Carbohydrate Research 408 (2015) 119–126

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Occurrence of glycine in the core oligosaccharides of *Hafnia alvei* lipopolysaccharides—identification of disubstituted glycoform

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ARTICLE INFO

Article history:
Received 23 September 2014
Received in revised form
17 November 2014
Accepted 22 November 2014
Available online 3 December 2014

Keywords:
Lipopolysaccharide
Endotoxin
Core oligosaccharide
Glycine
ESI

ABSTRACT

Endotoxins (lipopolysaccharides, LPS) are the main surface antigens and virulence factors of Gram-negative bacteria involved for example in the development of nosocomial infections and sepsis. They consist of three main regions: O-specific polysaccharide, core oligosaccharide, and lipid A. Bacteria modify LPS structure to escape the immune defence, but also to adapt to environmental conditions. LPS's structures are highly diversified in the O-specific polysaccharide region to evade bactericidal factors of immune system, but retain some common epitopes that are potential candidates for therapeutic strategies against bacterial infections. Common occurrence of glycine within the structure of LPS is a known phenomenon and was previously reported for variety of species. Since glycine residue substitutes mainly core oligosaccharide of LPS, especially inner core region, it was also considered as a part of common epitope for broad-reactive antimicrobial antibodies. Herein, we used multiple-stage electrospray ionisation mass spectrometry to identify glycine substitution in core oligosaccharide type characteristic for *Hafnia alvei* LPS, and isolated from five strains of different O-serotypes: 32, PCM 1190, PCM 1192, PCM 1200, and PCM 1209. The location of glycine in core oligosaccharide was determined in detail for LPS 1190 using ESI-MS^n. Three glycoforms were identified, including two mono-glycinylated and one diglycinylated core oligosaccharides.

1. Introduction

Lipopolysaccharide (LPS, endotoxin) is a main surface antigen of Gram-negative bacteria, called O antigen. LPS is built of lipid A substituted with core oligosaccharide (OS) that is further substituted with O-specific polysaccharide (O-PS) built of oligosaccharide repeating units (RU). In most of species core oligosaccharides contain outer core (hexose region) and inner core (heptose region). As a main virulence factor LPS constitutes pathogen associated molecular pattern and is the target for rapid response of innate immunity system. Lipid A is the most conservative region of LPS recognised by receptors present on the surface of target cells for LPS, such as CD14/TLR 4/MD2 receptor complex. The core OS and O-PS modulate activity of lipid A, influences persistence of LPS aggregates in the bloodstream and mask lipid A region. Among substituents modifying LPS structure and activity are various amino acids, *O*-acetyl groups, phosphate groups (P), pyrophosphate groups (PP) and ethanolamine (Etn). They were reported to occur in all regions of lipopolysaccharides and, besides *O*-acetyl groups and amino acids, are common substituents of the core OS. It is known that modifications of core OS and lipid A with amine-containing substituents (Etn, ester-linked amino acids, glycine) or the lack of P and PP represent adaptive mechanism of reduction of net negative charge of bacterial surface promoting cationic resistance against cationic antimicrobial peptides such as polymyxin B.[1] Thus such structural modifications affect virulence of pathogenic bacteria.

For years glycine (Gly) was identified among nonsugar substituents in a variety of LPS preparations.[2] The first reports described the presence of Gly in LPSs of *Escherichia coli*[3] and *Salmonella enterica* serovar typhimurium.[4] Afterwards Gly was found as integral constituent of core OS fractions isolated from LPS of *Escherichia coli*, *Salmonella* spp., *Hafnia alvei*, *Citrobacter* spp. and

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http://dx.doi.org/10.1016/j.carres.2014.11.017
0008-6215/

Shigella sonnei, *Vibrio cholerae*, *Thiobacillus* spp. and *Proteus* spp.[2] The first indication of the covalent linkage between core OS and Gly was based on GC–MS analyses.[5] Together with the development of modern analytical techniques, such as multiple stage electrospray ionisation mass spectrometry (ESI-MS^n), capillary electrophoresis (CE)-ESI-MS, and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) occurrence of Gly was further supported by detailed MS^n analyses. Described analytical techniques allowed for wide range screening of Gly substitution in LPSs of typeable and nontypeable *Haemophilus influenzae* strains,[6,7] where amino acid was predominantly identified in the inner core region of the core OS. Among other glycinylated examples are core OSs of lipopolysaccharides isolated from several strains of *Neisseria meningitidis*,[8] *Proteus mirabilis*,[9] *Shigella flexneri*,[10] *Campylobacter jejuni*[11] and *Plesiomonas shigelloides*.[12]

Presented results supplemented described data with information about occurrence of Gly in core OSs of *H. alvei* lipopolysaccharides isolated from strains 32, PCM (Polish Collection of Microorganisms) 1190, PCM 1192, PCM 1200 and PCM 1209. This bacterium is an opportunistic human pathogen which contributes to bacteremia, septicaemia in humans and animals, but also to acute cholecystitis, mixed hospital infections, appendicitis, community-acquired cholangitis and respiratory diseases.[13] Three core OS types (Fig. 1A–C) were identified to date for *H. alvei* LPS.[14–16] Five *H. alvei* LPSs selected herein are characterised by different O-serotypes, that is, different structures of O-PSs[15,17–20] and all contain the most common for *H. alvei* core OS (Fig. 1A) built of Glc_2–Hep_3–Kdo (Kdo, 2-keto-3-deoxy-octulosonic acid) hexasaccharide substituted by phosphate group (P) and pyrophosphoethanolamine (PPEtn).[15,21–23] Previously Gamian et al. reported the coexistence of the core OS and Gly constituents in core OS fractions obtained by mild acid hydrolysis of some *H. alvei* LPSs.[5] Additionally, Gly residue was found recently as integral constituent of the core region in clinical isolate *H. alvei* 2670 LPS,[16] which is characterised by the presence of nontypical core region (Fig. 1C). Data presented herein constitute state-of-the-art mass spectrometric analysis of different glycoforms of glycinylated core OSs of *H. alvei* LPS and leaded to identification of mono- and diglycinylated glycoforms, supporting previous results that glycine may be an essential substituent for LPS of this species.

2. Results and discussion

2.1. Isolation of lipopolysaccharide and core oligosaccharides

Lipopolysaccharides of *H. alvei* 32, PCM 1190, 1192, 1209, and 1200 were extracted from bacterial cells by the hot phenol/water method[24] and purified as previously described.[25] Poly- and oligosaccharides were released from lipid A by acidic hydrolysis of LPS and fractionated by gel chromatography on Bio-Gel P-10. According to previously published data, fractionation of poly- and oligosaccharides yielded five PS fractions containing O-specific polysaccharides with different numbers of RUs (PS1–PS5),[17,26] and two core OS fractions (OS1–OS2). Regardless of the strain, fraction OS1 contained common type of the core OS characteristic for most of *H. alvei* LPSs: Glc_2–Hep_3–Kdo hexasaccharide substituted with P and PPEtn (Fig. 1A).[15,21–23]

2.2. ESI-MS analyses of H. alvei core oligosaccharides

ESI-MS spectra obtained for core oligosaccharides (fractions OS1) isolated from *H. alvei* 32, PCM 1190, 1192, 1200, and 1209 contained a similar pattern of ions and indicated heterogeneity of this region (Fig. 2). Interpretation of observed $[M+2H]^{2+}$ ions is shown in Table 1. Taking into account previously published structure of the core OS of *H. alvei* PCM 1192,[15] ion at *m*/*z* 711.70 corresponded to characteristic and predominant for *H. alvei* LPS core oligosaccharide (Fig. 1A). Most of ions, with the highest abundance in the case of strain 1190, were attributed to Gly substitution. Both mono- (ion at *m*/*z* 740.21 and its dehydrated counterpart at *m*/*z* 731.19, ion at *m*/*z* 780.18) and diglycinylated (ion at *m*/*z* 768.71 and its dehydrated counterpart at *m*/*z* 759.68) glycoforms were observed (Table 1) with molecular mass of 1478.40 Da and 1535.40 Da, respectively. The heterogeneity was related to the substituents, such as Gly, P, and PEtn. Due to the high abundance of glycinylated glycoforms in LPS 1190, this strain was chosen for detailed MS^n analysis of glycine location in the core OS. Ions at *m*/*z*

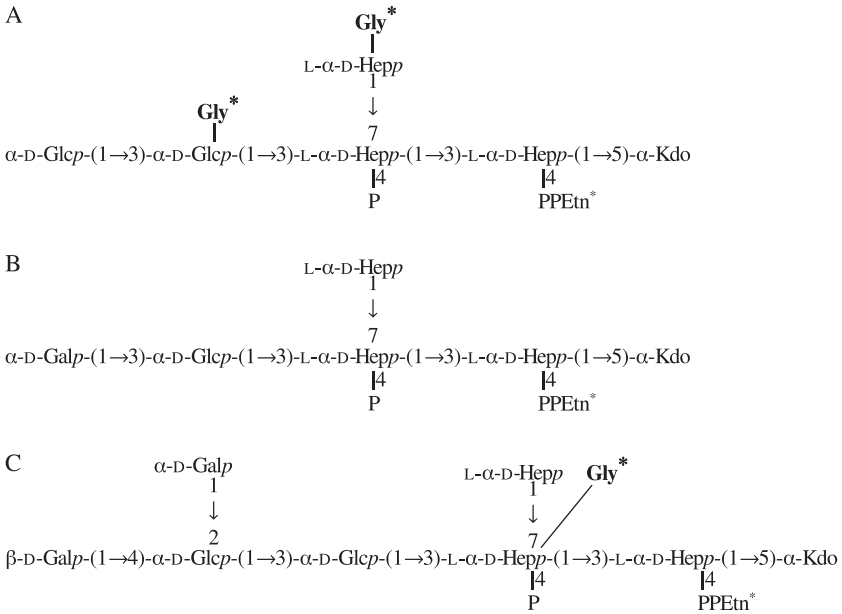

Figure 1. Structures of glycoforms identified in core OS fractions obtained by mild acid hydrolysis of *H. alvei* lipopolysaccharides. (A) Core OS type typical for *H. alvei* LPS. (B) The core OS identified for strains 1185 and 1204.[14] (C) The core OS identified for strain 2670.[16] The asterisk denotes nonstoichiometric substituents.