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Chemical structure of the cell wall-associated polysaccharide of *Bifidobacterium animalis* subsp. *lactis* LKM512

Yusuke Uemura · Mitsuharu Matsumoto

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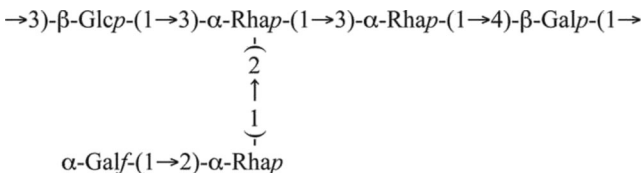
Abstract We have demonstrated that *Bifidobacterium animalis* subsp. *lactis* LKM512 had some probiotic properties *in vivo* and *in vitro*. To further understand their mechanisms, the chemical structure of the extracellular polysaccharide that constructs the cell envelope was determined. The strain was anaerobically cultured in MRS broth at 37 °C for 20 h, then the bacterial cells were harvested by centrifugation and washed. The cell wall-associated polysaccharide (CPS) was prepared from the cell wall component digested by lysozyme. The results of anion exchange and gel filtration chromatography showed that the polysaccharide was negatively charged and had a high molecular mass. The CPS was found to compose of galactopyranosyl, galactofuranosyl, glucopyranosyl and rhamnopyranosyl residues in the molar ratio of 1:1:1:3 by using methylation analysis with GC-MS and HPLC profiling. From the results of the structural characterization by 1 dimensional and 2 dimensional NMR spectroscopy, the polysaccharide was established to be a hexasaccharide repeating unit with the following structure:



Keywords Probiotics · *Bifidobacterium animalis* subsp. *lactis* · Polysaccharide · Methylation analysis · NMR spectroscopy

Y. Uemura (✉) · M. Matsumoto
Dairy Science and Technology Institute, Kyodo Milk Industry Co. Ltd., 20-1 Hirai, Hinode-machi, Tokyo 190-0182, Japan
e-mail: YusukeUEMURA999@gmail.com

M. Matsumoto
e-mail: m-matumoto@meito.co.jp

Introduction

Bifidobacteria is a part of the commensal species present in intestinal microbiota. They are generally utilized as a probiotics in food products to improve the gastrointestinal environment. The beneficial effect is expected to improve host resistance to pathological conditions such as irritable bowel syndrome [1], ulcerative colitis [2], diarrhea [3] and atopic dermatitis [4]. In the relationship between probiotics and intestinal environments, it is important to understand what biomolecules directly affect host gastrointestinal tract (GIT) and the other bacteria. One of the key regulators of probiotics-host and/or probiotics-other bacteria interactions are thought to be bacterial cell surface molecules.

Bacterial extracellular polysaccharides such as lipopolysaccharide (LPS), peptidoglycan and teichoic acid are ubiquitous components of the bacterial cell surface. These are immunologically important ligands capable of interacting with the host pattern recognition receptors (PRR) present in the gastrointestinal mucosa [5]. On the other hand, previous reports have shown that the probiotics, bifidobacteria and lactobacilli, can produce extracellular polysaccharide including a cell wall-associated (capsular) polysaccharide (CPS) and an excreted exopolysaccharide (EPS). These chemical structures show species- and strain-specific features [6, 7] and their detailed function in the host gut is unclear. In Gram-positive bacteria, CPS is present in the outer layer of the bacterial cell and can have direct contact with the host intestinal epithelial cells. Elucidation of the chemical structure of CPS leads to a better understanding of its probiotic effects.

The probiotic strain *Bifidobacterium animalis* subsp. *lactis* LKM512 (hereafter referred to as LKM512) exhibits the ability to adhere to intestinal mucin [8] and potent acid tolerance [9], and proliferate in the colon and alter intestinal bacterial composition [10]. Thus, we hypothesize that CPS is related to the colonization of LKM512 in the colon. In clinical trials, the consumption of yogurt containing LKM512 has been shown to result in decreased levels of acute inflammation markers in hospitalized elderly patients [11] and improved symptoms of adult patients with atopic dermatitis

[4]. Recently, we demonstrated that oral administration of this probiotic strain promoted longevity in mice [12]. These beneficial effects might be caused by CPS.

In this study, we analyzed the CPS on the surface of the LKM512 cell envelope and characterized its chemical structure by using methylation analysis and NMR.

Materials and methods

Bacterial growth conditions and purification of the polysaccharides

LKM512 was anaerobically cultured in MRS broth (Merck, USA) at 37 °C for 20 h. At a stationary phase (OD_{600}=0.6 to 1.0), the pellet containing bacterial cells was collected by centrifugation (5,000 rpm, 30 min, 4 °C) and washed with PBS (pH 7.2) three times. The pellet was treated with 20 μg/mL lysozyme (Wako, Japan) in 10 mM $NaHPO_4$ (pH 6.7) at 37 °C for 48 h to degrade the peptidoglycan. The suspension was centrifuged to separate the protoplast (Gram stained negatively) and the supernatant containing crude CPS.

The CPS was precipitated from the supernatant by the addition of two volumes of EtOH followed by an overnight incubation at 4 °C. After centrifugation, the polysaccharide precipitant was suspended in distilled water and lyophilized. These procedures were repeated twice. The lyophilized sample was treated with 50 μg/mL proteinase K (Sigma-Aldrich, USA) in 12.5 mM Tris–HCl buffer (pH 8.0) at 37 °C for 12 h. After inactivation of the enzyme by heating at 95 °C, the polysaccharide was precipitated again as described above. The precipitant resolved in distilled water was dialyzed in a membrane (6,000 to 8,000 Da cut off) for 2 days with two water changes daily, and then lyophilized. The dialyzed sample was lyophilized and stored at −20 °C until use.

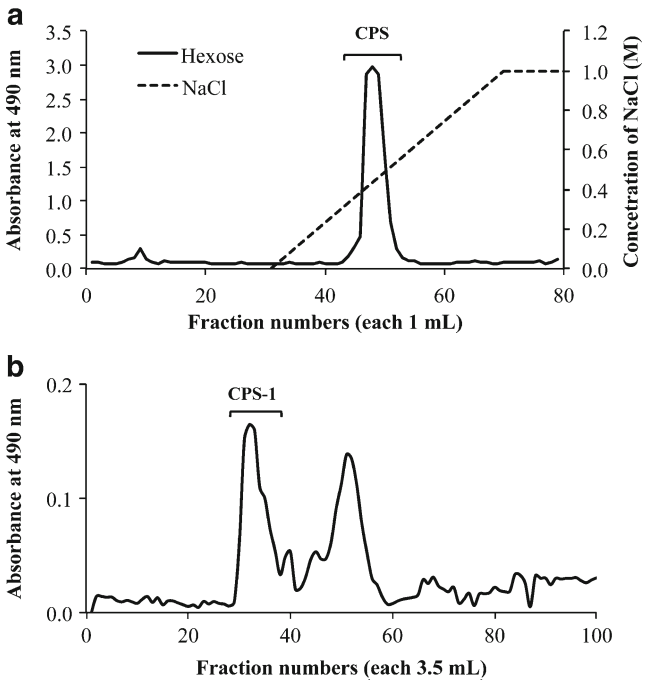


Fig. 1 Chromatographic separation of CPS from *B. animalis* subsp. *lactis* LKM512 on an anion exchange (a) and gel filtration (b) column. Each fraction was monitored using the phenol-H_2SO_4 method (490 nm)

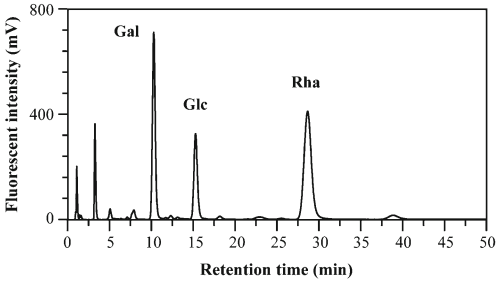


Fig. 2 HPLC profile of ABEE-labeled CPS-1 on a Honenpak C18 column. Each peak was monitored using a fluorescence detector (Ex.305 nm, Em.360 nm)

Further purification of the crude polysaccharide was performed using an anion exchange column HiTrap Q HP (5 mL; GE Healthcare, UK) equilibrated with 25 mM Tris–HCl buffer (pH 8.0). The adsorbed component was eluted by a linear gradient with 0 to 1 M NaCl at a flow rate of 5 mL/min and monitored by using the phenol-sulfuric acid method [13]. The adsorbed fraction was dialyzed and further purified on a gel filtration column HiPrep Sephacryl S-300 HR (1.6×60 cm; GE healthcare, UK) equilibrated with 50 mM $(NH_4)_2CO_3$ solution at a flow rate of 1.5 mL/min. The obtained peak was fractionated and lyophilized.

Quantification of phosphate content

The phosphate content of the purified polysaccharide was quantified using a PiBlue Phosphate Assay Kit (BioAssay Systems, USA). A standard curve was calculated with a phosphate standard. The absorbance of the reaction mixture was measured at 630 nm on a GENios plate reader (TECAN, Japan).

Compositional analysis of the monosaccharide

Monosaccharide composition of purified CPS was determined by using a 4-aminobenzoic acid ethyl ester (ABEE) labeling

Table 1 Methylation analysis of CPS-1

Retention time (min)	PMAA	Structural feature
8.2	3,2-Me_2-Rha	→2)-α-Rha*p*-(1→
8.6	2,4-Me_2-Rha	→3)-α-Rha*p*-(1→
8.8	2,3,5,6-Me_4-Gal	α-Gal*f*-(1→
10.1	4-Me-Rha	→2,3)-α-Rha*p*-(1→
11.3	2,3,6-Me_3-Gal	→4)-β-Gal*p*-(1→
11.7	2,3,6-Me_3-Glc	→3)-β-G1c*p*-(1→

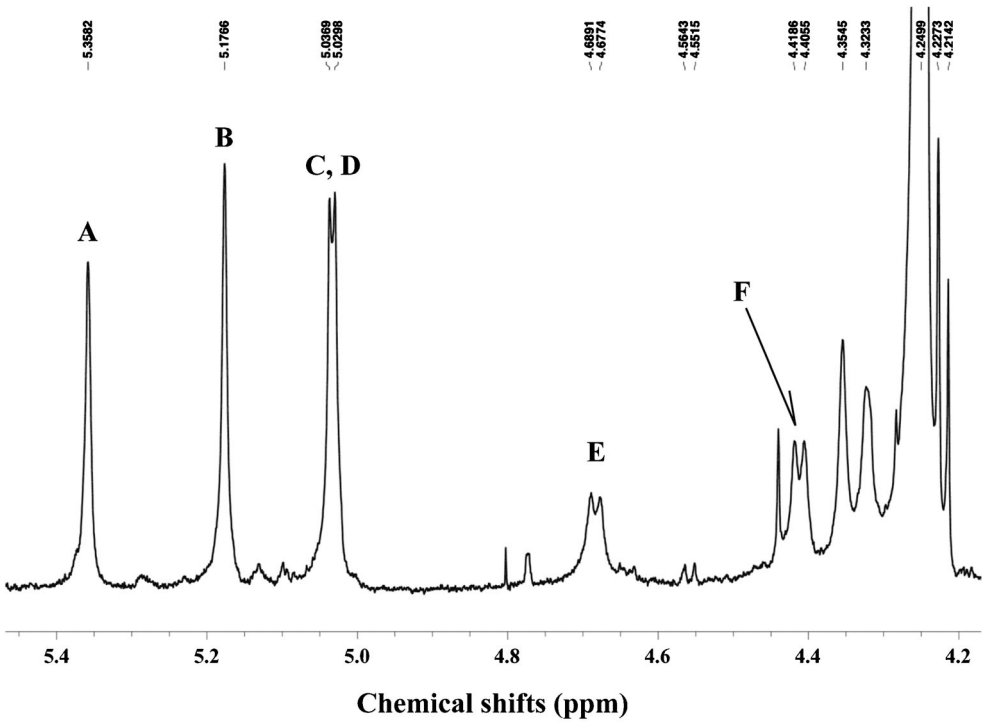


Fig. 3 600 MHz ^{1}H-NMR spectrum of the anomeric region of CPS-1. The spectrum was obtained in D_2O at 600 MHz with a Bruker AvanceIII spectrometer operated at a probe temperature of 70 °C

kit (J-Oilmils, Japan). After the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) (100 °C, 3 h) and acetylated with pyridine, the resulting monosaccharides were incubated with ABEE reagent (80 °C, 1 h). ABEE-labeled monosaccharides were extracted with water/chloroform (1/1, v/v), and the upper aqueous layer was analyzed on a Honenpak C18 column (75 mm×4.6 mm I.D.; J-Oil mils, Japan) on a Waters Alliance 2,695 HPLC system with a 474 scanning fluorescence detector (Ex:305 nm, Em:360 nm). Elution was done with 0.2 M borate-potassium buffer (pH 8.9)/acetonitrile (93/7, v/v). The retention time of the peaks was compared with nine ABEE-standards consisting of galacturonic acid (GalA) (retention time=7.6 min), galactose (Gal) (10.2 min), mannose (Man) (12.9 min), glucose (Glc) (15.3 min), ribose (Rib) (17.9 min), *N*-acetylglucosamine (GlcNAc) (22.7 min), fucose (Fuc)

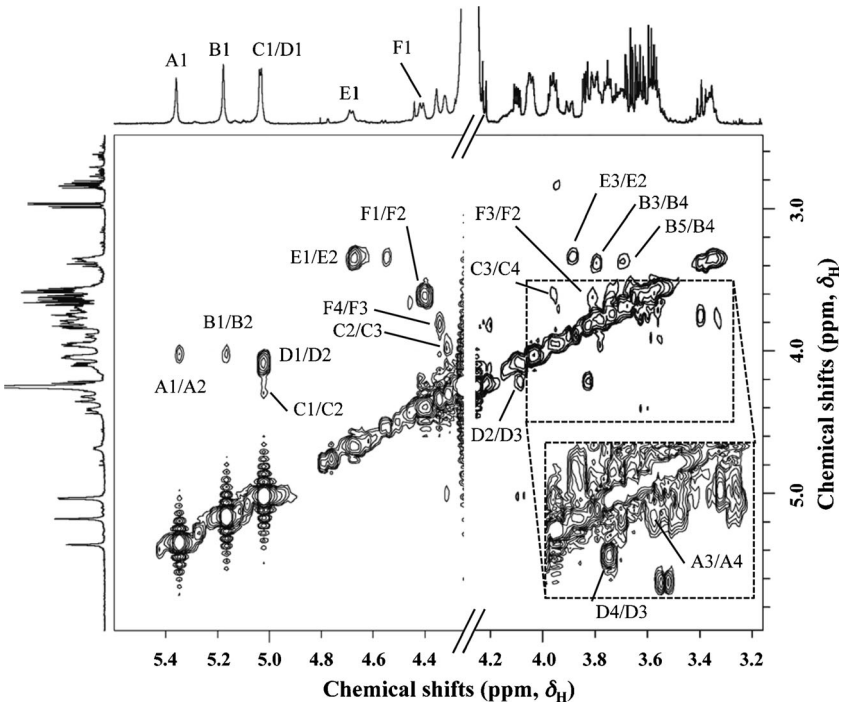


Fig. 4 600 MHz ^{1}H-^{1}H COSY spectrum of the anomeric and ring-proton region of CPS-1. The partial spectrum in the dot-line square shows weak signals

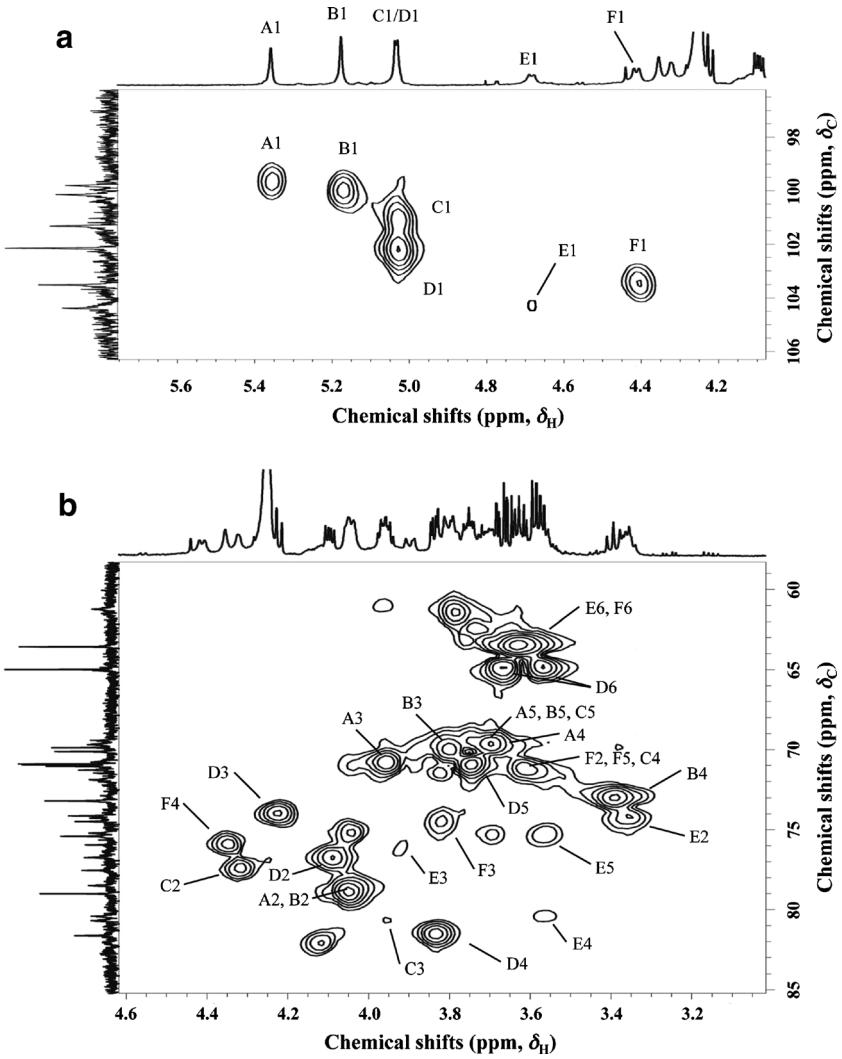


Fig. 5 600 MHz ^{1}H-^{13}C HSQC spectra of the anomeric (a) and the ring-proton region (b) of CPS-1. The appropriate part of the 1D ^{1}H-NMR spectrum is shown at the top of each spot. Correlated ^{13}C signals are indicated next to the relevant contours

(24.8 min), rhamonose (Rha) (28.4 min), and *N*-acetylgalactosamine (GalNAc) (39.0 min).

Methylation analysis

The partially methylated alditol acetate (PMAA) was prepared using a previously reported method [14] with minor modifications. Methylation was done with methyl iodide in NaOH-DMSO solution. Hydrolysis of permethylated polysaccharide was performed with 2 M TFA (100 °C, 3 h). The partially methylated monosaccharides were reduced with $NaBH_4$ and acetylated with pyridine, acetic anhydride and 4-*N*,*N*′-dimethylaminopyridine (DMAP). The PMAA was analyzed by GC-MS on Shimadzu GC-2010 and GCMS-QP2010

Table 2 ^{1}H- and ^{13}C-NMR chemical shifts of CPS-1

Residue	Chemical shifts of reporter groups (ppm, δ)												
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	C-1	C-2	C-3	C-4	C-5	C-6
A→3)-α-Rha*p*(1→	5.36	4.05	3.96	3.65	3.71	1.23	-	99.80	78.97	70.87	70.10	69.86	17.31
B→2)-α-Rha*p*(1→	5.18	4.04	3.80	3.39	3.71	1.25	-	100.14	78.97	70.10	73.16	69.66	17.12
C→2,3)-α-Rha*p*(1→	5.04	4.32	3.96	3.59	3.73	1.27	-	101.32	77.52	80.66	71.02	69.40	17.31
D α-Gal*f*-(1→	5.03	4.10	4.22	3.84	3.75	3.57	3.68	102.14	76.72	74.10	81.60	70.92	64.96
E→3)-β-Glc*p*(1→	4.68	3.36	3.87	3.56	3.56	3.59	3.66	104.38	74.25	76.70	80.37	75.37	63.54
F→4)-β-Gal*p*-(1→	4.41	3.62	3.81	4.35	3.62	3.59	3.66	103.50	70.92	74.45	75.92	71.02	63.54

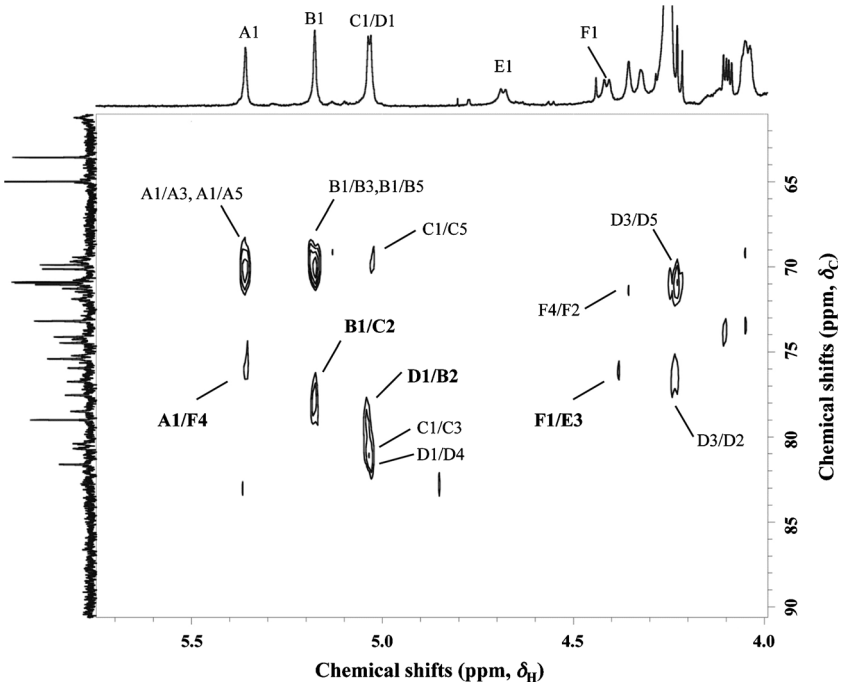


Fig. 6 600 MHz ^{1}H-^{13}C HMBC spectrum of the anomeric region of CPS-1. The inter-residue couplings, A1/F4, B1/C2, D1/B2 and F1/E3, are expressed in bold

equipped with a capillary column ZB-WAX (30 m× 0.25 mm×1.00 μm) (Phenomenex, USA). The temperature program of the instrument was 100 °C for 1 min, from 100 °C to 250 °C at 40 °C/min and 250 °C for 15 min. The GC peaks and MS fragments of PMAA were identified by previous study [15].

NMR studies of the polysaccharides

All spectra of the polysaccharide were recorded on a Bruker AvanceIII 600 spectrometer using a 5 mm probe. The sample was deuterium-exchanged by lyophilization from 99.9 % D_2O and measured with 1-dimensional (^{1}H and ^{13}C) and 2-dimensional (^{1}H-^{1}H correlated spectroscopy [COSY], ^{1}H-^{13}C heteronuclear single quantum coherence [HSQC], ^{1}H-^{13}C heteronuclear multiple quantum coherence [HMBC], and ^{1}H-^{1}H total correlation spectroscopy [TOCSY]) experiments at 70 °C (343 K) by reference to an internal acetone standard (δ_H=2.225). Data was then analyzed using TopSpin software.

Results and discussion

Purification, phosphate content and compositional analysis

The CPS of LKM512 was mostly adsorbed on a HiTrap Q HP column (Fig. 1a). CPS-1 was obtained from the subsequent gel filtration chromatography of the adsorbed fractions (Fig. 1b). The molecular mass of CPS-1 was calculated to be approximately 4×10^5 from the elution volume (112 mL) of the CPS-1 peak. A monitor at 280 nm showed that the fraction did not contain protein or peptide contaminants (data not shown). The fraction contained phosphate at a concentration of 6.3 nmol/mg. This value was lower than those of other bifidobacterial polysaccharides (28~336 nmol/mg) [16]. Cell wall components (e.g., polysaccharide and teichoic acid) of Gram-positive bacteria commonly contain phosphorus as muramic acid 6-phosphate of peptidoglycan through a phosphodiester linkage [17, 18]. For determination of monosaccharide composition, ABEE labeled components from the polysaccharides were compared with nine monosaccharide standards. CPS-1 contained Gal, Glc and Rha residues at a molar ratio of 1.9:1.0:2.9 calculated from the peak areas (Fig. 2). This CPS-1 result was very similar to that of the EPS from *B.animalis* subsp. *lactis* IPLA-R1 (strain IPLA-R1) [19].

Structural characterization of CPS-1 by methylation analysis and NMR

Methylation analysis of CPS-1 by GC-MS showed the presence of six and seven monosaccharides, respectively (Table 1). The GC peaks from CPS-1 were identical to PMAAs derived

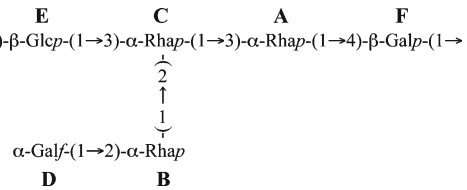


Fig. 7 A hexasaccharide repeating unit from *B. animalis* subsp. *lactis* LKM512 CPS-1

from 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol, 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-galactitol, 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-rhamnitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol.

The assignments of ^{1}H and ^{13}C chemical shifts of CPS-1 were determined by cross-checking ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC data with a previous report [19]. The anomeric region of ^{1}H-NMR in CPS-1 indicated six signals (δ 4.41–5.36) designated as **A** to **F** (Fig. 3). The integration values of **A**, **B**, **C**/**D**, **E** and **F** were 1.0, 1.2, 1.8, 0.7, and 1.1, respectively. Since the $J_{H\text{-}1,H\text{-}2}$ coupling constants of **E** (δ 4.68) and **F** (δ 4.41) were 7.0 and 7.9 Hz, respectively, these residues were supposed to have β-configuration [20]. In addition, the HSQC spectrum showed ^{1}H-^{13}C couplings of their H-1 signals in the anomeric region. These results are consistent with the fact that CPS-1 consisted of six types of monosaccharides. The aggregated characteristic doublets (integration value=9.3) at δ 1.23, 1.25, and 1.27 were consistent with three H-6 signals of 6-deoxy-6-methyl-hexose, which were supposed to be Rha residues from the results of the HPLC and GC-MS profiles. From this information and the composition of the anomeric protons in the ^{1}H-NMR spectrum, it was concluded that the CPS-1 repeating unit comprises one Glc, two Gal, and three Rha residues.

In the COSY spectrum, **A** H-1 (δ 5.36), **B** H-1 (δ 5.18), **C** H-1 (δ 5.04), **D** H-1 (δ 5.03), **E** H-1 (δ 4.68), and **F** H-1 (δ 4.41) showed cross-peaks with **A** H-2 (δ 4.05), **B** H-2 (δ 4.04), **C** H-2 (δ 4.32), **D** H-2 (δ 4.10), **E** H-2 (δ 3.36) and **F** H-2 (δ 3.62), respectively (Fig. 4). Furthermore, there were intra-residue cross-peaks in the COSY (Fig. 4) and the TOCSY (data not shown) spectra that assigned each signal from **A** (H-2/H-6, H-3/H-4, H-3/H-6, H-5/H-6), **B** (H-2/H-6, H-3/H-4, H-3/H-6, H-4/H-5, H-4/H-6, H-5/H-6), **C** (H-2/H-3, H-3/H-4, H-3/H-6, H-5/H-6), **D** (H-1/H-3, H-1/H-4, H-2/H-3, H-2/H-5, H-3/H-4, H-5/H-6), **E** (H-2/H-3, H-2/H-4), and **F** (H-1/H-3, H-2/H-3, H-3/H-4). As the H-4 resonance for Gal (δ 4.30 – 3.85) is substantially shifted to a lower field than that of a Glc (δ 3.75 – 3.45) [21], residues **E** and **F** were identified as Glc and Gal, respectively. Although there are very weak signals for **A** and **B** H-2/H-3 in the COSY spectrum, the H-3 signals (**A** δ 3.96 and **B** δ 3.80) were assigned by the presence of cross-peaks with **A** H-6 (δ 1.23) and **B** H-4 (δ 3.39), respectively. From these data, the HSQC correlation spectrum and comparison with the EPS from the IPLA R-1 strain, all ^{1}H and ^{13}C signals of CPS-1 were assigned (Fig. 5a and b, Table 2). As shown in the HMBC spectrum (Fig. 6), the cross-peaks, **A**(1→4)**F**, **B**(1→2)**C**, **D**(1→2)**B**, and **F**(1→3)**E**, were indicated as inter-residue linkages. The strong cross peak of **D** H-1/H-4 in the HMBC spectrum implied that residue **D is** the terminal galactofuranoside. Based on the information described above, the chemical structure of the repeating unit of CPS-1 could be predicted by the sequence: (→3)**E**(β1→3)[**D**(α1→2)**B**(α1→2)]**C**(α1→3)**A**(α1→4)**F**(β1→). Because the signals in the 1D/2D-NMR spectra of CPS-1 were very similar with those of the EPS from strain IPLA-R1, it was concluded that the repeating unit structure of CPS-1 is likely to be the sequence. Nonetheless, since the signals from **A** H-3 (δ 3.96), **A** H-4 (δ 3.65), **E** H-3 (δ 3.87), and **E** C-3 (δ 76.70) shifted at a lower magnetic field than those of the EPS of strain IPLA-R1, both chemical structures were partially different in the substitutions, i.e., Rha(α1→3)Rha and Gal(β1→3)Glc in CPS-1. However, the characteristic HSQC signal (δ_H 3.7 δ_C 75.5) in Fig. 5 is likely to be identical with residue E3 observed in IPLA-R1. In conclusion, it is evident that the CPS-1 of LKM512 is a polysaccharide mixture of the repeat structures found in Fig. 7 and in EPS of IPLA-R1.

The structural differences of extracellular polysaccharides produced by a probiotic species were previously reported in the cases of *B. breve* [16] and *Lactobacillus casei* [22]. The polysaccharide produced by *B. animalis* subsp. *lactis* was found to have high Rha residue content and the presence of terminal Gal furanoside compared with those of other bifidobacterial species, such as *B. breve* [16], *B. longum* [23] and *B. bifidum* [24]. Among bacterial strains, genetic features may affect phenotypic characters such as the biosynthesis of extracellular polysaccharides. Although the unique structure of CPS-1 may contribute to its ability to regulate biological and physiological functionalities in the host GIT, it will be necessary to perform further research using in vitro and in vivo assays to reveal these mechanisms.

Finally, the above results led to the conclusion that the novel structure of the CPS produced by LKM512 contains a hexasaccharide repeating unit, as shown in Fig. 7.

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