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Fractionation and analysis of lipopolysaccharide-derived oligosaccharides by zwitterionic-type hydrophilic interaction liquid chromatography coupled with electrospray ionisation mass spectrometry

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ABSTRACT

Lipopolysaccharide (LPS, endotoxin) is a main surface antigen and virulence factor of Gram-negative bacteria. Regardless of the source of LPS, this molecule, isolated from the smooth forms of bacteria, is characterised by a general structural layout encompassing three regions: (i) an O-specific polysaccharide (O-PS) – a polymer of repeating oligosaccharide units, (ii) core oligosaccharide (OS), and (iii) the lipid A anchoring LPS in the outer membrane of the cell envelope of Gram-negative bacteria. Structural analysis usually requires degradation of LPS and further efficient separation of various poly- and oligosaccharide glycoforms.

The hydrophilic interaction liquid chromatography (HILIC) was shown as an efficient technique for separation of labelled or native neutral and acidic glycans, glycopeptides, sialylated glycans, glycosylated and nonglycosylated peptides. Herein we adopted ZIC® (zwitterionic stationary phase covalently attached to porous silica)-HILIC technology in combination with electrospray ionisation mass spectrometry to separate different LPS-derived oligosaccharides. As a result three effective procedures have been developed: (i) to separate different core oligosaccharides of *Escherichia coli* R1 LOS, (ii) to separate RU-[Hep]-Kdo oligosaccharides from core OS glycoforms of *Hafnia alvei* PCM 1200 LPS, and (iii) to separate Hep and Kdo-containing mono, di-, tri- and tetrasaccharides of *H. alvei* PCM 1200 LPS. Moreover, some of developed analytical procedures were scaled to semi-preparative protocols and used to obtain highly-purified fractions of the interest in larger quantities required for future evaluation, analysis, and biological applications.

1. Introduction

Lipopolysaccharide (LPS, endotoxin) is a main surface antigen of Gram-negative bacteria. LPS protects microorganisms against the defensive mechanisms of the infected host, bile acid and hydrophobic antibiotics. Lipopolysaccharides are extensively studied within the field of microbiology, biochemistry, immunology, and genetics, since they play a significant role as a virulence factor of Gram-negative bacteria and are powerful activators of innate immune response involving CD14/TLR4/MD2 receptor complex. As a consequence of its activity, LPS is a main causative agent involved in development of Gram-negative sepsis and septic shock.[1]

Regardless of the source of LPS, this molecule, isolated from the smooth forms of bacteria, is characterised by a general structural layout encompassing three regions: (i) an O-specific polysaccharide (O-PS) – a polymer of repeating oligosaccharide units, characterised by a high structural variability determining serological specificity of LPS (O serotype), (ii) core oligosaccharide (OS) – a region of limited variability within a species, and (iii) lipid A – a glycolipid region anchoring LPS in the outer membrane of the cell envelope of Gram-negative bacteria. Lipid A is linked with a core OS by acid labile ketosidic linkage between the 2-keto-2-deoxy-D-*manno*-octulosonic (Kdo) residue of the core OS and lipid A. The presence of this linkage enables delipidation of LPS by mild acid hydrolysis (lipid A removal) releasing of poly- and oligosaccharides that are subjects for structural analysis of O-PS and core OS.[2–5] The

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de-N,O-acylation of LPS using anhydrous hydrazine and/or aqueous 4 M KOH treatment is another degradation method with preservation of all ketosidic linkages.[5–8] Combination of both of these methods with lipid A analysis leads to complete structure elucidation of LPS, including identification of most of labile substituents and linkages between lipid A, core OS and O-PS.

Regardless of delipidation method of LPS, the mixture of poly- and oligosaccharides are usually fractionated before further structural analysis employing NMR spectroscopy and mass spectrometry, to separate high-molecular weight O-PSs from low-molecular weight fractions containing core OS glycoforms and short O-PS fragments. However collected fractions are still characterised by moderate variability with reference to one strain. Above all, number of RUs and nonstechioimetric substituents, such as amino acids, O-acetyl groups, methyl and methyl phosphate groups, define a variability of O-PS fractions. Heterogeneity of core OS is attributed to sugar composition, phosphate groups (P), pyrophosphate groups (PP), ethanolamine (Etn), carbamoyl groups, amino acids (i.e. glycine, Gly), and O-acetyl groups (OAc).[9] Efficient separation of described glycoforms facilitates structural analysis by NMR spectroscopy and enables further identification of structure–activity relationships.

Various chromatographic methods have been used for fractionation of LPS-derived poly- and oligosaccharide mixtures, including (i) size exclusion chromatography (SEC) utilising for example Biogels (Bio-Rad, USA), Sephadex G-50,[4] Toyopearl HW-40 (Tosoh Bioscience LLC, Japan), (ii) ion-exchange chromatography, including high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD).[6–8,10] Despite the variety of commercially available chromatographic columns, SEC does not enable for precise separation of individual core OS glycoforms of similar molecular mass from complex mixtures. Of all these techniques only HPAEC-PAD provides effective separation of poly-, oligo-, and monosaccharides; nevertheless it requires strong alkaline conditions (for most of applications), dedicated equipment, and desalting step to be on-line coupled with mass spectrometer. HPAEC-PAD-based protocols cannot be easily supported by on-line MS monitoring.[11,12] Additionally alkaline conditions may cause the loss of some labile constituents of core OS such as O-acetyl groups and ester-linked glycine.

Herein we proposed to complement described methods with hydrophilic interaction chromatography (HILIC) commonly used in glycomics for analysis of glycoprotein glycans, glycopeptides and nucleotide sugars.[13,14] The HILIC [including zwitterionic stationary phase covalently attached to porous silica (ZIC®)-HILIC] is used as a tool to change the selectivity or to improve peak resolution for polar and hydrophilic compounds such as carbohydrates, metabolites, acids and bases, organic and inorganic ions, metal complexes, amino acids, peptides and protein digests.[15] HILIC was shown as efficient technique for separation of labelled or native neutral and acidic glycans, glycopeptides, sialylated glycans, glycosylated peptides from nonglycosylated peptides.[13] It seems to be a powerful technique in large-scale glycomics and glycoproteomics such as the analysis of entire glycoproteomes at the glycopeptide level.[14]

We used ZIC®-HILIC coupled with electrospray ionisation mass spectrometry (ESI-MS) for analytical and semi-preparative fractionation of oligosaccharides released after mild acid hydrolysis of well characterised LPS of *Escherichia coli* R1 and *Hafnia alvei* PCM 1200.[2,10,16,17] The method offers a new analytical and separation tool for structural analysis of some complex LPS-derived oligosaccharides.

2. Results

2.1. Lipopolysaccharides and core oligosaccharides isolation

Lipopolysaccharides of rough *E. coli* R1 and smooth *H. alvei* PCM 1200 were extracted from bacterial cells by the hot phenol/water method[18] and purified as previously described.[19] Poly- and oligosaccharides were released from lipid A by acidic hydrolysis of LPS and fractionated by gel chromatography on Bio-Gel P-10.

The Bio-Gel P-10 fractionation of oligosaccharides isolated from *E. coli* R1 LPS yielded two fractions (EcOS1 and EcOS2). Fraction EcOS1 contained all expected glycoforms of the core OS.[10,20,21] The fraction EcOS2 contained degradation products of LPS such as Kdo-containing mono- and low-molecular weight oligosaccharides (data not shown). Negative-ion mode ESI-MS analysis of EcOS1 fraction was performed indicating the presence of various core OS glycoforms (Fig. 1A). Interpretation of observed ions, $[M-2H]^{2-}$ and $[M-3H+Na]^{2-}$ including dehydrated variants was shown in Table 1 and was in agreement with previously published data.[10,20,21] Backbone structure of identified glycoforms contained $Hex_5{\cdot}Hep_3{\cdot}Kdo$ nonasaccharide. Heterogeneity of the EcOS1 was attributed to the presence of additional substituents such as P, PP, HexN, and PPEtn.

Fractionation of *H. alvei* LPS 1200-derived poly- and oligosaccharides on Bio-Gel P-10 was previously described and five PS fractions containing O-specific polysaccharides with different number of RUs (PS1-PS5)[17,22] and two core OS fractions (HaOS1 and HaOS2)[2] were isolated as expected and verified by ESI-MS analysis (Fig. 3A). Interpretation of observed $[M-2H]^{2-}$ ions and dehydrated variants thereof was shown in Table 2. The HaOS1 fraction contained the mixture of various previously described glycoforms of the core OS built of $Glc_2{\cdot}Hep_3{\cdot}Kdo_1$ hexasaccharide substituted P, PPEtn,[16,23] and Gly[2] and one repeating unit of the O-PS linked to the Hep-Kdo disaccharide (RU-[Hep]-Kdo).[17] The fraction HaOS2 contained the mixture of low-molecular weight compounds, mono-, di-, tri-, and tetrasaccharides built of Hep and Kdo residues (Table 3), and represented products of mild acid hydrolysis of labile regions, mainly ketosidic linkages, present in the outer and inner core of native LPS 1200.[17]

2.2. Analytical and semi-preparative ZIC®-HILIC-based separation of E. coli R1 core OS glycoforms

To separate different core OS glycoforms isolated from *E. coli* R1 LPS (fraction EcOS1), analytical separation protocol was optimised using SeQuant® ZIC®-HILIC analytical column coupled with ESI-IT-MS and resulted in development of a method with 90–40% gradient of acetonitrile in 0.1% formic acid. The protocol resulted in effective separation of four fractions (I-IV), as observed on basic peak chromatogram (BPC) (Fig. 1B). The spectrum of fraction I showed $[M-2H]^{2-}$ ions attributed to the $Hex_5{\cdot}Hep_3{\cdot}Kdo{\cdot}$nonasaccharide substituted with two and three P (ions at *m/z* 891.4 and 931.3 and respective dehydrated variants) (Fig. 1C). Fraction II contained mostly $Hex_5{\cdot}Hep_3{\cdot}Kdo$ nonasaccharide substituted with PPEtn and P or PP (ions at *m/z* 952.9 and 992.8) (Fig. 1D). Fraction III contained $HexN{\cdot}Hex_5{\cdot}Hep_3{\cdot}Kdo$ decasaccharide substituted with one or two P (ions at *m/z* 931.9, 971.8) (Fig. 1E). Fraction IV contained $HexN{\cdot}Hex_5{\cdot}Hep_3{\cdot}Kdo$ decasaccharide substituted with PPEtn (ions at *m/z* 993.4) and some traces of monophosphorylated $Kdo{\cdot}Hep_3{\cdot}Hex_5{\cdot}HexN$ decasaccharide (the ion at *m/z* 922.9) (Fig. 1F).

Semi-preparative separation LC-MS protocol for crude core OS fraction obtained directly after hydrolysis of LPS (the mixture of EcOS1 and EcOS2) was optimised using SeQuant® ZIC®-HILIC semi-preparative column and resulted in development of a method with 62–40% gradient of acetonitrile in 0.1% formic acid (Fig. 2). The fractionation yielded 6 fractions (I–VI) identified on the basis of BPC chromatogram of $[M-2H]^{2-}$ ions and their dehydrated variants attributed to core OS glycoforms. Since semi-preparative procedure was done for crude OS fraction obtained after mild acid hydrolysis, fraction I contained Kdo-containing mono- and low-molecular weight oligosaccharides (data not shown). The procedure provided separation of diphosphorylated $Hex_5{\cdot}Hep_3{\cdot}Kdo$ nonasaccharide (fraction II, ion at *m/z* 891.2) (Fig. 2B), the mixture of di- and triphosphorylated glycoforms substituted with Etn (in form of PPEtn)