1993; Reuhs et al., 1993). The preparative PAGE procedure described here permits, in basically one step, the preparative isolation of very narrow size ranges of CPS, EPS, or LPS from bacterial strains representative of a wide range of Gram-negative bacteria. Thus, this method should greatly facilitate characterizing the role that these molecules play in determining plant- and animal-microbe interactions.

Materials and methods

Isolation of the crude polysaccharide fraction
Rhizobium fredii was grown and extracted by a modified hot phenol-water procedure as previously reported (Reuhs et al., 1993), as were Rhizobium meliloti (Reuhs et al., 1993), and Rhizobium leguminosarum (Carlson et al., 1987). Proteus mirabilis and Salmonella enteriditis were grown and crude extracts prepared as previously described (Darveaux and Hancock, 1983; Gygi et al., 1995). In each case the crude polysaccharide extract was dialyzed and lyophilized. If neutral polysaccharides are present, as is the case for \( R.fredii \), they were removed by DEAE-Sephadex column chromatography prior to preparative PAGE. Neutral polysaccharides from \( R.fredii \) were removed by dissolving up to 200 mg of the crude polysaccharide extract in \( H_2O \) and applied to a 3 cm \( \times \) 15 cm DEAE-Sephadex column. The column was washed with 50 ml of \( H_2O \), and the bound fraction was eluted with 200 ml of a 0.0 to 1.0 M ammonium formate gradient. Fractions were assayed by colorimetric procedures for hexose (York et al., 1985), uronic acid (York et al., 1985), and Kdo (York et al., 1985), and by analytical PAGE. The neutral polysaccharide fraction from \( R.fredii \) was described in a previous report (Reuhs et al., 1993) and was not analyzed further for this study. The acidic fraction was pooled, dialyzed against \( H_2O \), and lyophilized. The \( R.fredii \) acidic polysaccharide preparation, as well as the crude polysaccharide extracts from the other bacteria, were further purified by preparative PAGE as described below.

Preparative gel electrophoresis

The crude polysaccharide extracts, up to 100 mg, were electrophoresed on the model 491 Prep Cell (Bio-Rad, Richmond, CA) in two stages. In the first stage, electrophoresis was performed in the absence of DOC. This was followed by a second stage in which DOC was added to the running buffer. A 5 cm, 15% polyacrylamide gel (25 ml of 30% acrylamide/bis (30% T/2.67% C), 10 ml of 1.88 M Tris base, pH 8.8, and 15 ml of \( H_2O \) was cast according to the manufacturer's recommendations. A sample of the polysaccharide preparation was dissolved in 2 ml of sample buffer (0.125 M Tris base, 10% glycerc, pH 6.8), loaded onto the gel, and run at a constant voltage of 200 V. During the first stage of the electrophoretic run the running buffer (0.29 M glycine, and 0.037 M Tris base) did not contain DOC. Fractions (10 ml) were collected for up to 24 h at an elution rate of 1 ml/min. The nonidentogen running buffer was subsequently sponed off from the upper running buffer reservoir and replaced with DOC-running buffer (0.29 M glycine, 0.037 M Tris base, and 0.25% DOC). Fractions were collected for up to an additional 24 h. Buffer salts and DOC were removed by dialysis as previously described (Reuhs et al., 1993).

Analytical polyacrylamide gel electrophoresis (PAGE)

Fractions from preparative gel electrophoreoses were analyzed by analytical PAGE as follows: 50 \( \mu \)l aliquots from every 10th fraction were dried under vacuum and dissolved in 10 \( \mu l \) of sample buffer, and 1 \( \mu l \) was loaded onto an 18% analytical gel. PAGE was performed using DOC-running buffer. The gels were fixed in 100 ml of 0.01% Alcian blue in EAW (40% ethanol, 5% acetic acid, in water) overnight, followed by silver staining (Reuhs et al., 1993). Alcian blue is a cationic dye which allows the visualization of acidic polysaccharides by silver-staining (Corzo et al., 1991; Reuhs et al., 1993).

Composition analysis

Glycosyl components of the various fractions were characterized by the preparative and analysis of either aldol acetates or of trimethylsilyl (TMS) methyl glycosides. The aldol acetates and TMS methyl glycosides were prepared as previously described (York et al., 1985), and analysis was performed by gas liquid chromatography (GLC) and by combined GLC-mass spectrometry (MS). A 30 m SP2330 capillary column from Supelco (Bellefonte, PA) and a 30 m DB-1 capillary column from J & W Scientific (Folsom, CA) were used for the analysis of the aldol acetates and TMS methyl glycosides, respectively.

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