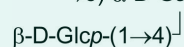
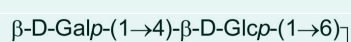


Structures of cell-wall glycopolymers of *Lactococcus lactis* BIM B-1024

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Abstract

Glycopolymers of two types were isolated from the cell wall of *Lactococcus lactis* BIM B-1024 by stepwise extraction with cold and hot 10% CCl₃CO₂H and separated by anion-exchange gel chromatography. The following structures of the glycopolymers were established by sugar analysis, dephosphorylation with 48% HF, 1D and 2D NMR spectroscopy, and ESI-MS:



Polysaccharides with the same or similar structures to PSI have been found earlier in various *Lactobacillus* species, whereas, to our knowledge, the structure of PSII is new.

Introduction

Lactic acid bacteria possesses probiotic properties and are beneficial to human health including the following activities: production of antimicrobial substances (bacteriocins, organic acids) and adherence to gut mucosa, enhancement of mucosal and host immunity; prevention of diarrhea, colon cancer, hypercholesterolemia and general improvement of the gastrointestinal microflora (1-2). The *Lactococcus* genus includes 5 species relating to a group of mesophilic lactic acid bacteria. *Lactococcus lactis* is the only-begotten one which has industrial importance. Lactococci are gram-positive, non-motile, catalase-negative, microaerophilic, non-spore forming bacteria and are able to excreting extracellular polysaccharide substances (3-4). Green plants are considered as the natural habitat for *L. lactis*, but frequently these microorganisms are also isolated from milk and dairy products. Lactococci and other lactic acid bacteria are currently used in biotechnology for production of a wide variety of fermented foods, especially meat and vegetables. *L. lactis* strains are also used in manufacturing of fermented dairy products, such as cream, butter, cheese (5-6). The problem of bacteriophage infection in factories is therefore important because of great economic damage (7).

The probiotic properties of different lactic acid bacteria are closely related to their biologically active glycoconjugates, which are structural molecules of the bacterial cell envelope. These substances are represented by polysaccharides, glycoproteins, teichoic and lipoteichoic acids, along with and in complexes with proteins (8-9). Some strains of *L. lactis* produce thermostable proteins bacteriocins with bactericidal properties

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against closely related bacteria (10). These compounds prevent the growth of pathogens that makes them promising for use as probiotics (11-12). Furthermore, *L. lactis* is able to reduce the level of cholesterol (13). Another practical application of lactococci is live vaccines (14-15). Exopolysaccharides producing by lactic acid bacteria stimulate the immunity of host (16-18).

In this study, we isolated and studied structures of exopolysaccharides from *Lactococcus lactis* 1024-BIM, which appears antibiotic susceptible according to the European Food Safety Authority (EFSA) requirements and is perspective for application as probiotic and live vaccines.

Materials and Methods

Isolation of polysaccharides

A cell wall preparation was obtained by cell disintegration using a UZDN_1 ultrasonic disintegrator as described (19). Polysaccharides were extracted from the cell walls with 10 % TCA (1:10 w/v) at 4 °C for 48 h. The mixture was centrifuged, the supernatant was dialyzed against distilled water and lyophilized to give CE. The sediment was extracted with 10 % TCA at 100 °C for 5 min, the supernatant was dialyzed against distilled water and lyophilized to give HE. Both polysaccharide preparations obtained were dissolved in pyridinium acetate buffer and applied to a column (80 × 1.6 cm) of TSK HW-40 (S) using 1% AcOH as eluent and monitoring with Knauer differential refractometer.

The polysaccharide preparations (22 mg) from cold and hot extracts were combined and fractionated by anion-exchange chromatography on a column (20 × 1 cm) of DEAE-Trisacryl M using a stepwise gradient of 0.005, 0.01, 0.1, 0.25, and 0.5 M hydrogen sodium phosphate pH 6.3. As result, two polysaccharides were obtained: PSI eluted in 0.005 M buffer (6.1 mg) and PSII eluted in 0.1 M buffer (10.4 mg).

Chemical methods

Hydrolysis was performed with 2 M CF₃CO₂H (120 °C, 2 h), the monosaccharides were analyzed by GLC as the alditol acetates on an Ultra 2 capillary column using a Hewlett–Packard 5880 instrument and a temperature gradient of 180 °C (1 min) to 290 °C at 10 °C min⁻¹. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (S)-2-octanol as described (20).

PSII was treated with 48 % HF at 4 °C for 16 h, and the products were fractionated on a TSK HW-40 column as described above.

NMR spectroscopy

NMR spectra were recorded for solutions in 99.95% D₂O after deuterium-exchange by freeze-drying from 99.9% D₂O. Spectra were measured on Bruker DRX-500 and Bruker Avance II 600 spectrometers with 5 mm broadband inverse probehead at 30 °C. Sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d₄ (δ_H 0.0, δ_C -1.6) was used as internal standard for calibration. 2D NMR

experiments were performed using standard Bruker pulse programs. The 2D TOCSY and ROESY spectra were recorded with a 60 ms duration of MLEV-17 spin-lock and a 150 ms mixing time, respectively. The gradient-selected ¹H,¹³C HMBC spectrum was recorded with a 60 ms delay for evolution of long-range spin couplings.

Mass spectrometry

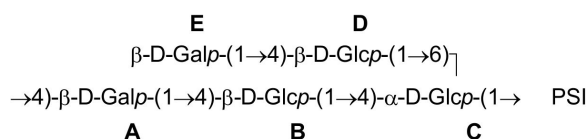
High-resolution ESI mass spectra were measured on a Bruker micrOTOF II instrument (21). The measurements were done in positive or negative ion modes (interface capillary voltage -4500 V or 3200 V, respectively); mass range from *m/z* 50 to *m/z* 3000 Da. Internal calibration was done with Electrospray Calibrant Solution (Fluka). A syringe injection was used for 1:1 acetonitrile/water solutions (flow rate 3 mL min⁻¹). Nitrogen was applied as a dry gas; interface temperature was set at 180 °C.

Results and Discussion

Polysaccharides were isolated from disintegrated cells of *L. lactis* BIM B-1024 by stepwise extraction with 10% CCl₃CO₂H first at 4 °C for 48 h and then at 100 °C for 5 min. The cold extract and hot extract were separately dialyzed, lyophilized, and purified by GPC on TSK HW-40 (S). Sugar analysis by GLC of the alditol acetates revealed similar composition of both extracts, which contained ribitol (Rib-ol), glucose, galactose, and GalNAc. The D configuration of the monosaccharides was determined by GLC of the acetylated (S)-2-octyl glycosides (22).

The NMR spectra suggested that both extracts are mixtures of polysaccharides, which were separated by anion-exchange chromatography on DEAE-Trisacryl M using a stepwise gradient of 0.005 to 0.5 M hydrogen sodium phosphate pH 6.3. As result, two polymers were obtained: a neutral polysaccharide PSI and an acidic polysaccharide PSII, which were studied by 1D and 2D NMR spectroscopy.

The ¹H and ¹³C NMR spectra of PSI showed signals for anomeric atoms at δ_H 4.46-4.92 and δ_C 101.1-104.6 of five monosaccharide residues designated units A-E. Tracing connectivities in the 2D ¹H,¹H COSY, 1D and 2D ¹H,¹H TOCSY, and ¹H,¹³C HSQC spectra enabled assignment of the ¹H and ¹³C NMR signals of PSI (Table 1). The ¹H and ¹³C NMR chemical shift data combined with data of linkage and sequence analyses by 1D NOE, 2D ROESY, and 2D ¹H,¹³C HMBC experiments (not shown) revealed the following structure of PSI:



This structure has been established earlier for an exopolysaccharide of *Lactobacillus delbrueckii* subsp *bulgaricus* 291 (23). Also, exopolysaccharides with similar structures differing in O-acetylation of one of the glucose residue or

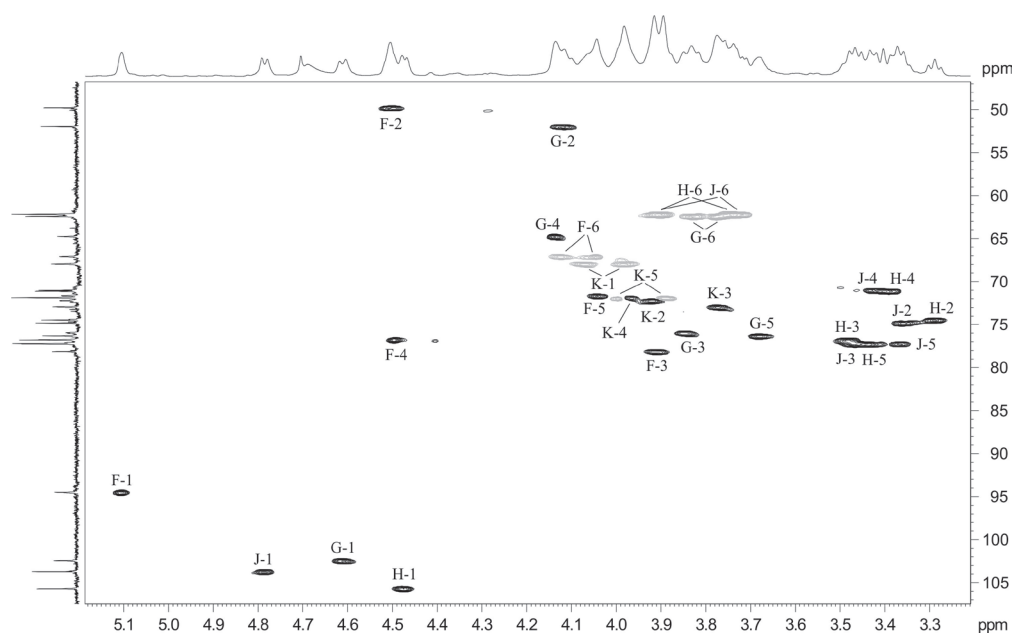


Figure 1. Parts of a 2D ^1H , ^{13}C edHSQC spectrum of PSII. The corresponding parts of the ^1H NMR spectrum are shown along the axes. Cross-peaks of transglycosidic protons are annotated in bold face. Numbers refer to protons of sugar units designated as shown in Table 1.

Table 1. ^1H and ^{13}C NMR chemical shifts (δ , ppm) of the PSI, PSII and oligosaccharide obtained after dephosphorylation of PSII (OS1)

Sugar residue		H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
PSI							
$\rightarrow 4$)- β -D-Galp-(1 \rightarrow)	(A)	4.52 104.6	3.59 72.0	3.77 73.2	4.03 78.6	3.80 76.7	3.94/3.84 61.4 ^c
$\rightarrow 4$)- β -D-Glcp-(1 \rightarrow)	(B)	4.63 103.4	3.37 74.2	3.68 75.5	3.68 79.7	3.61 76.1	3.84/3.99 61.2 ^c
$\rightarrow 4,6$)- α -D-Glcp-(1 \rightarrow)	(C)	4.92 101.1	3.61 72.8	3.86 72.5	3.80 79.1	4.40 70.8	4.16/4.00 68.4
$\rightarrow 4$)- β -D-Glcp-(1	(D)	4.55 103.5	3.38 74.0	3.68 75.6	3.68 79.7	3.61 76.0	3.84/3.99 61.3 ^c
β -D-Galp-(1	(E)	4.46 104.2	3.56 72.2	3.67 73.8	3.94 69.8	3.74 76.7	3.76/3.79 62.3
PSII							
$\rightarrow 3,4,6$)- α -D-GalpNAc-(1 \rightarrow)	(F)	5.11 94.4	4.50 49.9	3.91 78.2	4.49 76.8	4.04 71.7	4.05/4.12 67.1
$\rightarrow 3$)- β -D-GalpNAc-(1 \rightarrow)	(G)	4.62 102.5	4.12 52.1	3.84 76.0	4.13 64.7	3.68 76.3	3.77/3.82 62.4
β -D-Glcp-(1 \rightarrow)	(H)	4.47 105.7	3.29 74.5	3.48 76.9	3.39 71.1	3.42 77.3	3.73/3.90 62.1
β -D-Glcp-(1 \rightarrow)	(J)	4.78 103.8	3.36 74.9	3.48 77.2	3.42 71.1	3.37 77.3	3.75/3.90 62.1
$\rightarrow 5$)-Rib-ol-1-P-(O \rightarrow)	(K)	3.98/4.06 68.0	3.94 72.3	3.77 73.0	3.92 72.2	3.89/4.00 72.0	
Oligosaccharide 1							
$\rightarrow 3,4$)- α -D-GalpNAc-(1 \rightarrow)		5.09 95.1	4.50 49.8	3.93 78.0	4.49 76.7	4.08 71.7	3.79 62.0
$\rightarrow 3$)- β -D-GalpNAc-(1 \rightarrow)		4.59 102.5	4.37 49.5	3.82 76.3	4.13 64.9	3.66 76.4	3.84 61.8
β -D-Glcp-(1 \rightarrow)		4.49 105.8	3.32 74.3	3.49 76.8	3.40 71.1	3.44 77.1	3.73/3.92 62.1
β -D-Glcp-(1 \rightarrow)		4.83 103.3	3.37 74.7	3.50 77.2	3.43 71.1	3.41 77.0	3.73/3.92 62.1
$\rightarrow 5$)-Rib-ol		3.89/3.99 71.9	3.89 72.1	3.72 73.2	3.81 73.2	3.89/4.00 72.0	

containing an additional galactose residue have been found in *Lactobacillus lactis* subsp. *cremoris* B891 (24) and *Lactobacillus helveticus* K16 (25), respectively.

The ¹H NMR spectrum of PSII showed signals for four anomeric protons at δ 4.47-5.11, other sugar ring protons at δ 3.29-4.50, and two *N*-acetyl group at δ 2.02-2.09. The ¹³C NMR spectrum of PSII contained signals for four anomeric carbons at δ 94.4-105.7, a number of OCH₂ groups (C-6 of hexoses and GalNAc, C-1 and C-5 of Rib-ol) at δ 62.1-72.0, nitrogen-bearing carbons at δ 49.8-51.9, other sugar ring and ribitol carbons at δ 64.7-78.1, and two *N*-acetyl groups δ 23.4-23.8 (CH₃) and 176.0 (CO). The ³¹P NMR spectrum of PSII showed signal for a phosphate group at δ 1.0.

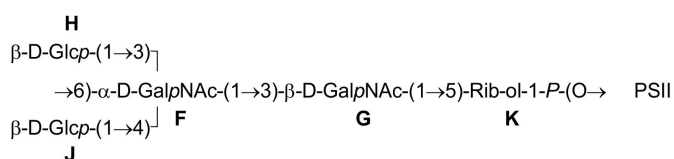
The NMR spectra of PSII were assigned using ¹H, ¹H COSY, TOCSY, ROESY, H-detected ¹H, ¹³C HSQC, HSQC-TOCSY, and ¹H, ³¹P HMBC experiments (Table 1), and it was found that PSII consists of repeating units containing two glucose and two GalNAc residues and ribitol designated units **F-K**. The a-linkage of GalpNAc (**F**) and b-linkages of GalpNAc (**G**) and both Glcp (**H** and **J**) were established by characteristic H-1 and C-5 chemical shifts (Table 1) and the presence of H-1/H-2 cross-peaks for unit **F** and H-1/H-5 cross-peaks for units **G-J** in the ROESY spectrum (Table 2).

The C-1-C-5 chemical shifts of Glcp (**H**) and Glcp (**J**) (Table 1) were similar to those of non-substituted b-Glcp (22) and, hence, both glucose residues occupy the terminal positions in side chains. Downfield displacements were observed for the signals of C-3, C-4 and C-6 of GalpNAc (**F**), C-3 of GalpNAc (**G**), C-1 and C-5 of ribitol (**K**), as compared with their positions in the corresponding non-substituted compounds (22). These shifts defined the positions of substitution of the sugars and ribitol in the repeating unit.

The sequence of the monosaccharides was determined by

correlations between the anomeric protons and protons at the linkage carbons or the linkage carbons revealed by the ROESY and HMBC experiments, respectively (Table 2). The ¹H, ³¹P HMBC spectrum showed correlations between a phosphate group and H-1 of ribitol (**K**) at δ 1.0/3.98, 4.06 and H-6 of GalNAc (**G**) at δ 1.0/4.05 and 1.0/4.12. Phosphorylation of ribitol (**K**) at O-1 was confirmed by a low-field position of the signal for C-1 at δ 68.0 as compared with its position at δ 63.8 in non-substituted ribitol (26).

Therefore, the repeating unit of PSII is a branched tetrasaccharide-ribitol phosphate having the following structure:



In addition to the major signals tabulated in Table 1, the NMR spectra showed a number of minor signals, which were not assigned owing to their low intensities. These signals may belong to monosaccharides from terminal oligosaccharide units of PSII.

Treatment of PSII with 48% HF afforded dephosphorylated oligosaccharides, which were fractionated by GPC on TSK HW-40 (S) and studied by 1D and 2D NMR spectroscopy (for assigned ¹H and ¹³C NMR chemical shifts see Table 2) and high-resolution ESI MS. Two major compounds were identified as tetrasaccharide-ribitol (**1**) (Hex₂HexNAc₂Rib-ol,

(M+Na)⁺ ion peak at *m/z* 905.3201, calculated *m/z* 905.3221) and tetrasaccharide (**2**) (Hex₂HexNAc₂, (M+Na)⁺ ion peak at *m/z* 771.2617, calculated *m/z* 771.2642). Oligosaccharide **2** resulted evidently from overhydrolysis of a glycosidic linkage during dephosphorylation. Structures of compounds **1** and **2**

Table 2. Correlations for anomeric proton in the 2D ROESY and ¹H, ¹³C HMBC spectra of PSII

H-1 in sugar residue (d)	Correlations to atoms in sugar residue (d)	
	ROESY	HMBC
F H-1 (5.11) C-1 (94.4)	F H-2 (4.50), G H-4 (4.13), H-3 (3.84)	G C-3 (76.0), F C-5 (71.7), C-3 (78.2) G H-3 (3.84), F H-5 (4.04), H-4 (4.49), H-1 (5.11)
G H-1 (4.62) C-1 (102.5)		K C-5 (72.0) K H-5 (3.89/4.00), G H-1 (4.62), H-2 (4.12), H-4 (4.13), H-5 (3.68)
H H-1 (4.47) C-1 (105.7)	H H-3 (3.48), H-5 (3.42), H-2 (3.29), H-4 (3.39), F H-3 (3.91)	F C-3 (78.2) F H-3 (3.91), H H-1 (4.47), H-3 (3.48), H-5 (3.42), H-2 (3.29)
J H-1 (4.78) C-1 (103.8)	F H-4 (4.49), J H-3 (3.48), H-4 (3.42), H-5 (3.37)	F C-4 (76.8), J C-2 (74.9) F H-4 (4.49), J H-1 (4.78), H-3 (3.48), H-5 (3.37)

confirmed the structure of PSII.

To the best of our knowledge, the PSII structure is unique among known bacterial polysaccharides. Earlier, a cell wall polysaccharide having a similar structure of the main chain but with only one side chain of a single galactose residue has been reported in *Lactococcus lactis* subsp. *cremoris* H414 (27).

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Conflict of interest statement

The authors declare no conflict of interest.

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