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Note

The structure of O-polysaccharides isolated from plant pathogenic bacteria *Pectobacterium wasabiae* IFB5408 and IFB5427

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ABSTRACT

O-Polysaccharides were isolated from the lipopolysaccharides of two strains of plant pathogenic bacteria *Pectobacterium wasabiae* isolated in Poland in 2013 (IFB5408 and IFB5427). The purified polysaccharides were analyzed using 1D and 2D NMR spectroscopy (^{1}H, DQF-COSY, TOCSY, ROESY, HSQC, HSQC-TOCSY, and HMBC) and the chemical methods. Sugar and methylation analyses of native polysaccharides, absolute configuration assignment of constituent monosaccharides together with NMR spectroscopy data revealed that the chemical structures of both O-polysaccharides are the same.

Pectobacterium wasabiae (formerly *Erwinia carotovora* subsp. *wasabiae*) strains were firstly isolated from Japanese horseradish (*Eutrema wasabi*).[1] In 2010, Pitman et al. proved that they are capable of causing disease symptoms on potato (*Solanum tuberosum* L.).[2] Milestone in the history of this species was the reclassification of *Pectobacterium carotovorum* subsp. *carotovorum* SCC3193 reference strain used in molecular biology research to *Pectobacterium wasabiae*.[3] Recently performed screening of the European collections of plant pathogenic bacteria indicated that the strains of *P. wasabiae* have been causing disease symptoms on potato in Europe since the 60s of the 20th century, but were wrongly assigned to highly virulent *Pectobacterium carotovorum* subsp. *carotovorum*.[4–6]

P. wasabiae, a Gram-negative bacterium classified to the *Enterobacteriaceae* family, is one of the causative agents of soft rot and blackleg of potato and soft rot of economically important plants.[2,6] Strains of *P. wasabiae* cause different types of disease symptoms such as plant wilting, stem and tuber soft rot and blackleg of potato plants stems. Development of disease symptoms, caused by bacteria from the genus *Pectobacterium* on potato, is the result of plant–microbe interaction and depends on cultivar susceptibility, strain virulence and the presence of favorable environmental conditions.[7,8] The main virulence factors of pectinolytic bacteria are plant cell wall degrading enzymes (PCWDE) such as pectinases, cellulases and proteases, which are secreted via type II secretion system.[9] Due to the activities of PCWDE, *P. wasabiae* can degrade plant cell wall components and cause maceration of the plant tissue.

The other factors that play an important role in the disease symptoms development are lipopolysaccharides (LPS), which are important for bacterial adhesion and attachment to plant cells, and what affects the later interaction. It has been shown that LPS-deficient mutant of closely related *Pectobacterium atrosepticum* exhibits lower virulence than the wild type strain of *P. atrosepticum*.[10] These data indicated that LPS plays a role in the virulence of pectinolytic bacteria and in their ability to cause disease symptoms.

In this study the structure of O-polysaccharides (OPS) of LPS produced by two strains of *P. wasabiae* (IFB5408 and IFB5427) is described. The strain of *P. wasabiae* IFB5408 was isolated in Northern Poland in 2013 from potato plant exhibiting the symptoms of blackleg and soft rot, while the other strain IFB5427 originated from Southern Poland and was obtained in the same year from asymptomatic weed present in the potato fields.

The LPS was isolated from the cells of *P. wasabiae* IFB5408 using phenol-water extraction, then purified by ethanol precipitation. The lipid A part was cleaved off (mild acidic hydrolysis) and removed by centrifugation. The supernatant was lyophilized, dissolved in water and fractionated using gel permeation chromatography (GPC). Similar fractions were pooled together and preliminary analyzed by ^{1}H NMR. The high molecular mass OPS was structurally characterized using chemical methods and NMR spectroscopy. The sugar analysis identified three monosaccharides: Gal, GalN, and ManN in the molar ratio ~2 : 1 : 1. The substitution positions of the monosaccharides were

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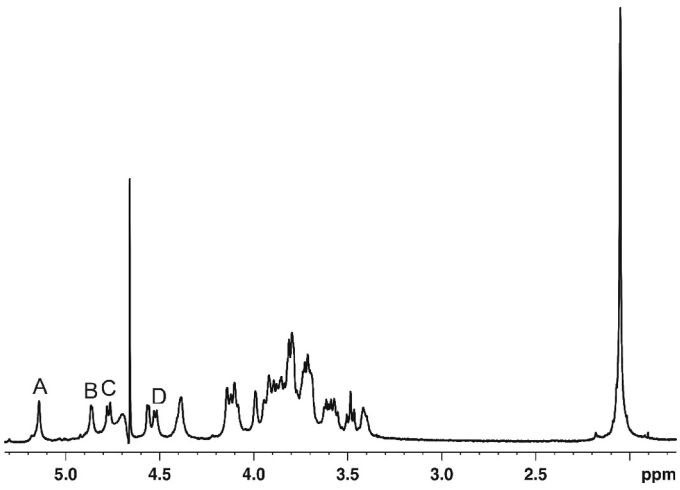


Fig. 1. ^{1}H NMR spectrum of the OPS isolated from *P. wasabiae* IFB5408. The letters refer to the monosaccharide residues as defined in Table 1.

assigned using methylation analysis (methylation, hydrolysis, reduction, acetylation). The results of gas–liquid chromatography coupled with mass spectrometry (GLC–MS) analysis of partially methylated alditol acetates revealed the presence of four derivatives in the repeating unit of the OPS: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-hexitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-hexitol, 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-hexitol, and 1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-2-*N*-methylacetamido-hexitol.

The results of sugar and methylation analyses together with the lack of carbon atom signals in the region of δ 82–88 of ^{13}C NMR spectrum (the pyranoside form of the rings of all monosaccharides) identified the presence of →4-Gal, →3,4-Gal, →3-HexN, and *t*-HexN in the repeating unit of the OPS. Moreover, GLC analysis of synthesized (*S*,*R*)- and (*S*)-but-2-yl glycosides identified D absolute configuration of all monosaccharides.

^{1}H NMR spectrum (Fig. 1) showed four signals of anomeric protons at δ: 5.141 (**A**), 4.865 (**B**), 4.771 (**C**), and 4.519 (**D**) in the ratio 1 : 1 : 1 : 1. There was also a characteristic signal of two methyl groups of N-acetyl groups at δ ~2.05, as well as of remaining carbohydrate ring protons in the region of δ 3.41–4.56. TOCSY spectrum showed four spin systems: **A** with seven protons and remaining **B**–**D** with four protons each. The order of protons in the spin systems was assigned using DQF–COSY spectrum. The remaining chemical shifts of H-5 and H-6 protons of residues **B**–**D**, as well as all of ^{13}C chemical shifts of carbon atoms were deduced from heteronuclear ^{1}H–^{13}C HSQC, HMBC and HSQC–TOCSY spectra. All ^{1}H and ^{13}C chemical shifts of the OPS are listed in Table 1.

The glycosylation positions of the monosaccharides were determined by identification of carbon atoms with higher chemical shifts in comparison to unsubstituted resides: C-3 and C-4 of residue **B**, (δ 78.21 and 76.44), C-3 of residue **C** (δ 80.98), C-4 of residue **D** (δ 78.15). The remaining residue **A** was identified as a terminal residue.

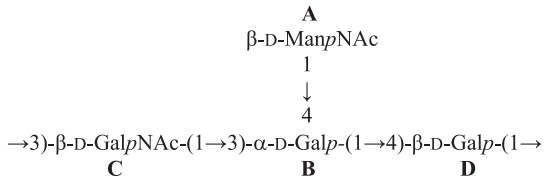
The anomeric configurations of constituent monosaccharide residues were assigned using $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants obtained from HSQC experiment recorded without decoupling.

The value of 171 Hz for residue **B** identified α anomeric configuration. The values 167 Hz for residue **A**, 161 Hz for residue **C** and 162 Hz for residue **D** revealed their β anomeric configuration. Moreover, $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constants confirmed β anomeric configuration of residues **C** and **D** (respectively 9.0 Hz and 8.0 Hz), and α anomeric configuration of residue **B** (3.5 Hz).

The residues **B**, **C** and **D** possessed the *galacto*-configuration and were identified as →3,4)-α-D-Gal*p*, →3)-β-D-Gal*p*NAc, and →4)-β-D-Gal*p*, respectively. Residue **A** (*manno*-configuration) was identified as the terminal β-D-Man*p*NAc.

The sequence of monosaccharides in the repeating unit of the OPS was based on the interpretation of HMBC and ROESY spectra. The following *inter*-glycosidic correlations were observed in HMBC spectrum (Fig. 2): H-1 of **A** with C-4 of **B**, H-1 of **B** with C-4 of **D**, H-1 of C with C-3 of **B**, H-1 of D with C-3 of **C**, as well as C-1 of **A** with H-4 of **B**, C-1 of **B** with H-4 of **D**, C-1 of **C** with H-3 of **B**, and C-1 of **D** with H-3 of **C**. Moreover, HMBC spectrum revealed information about the substitution of nitrogen atoms of Man*p*N (**A**) and Gal*p*N **(C)** by two N-Ac groups (Table 1). ROESY spectrum (Fig. 3) confirmed the sequence of the monosaccharides obtained from HMBC experiment. The following strong *inter*-residual NOE proton contacts were observed: **A**1/**B**4, **B**1/**D**4, **C**1/**B**3, and **D**1/**C**3.

All the information obtained from chemical analyses and NMR spectroscopy allowed to determine the structure of the repeating unit of *P. wasabiae* IFB5408 O-chain as the following.



Identical chemical structure was also assigned for the OPS isolated from *P. wasabiae* IFB5427.

According to our best knowledge, we have for the first time structurally characterized the O-polysaccharides isolated from *P. wasabiae*.

Table 1
^{1}H and ^{13}C NMR data of the OPS isolated from *P. wasabiae* IFB5408. Underlined values indicate the position of substitution

Residue [$^{1}J_{C\text{-}1,H\text{-}1}$; $^{3}J_{H\text{-}1,H\text{-}3}$ (Hz)]	Chemical shifts (ppm) ^{1}H and ^{13}C					
	H1	H2	H3	H4	H5	H6
	C1	C2	C3	C4	C5	C6
β-D-Man*p*NAc (A)	5.141	4.561	3.864	3.483	3.407	3.902/3.798
[167; <1.5]	100.28	54.34	73.24	68.21	77.16	61.73
N-Ac	—	2.044				
	176.54	22.98				
→3,4)-α-D-Gal*p* **(B)**	4.865	3.909	4.111	4.384	4.395	3.731/3.611
[171; 3.5]	101.59	69.58	<u>78.21</u>	<u>76.44</u>	71.23	61.33
→3)-β-D-Gal*p*NAc **(C)**	4.771	4.098	3.936	4.143	3.698	3.793/3793
[161; 9.0]	103.52	52.85	<u>80.98</u>	69.33	75.90	62.27
N-Ac	—	2.054				
	176.08	22.76				
→4)-β-D-Gal*p* **(D)**	4.519	3.568	3.691	3.989	3.727	3.852/3.809
[162; 8.0]	106.11	71.76	73.24	<u>78.15</u>	76.33	61.26