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Structural characterization of the extracellular polysaccharide produced by *Bifidobacterium longum* JBL05

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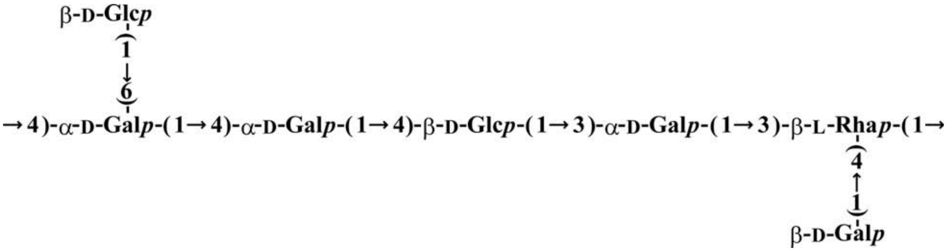
ABSTRACT

The extracellular polysaccharide produced by *Bifidobacterium longum* JBL05 was found to be composed of D-galactopyranosyl, D-glucopyranosyl and L-rhamnopyranosyl residues in the molar ratio of 4:2:1. Methylation analysis coupled with GC–MS, and both 1D and 2D NMR spectroscopy showed that the polysaccharide has a heptasaccharide repeating unit with the following structure:



$$\rightarrow 4)\text{-}\overset{\begin{array}{c}\beta\text{-D-Glc}p\\ 1\\ \downarrow\\ 6\end{array}}{\alpha\text{-D-Gal}p}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\underset{\begin{array}{c}4\\ \uparrow\\ 1\\ \beta\text{-D-Gal}p\end{array}}{\beta\text{-L-Rha}p}\text{-}(1\rightarrow$$

1. Introduction

Bifidobacteria and lactic acid bacteria (LAB), which are common in the human intestine, are used as probiotics to alter the intestinal microflora and to treat diarrhea, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Santosa, Farnworth, & Jones, 2006). These bacteria have anti-tumor (Commane, Hughes, Shortt, & Rowland, 2005), immunostimulatory (Liong, 2007) and anti-allergenic (Isolauri, Salminen, & Ouwehand, 2004; Ouwehand, 2007) properties. Extracellular polysaccharides (EPSs) produced by these microorganisms are well known for the important roles they play in these biological functions (Chabot et al., 2001; Kitazawa et al., 1998; Vinderola, Perdigon, Duarte, Farnworth, & Matar, 2006).

There are many published reports on the structural characteristics, fermentation, biosynthesis and functional properties of LAB EPSs, but only a few on Bifidobacteria EPSs (Ricciardi & Clementi, 2000; Ruas-Madiedo, Hugenholtz, & Zoon, 2002). Some EPSs produced by *Bifidobacterium longum* have specific functions, such as adhesion to the inner surfaces of the intestine (Abbad Andaloussi, Talbaoui, Marczak, & Bonaly, 1995), promotion of colonization (Roberts et al., 1995) and strong anti-mutagenesis effects (Sreekumar & Hosono, 1998). The specific function of a particular EPS is probably determined by its chemical structure. Therefore, characterization of the chemical structures of these polysaccharides is important to understanding both the properties of EPSs, and the microorganism that produces EPSs, Bifidobacteria.

Herein, we report on the structure of the EPS produced by *B. longum* JBL05.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Bifidobacterium longum JBL05 was isolated from the human intestine. It was identified as Bifidobacteria based on the taxonomic characteristics of the phenotype. This microorganism was

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deposited with the National Institute of Technology and Evaluation (NITE), and the accession number was NITE BP-82. The EPS was produced by culturing the microorganism in enzymatically degraded skim milk containing 3% cultivator (Yaizu Suisankagaku Industry Co., Ltd.), 2.5% lactose and 0.2% sodium ascorbate at 37 °C for 40 h under anaerobic conditions.

2.2. Isolation and purification

Microorganisms were removed from the culture medium by centrifugation of the slurry resulting from dilution with Milli-Q water (MQ) at 13,700*g* for 10 min. Twenty percent (v/v) EtOH was added to the cell-free medium with incubation overnight at 4 °C. After removal of the precipitates by centrifugation at 13,700*g* for 30 min, the EPS in the supernatant was precipitated by the addition of EtOH at 60% (v/v). The precipitated EPS was collected by centrifugation at 13,700*g* for 20 min, redissolved in MQ and then freeze–dried. These procedures were repeated three times.

The freeze–dried sample was redissolved in MQ and applied to an anion-exchange column (DEAE–Sephadex A-50, ID 6 × 25 cm, Amersham Biosciences) under four elution conditions: isocratic (H_2O), and, a step-wise NaCl gradient, 0–0.07, 0.07–0.14 and 0.14–0.34 M NaCl. For each elution condition, the injected samples were divided into 30–100 fractions using a fraction collector and the carbohydrate concentration was determined by the phenol–sulfuric acid method (Hodge & Hofreiter, 1962). A fraction containing EPS eluted with 0.07–0.34 M NaCl was concentrated and applied to a gel-filtration chromatography column (ID 5 × 67 cm) packed with TOYOPEARL HW65S (Tosoh). MQ was used as the eluent. The EPS fraction was freeze–dried and used as a native EPS sample.

The sonicated EPS was prepared using an ultrasonic homogenizer (US-300T, Nihonseiki) before being applied to the DEAE column.

2.3. Preparation of pyruvate-free EPS and partially acid-hydrolyzed EPS

The sonicated pyruvate-free EPS was prepared by heating the sonicated EPS (3–5 mg/ml in 1 mM oxalic acid, 0.1 M sodium chloride, pH 3.0) for 2 h at 95 °C, as described by Holzwarth and Ogletree (1979). Subsequently, the solution was neutralized with NaOH, dialyzed against MQ and freeze-dried.

The partially acid-hydrolyzed EPS was prepared by hydrolysis of pyruvate-free EPS with trifluoroacetic acid (TFA) for 1 h at 80 °C. After drying, it was dissolved in MQ and EtOH was added. The solution was stored at 4 °C overnight and, subsequently, the resulting precipitate was collected and freeze-dried.

2.4. Molecular weight determination

The weight-average molecular weight of these EPSs was estimated using gel permeation chromatography (GPC) in conjunction with DAWN-DSP multi-angle laser-light scattering (MALLS) (Wyatt Technology). The samples (100 µl, 0.2 wt%) were injected into the SB-806 M HQ column (ID 8 × 300 mm, Shodex) and eluted with 0.1 N sodium nitrate at a flow rate of 1.0 ml/min. Samples were filtered (pore size 0.45 µm) prior to injection. The refractive index increment, d*n*/d*c*, was assumed to be 0.145. Data were collected and processed using Astra (version 5.3.4.11) software (Wyatt Technology). Number-average molecular weight (M_n) and weight-average molecular weight (M_w) were directly calculated according to the definition of M_n and M_w using molecular weight and RI signal values at each elution volume.

2.5. Sugar composition analysis

The polysaccharide samples were hydrolyzed with 90% formic acid (100 °C, 6 h), and then with 2 M TFA (120 °C, 6 h). The sugar composition was analyzed by HPLC using an ION-300 column (ID 7.8 × 300 mm, Tokyo Kasei Kogyo).

The sugars were converted to alditol acetates by reduction, followed by treatment with acetic anhydride in an equal volume of pyridine (120 °C, 2 h), and then analyzed using GC–MS with an electron impact ionization detector and a DB225 column (ID 0.25 mm × 30 m, J&W Scientific).

2.6. Methylation analysis

The partially acid-hydrolyzed EPS was methylated twice using the method of Ciucanu and Kerek (1984). The EPS (3 mg) was dried at 60 °C under a vacuum overnight, and then was dispersed in 0.3 ml DMSO. After bubbling with N_2 and stirring until dissolved, 30 mg of solid sodium hydroxide and 0.15 ml of methyl iodide were added, and the solution was heated at 110 °C for 2 h. The reaction was stopped by the addition of MQ, and the permethylated polysaccharide was extracted with chloroform. The permethylated polysaccharide was then hydrolyzed and converted to partially methylated alditol acetates (PMAAs) using the procedure described in the sugar composition analysis section. The PMAAs were analyzed using GC–MS, as described above for the sugar composition analysis. The PMAAs were identified by comparison with the mass spectra reported by Jansson, Kenne, Liedgren, Lindberg, and Lonngren (1976).

2.7. Pyruvic acid and phosphoric acid analyses

The pyruvic acid content of the polysaccharide samples was determined as reported previously (Duckworth & Yaphe, 1970; Hadjivassiliou & Rieder, 1968). Five milligrams of EPS was hydrolyzed at 100 °C for 4 h with 3 ml of 0.04 N oxalic acid to cleave the pyruvic acid. After cooling, the reaction solution was neutralized with calcium carbonate and centrifuged. Phosphate buffer (0.5 ml of 0.2 M, pH 7.0) was added to the collected supernatant, and MQ was added to make a final volume of 10 ml. One milliliter of 1 M triethanolamine was added to 2 ml of this solution in a square quartz cuvette. Then, 50 µl of a 1.0% (w/w) solution of NADH (prepared with 1.0% (w/w) $NaHCO_3$) was added, and the absorbance at 340 nm was measured. Fifteen microliters of lactate dehydrogenase was added and the absorbance was measured in the same way. The pyruvic acid content was estimated from the decrease in NADH absorbance. The amount of phosphorus was determined using the method of Fiske and Subbarow (1925).

2.8. NMR spectroscopy

NMR spectra were recorded with a JEOL 500 MHz spectrometer (JNM-A500) at a probe temperature of 80 °C. All exchangeable H of the samples were replaced by D before obtaining the spectra in deuterium oxide. Chemical shifts were calibrated using coaxial NMR tubes with (3-trimethylsilyl)-propane sulfonic acid sodium salt (DSS, $\delta_H = 0.00$, $\delta_C = 0.00$) in the inner tube.

The 2D ^1H–^1H correlated spectroscopy (COSY), ^1H–^1H total correlation spectroscopy (TOCSY), ^1H–^{13}C heteronuclear single quantum coherence (HSQC) and ^1H–^{13}C heteronuclear multiple quantum coherence (HMBC) measurements were used to assign signals and to determine the sequence of sugar residues.

3. Results and discussion

3.1. Molecular weight and composition analyses

The yield of EPS obtained from the batch culture was approximately 1.3 g/L. Fig. 1 represents a GPC curve for the native EPS together with the plot of molecular mass vs elution volume. Table 1 shows the M_w and polydispersity index (M_w/M_n) determined by GPC–MALLS. It is clear that M_w decreases by sonication and partial acid hydrolysis.

The native EPS was composed of D-glucose, D-galactose and L-rhamnose in a molar ratio of 2:4:1 according to the results of the sugar composition analysis using HPLC and GC. This sugar composition was not changed by sonication, depyruvation and hydrolysis.

The molar ratio of pyruvate to the seven sugar repeating unit for the native EPS was 0.74. Phosphoric acid, which is part of the composition of some EPSs (Nakajima, Hirota, Toba, Itoh, & Adachi, 1992), was not detected.

3.2. Methylation analysis

Methylation analysis for the partially acid-hydrolyzed EPS showed the presence of seven sugar residues. These were identified as alditol acetates derived from 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl D-glucose; 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,6-tri- and 2,3-di-*O*-methyl-D-galactose; 2-mono-*O*-methyl-L-rhamnose (Table 2). The number of different sugar residues was consistent with the results of the sugar composition analysis by HPLC and GC.

3.3. NMR spectroscopy

As shown in Fig. 2a, the 1D ^{1}H spectrum of the sonicated EPS was complicated and much more difficult to analyze than the spectra of both pyruvate-free EPS (Fig. 2b) and partially acid-hydrolyzed EPS (Fig. 2c), which had sharper and well-separated peaks. The NMR spectrum for the pyruvate-free EPS is very similar to that of the partially acid-hydrolyzed EPS. Actually, the partially acid-hydrolyzed EPS was found to have no pyruvate residue.

The complete assignment of the ^{1}H and ^{13}C chemical shifts of the partially acid-hydrolyzed EPS (Table 3) was carried out by means of 2D ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC experiments.

The anomeric region (δ 4.2–5.5) of the 1D ^{1}H NMR spectrum of the partially acid-hydrolyzed EPS (Fig. 2c) contained a total of nine peaks. Seven peaks were ascribed to anomeric protons (H-1) of sugar residues arbitrarily labeled **A** to **G**. The other two peaks were identified as H-5 in residue **C** and H-2 in residue **F** (abbreviated **C** H-5, **F** H-2 in the following). The 1D ^{13}C NMR and HSQC spectra showed seven signals in the anomeric region ranging from δ 92 to δ 110. These results are consistent with the fact that the EPS consisted of seven types of monosaccharides.

Table 1
The weight-average molecular weight determined using GPC–MALLS.

Samples	M_w	Polydispersity index (M_w/M_n)
Native EPS	5.4×10^5	1.6
Sonicated EPS	1.5×10^5	1.3
Pyruvate-free EPS	6.3×10^4	1.4
Partially acid-hydrolyzed EPS	1.3×10^4	1.3

Table 2
Methylation analysis of the pyruvate-free EPS.

Partially methylated alditol acetate	t_R[a] (min)	Structural feature	Molar ratio
2,3,4,6-Me$_4$-Glc	1.00	β-D-Glc*p*-(1→	1.00
2,3,4,6-Me$_4$-Gal	1.07	β-D-Gal*p*-(1→	0.99
2-Me-Rha	1.17	→3,4)-β-L-Rha*p*-(1→	0.96
2,4,6-Me$_3$-Gal	1.35	→3)-α-D-Gal*p*-(1→	1.01
2,3,6-Me$_3$-Glc	1.39	→4)-β-D-Glc*p*-(1→	1.15
2,3,6-Me$_3$-Gal	1.42	→4)-α-D-Gal*p*-(1→	1.06
2,3-Me$_2$-Gal	1.95	→4,6)-α-D-Gal*p*-(1→	1.10

[a] Relative retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl D-glucitol.

Based on their chemical shift and the value of the coupling constant for anomeric signals in the ^{1}H NMR spectrum, residues **A**, **B** and **C** were determined to have α-configurations (the values of $J_{1,2}$ were 3–4 Hz), and residue **G** was determined to have β-configuration (the values of $J_{1,2}$ were 7.9 Hz) (Faber, Kamerling, & Vliegenthart, 2001; van Casteren, Dijkema, Schols, Beldman, & Voragen, 2000). Residues **D** and **E** were determined to have β-configurations based on both the chemical shift and $^1J_{C\text{-}1,H\text{-}1}$ value, which was approximately 160 Hz (Faber, Zoon, Kamerling, & Vliegenthart, 1998; Faber et al., 2001). Furthermore, residue **F** was also determined to have a β-configuration based on the $^1J_{C\text{-}1,H\text{-}1}$ value (166 Hz) of the overlapping **C** C-1 and **F** C-1 peaks (Gorshkova, Kalmykova, Isakov, & Ovodov, 1986). In addition, the chemical shift in **F** H-5 at δ 3.54 also indicates the β-configuration (Jansson, Lindberg, Wimalasiri, & Dankert, 1993; van Casteren et al., 2000).

In the TOCSY spectrum, **A** H-1 (δ 5.41) and **B** H-1 (δ 5.26) showed crosspeaks with **A** H-2, -3, -4 and **B** H-2, -3, -4, -5 (Fig. 4). The other protons **A** H-5, -6 and **B** H-6 were assigned in series from H-1 of **A** and **B** using COSY (Fig. 3). **C** H-1 (δ 4.98)

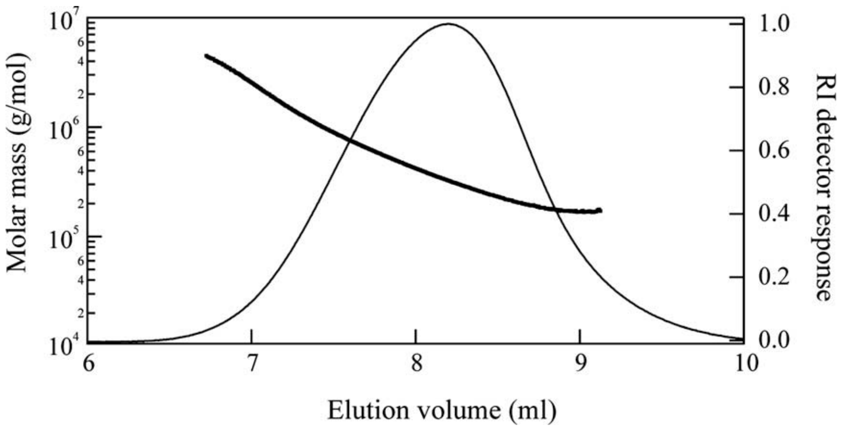


Fig. 1. The chromatogram and plot of molecular mass vs elution volume obtained by GPC–MALLS experiments for the native EPS.

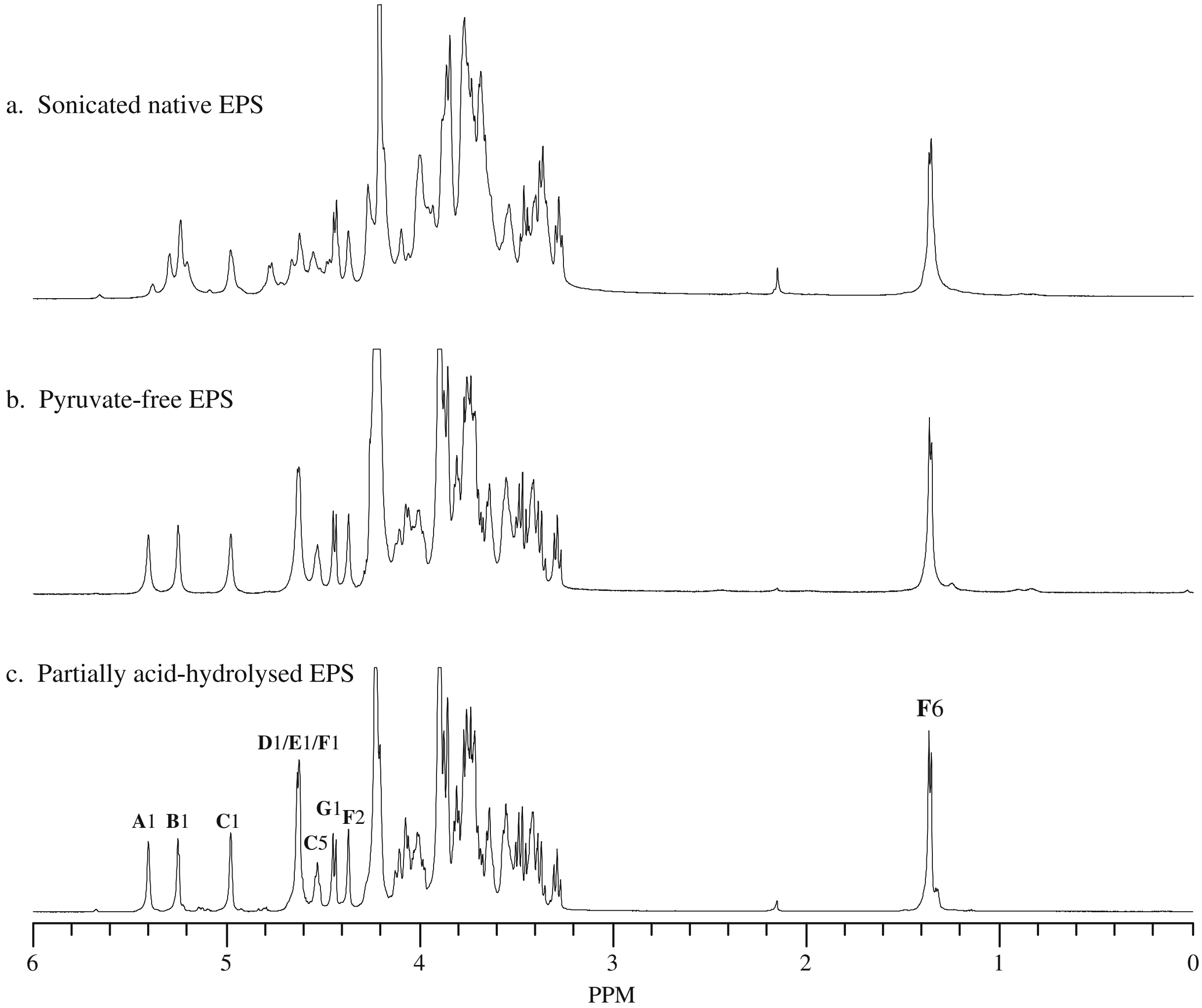


Fig. 2. Comparison of 1D ^{1}H NMR spectra of sonicated native EPS (a), pyruvate-free EPS (b), and partially acid-hydrolyzed EPS (c) produced by *B. longum* JBL05 recorded at 500 MHz in D_2O at 80 °C. **A**1 corresponds to the peak of **A** H-1, etc.

and **C** H-5 (δ 4.54) showed crosspeaks with **C** H-2, -3, -4 and **C** H-6a, -6b in the TOCSY spectrum. The correlation peak between **C** H-4 and **C** H-5 was observed in the COSY spectrum. Then the crosspeaks of (1) **D** H-1 (δ 4.64) and **D** H-2, -3, -4, (2) **E** H-1 (δ 4.64) and **E** H-2, -3, -4, -5 were assigned in the TOCSY spectrum. **G** H-1 (δ 4.45) showed crosspeaks with H-2, -3, -4, -5, -6a and -6b of residue **G** in the TOCSY spectrum.

Table 3
Chemical shifts (ppm) of ^{1}H and ^{13}C NMR signals for the partially acid-hydrolyzed EPS, recorded in D_2O at 80 °C. Coupling constants (Hz) are included in parentheses.

Residue	H-l	H-2	H-3	H-4	H-5	H-6
A →4)-α-D-Gal*p*-(1→	5.41 (3.7)	3.90	3.90	4.08	4.02	3.83, 3.83
B →3)-α-D-Gal*p*-(1→	5.26 (3.7)	4.05	4.12	4.21	4.21	3.72, 3.72
C →4,6)-α-D-Gal*p*-(1→	4.98 (<4.0)	3.86	3.86	4.23	4.54	3.76, 3.99
D β-D-Gal*p*-(1→	4.64	3.50	3.56	3.91	3.64	3.73, 3.78
E →4)-β-D-Gal*p*-(1→	4.64	3.42	3.76	3.64	3.56	3.76, 3.89
F →3,4)-β-L-Rha*p*-(1→	4.64	4.38	3.91	3.91	3.54	1.37
G β-D-Glc*p*-(1→	4.45 (7.9)	3.30	3.48	3.38	3.43	3.69, 3.89
	C-1	C-2	C-3	C-4	C-5	C-6
A →4)-α-D-Gal*p*-(1→	102.6	71.7	72.1	81.5	74.7	63.2
B →3)-α-D-Gal*p*-(1→	95.6	69.9	82.5	71.9	73.5	63.8
C →4,6)-α-D-Gal*p*-(1→	103.1	71.3	72.4	82.1	72.4	70.4
D β-D-Gal*p*-(1→	105.6	73.9	77.6	71.5	77.6	63.7
E →4)-β-D-Glc*p*-(1→	106.4	76.0	78.7	79.8	76.3	63.6
F →3,4)-β-L-Rha*p*-(1→	103.1	68.2	77.4	78.4	74.1	19.8
G β-D-Glc*p*-(1→	105.1	76.0	78.6	72.6	78.7	64.0

In addition, the crosspeaks between **F** H-5 and **F** H-6 of methyl group (δ 1.37, $J_{5,6}$ 6.1 Hz) in the COSY spectrum showed that residue **F** was assigned to L-rhamnose.

In the HMBC spectrum (Fig. 5), some crosspeaks were observed between H-1 and a ^{13}C peak in different residues: **A** H-1 and **E** C-4 (δ 79.8), **B** H-1 and **F** C-3 (δ 77.4), **C** H-1 and **A** C-4 (δ 81.5), **G** H-1 and **C** C-6 (δ 70.4) were linked.

These HMBC data revealed the existence of two sequences in the structure: (1) **G**(1 → 6)**C**(1 → 4)**A**(1 → 4)**E**(1 → and (2) **B**(1 → 3)**F**. Additionally, the crosspeaks between **B** H-3 and **E** C-1 (δ 106.4), were observed in the HMBC (data not shown).

Then, the group of overlapping anomeric proton peaks, H-1 in **D**, **E** and **F**, was correlated with four distinct carbon crosspeaks, C-2, -3, -4 in **F** and C-4 in **C**. Based on the results of methylation analysis, COSY and HMBC, residue **F** had a 1,3,4-linkage, and one glycosidic linkage from **F** C-3 to **B** C-1. Additionally, the crosspeak between **F** C-2 and the overlapping proton peaks, H-1 of **D**, **E** and **F**, was assigned to the intra-residue crosspeak in **F** between **F** C-2 and **F** H-1 because none of the residues had a glycosidic linkage at the C-2 position, based on methylation analysis. The remaining crosspeaks between anomeric protons, H-1 of **D**, **E** and **F** and **F** C-4 and/or **C** C-4 probably result from a glycosidic linkage. It is possible that

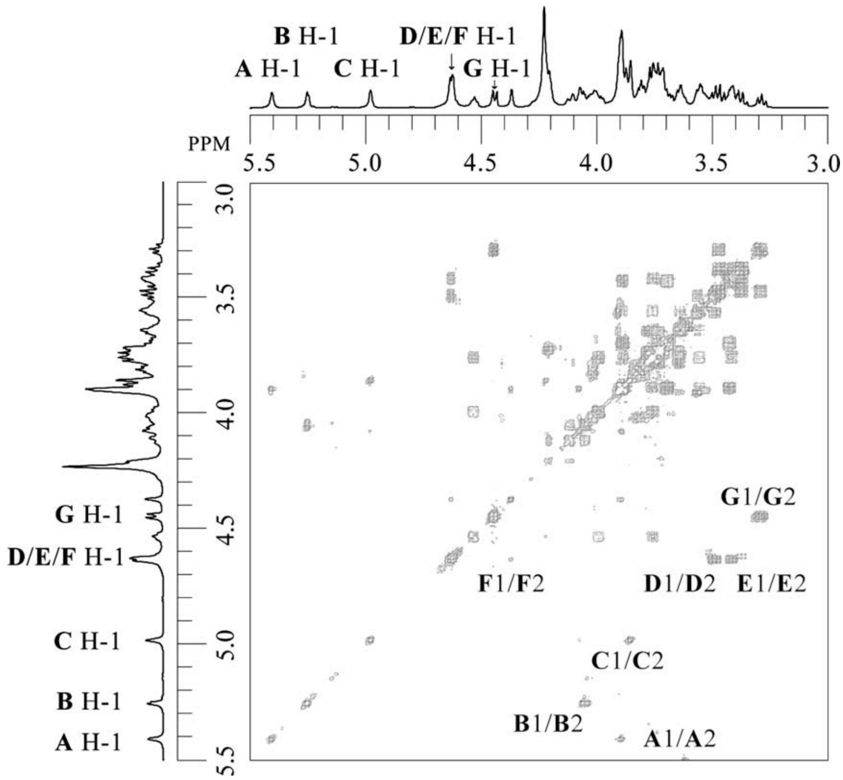


Fig. 3. 2D $^1H–^1H$ COSY spectrum of the partially acid-hydrolyzed EPS produced by *B. longum* JBL05, recorded in D_2O at 80 °C. **A**1/**A**2 corresponds to the crosspeak of A H-1 and **A** H-2, etc. A CH_3 signal was observed at δ 1.37, but is not shown in this figure.

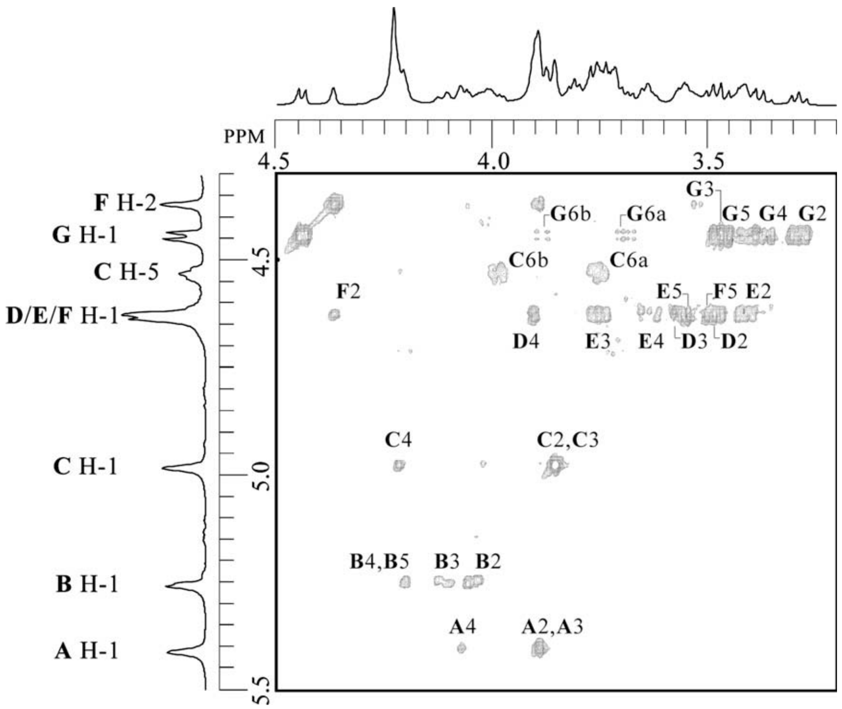


Fig. 4. 2D $^1H–^1H$ TOCSY spectrum of the partially acid-hydrolyzed EPS produced by *B. longum* JBL05, recorded in D_2O at 80 °C. **A**2 corresponds to the crosspeak of **A** H-1 and **A** H-2, etc.

residues **F** C-4 and/or **C** C-4 are connected to C-1 of **D**, **E** and/or **F** by a glycosidic linkage.

The crosspeaks between **C** H-4 and the overlapping peaks of **C** C-1 and **F** C-1 indicate that residues **C** and **F** are connected by a 1,4-linkage. Furthermore, the crosspeaks in HMBC observed between (1) **C** H-6 and **G** C-1, (2) **E** H-4 and **A** C-1 and (3) **A** H-4 and the overlapping peak of **C** C-1 and **F** C-1 were consistent with the above results. Finally, the correlation peaks found in the HMBC spectrum between the overlapping peaks of (1) **D** H-4, **F** H-4 and **F** H-3 and (2) **D** C-1 indicate the presence of a **F**(4 → 1)**D** linkage.

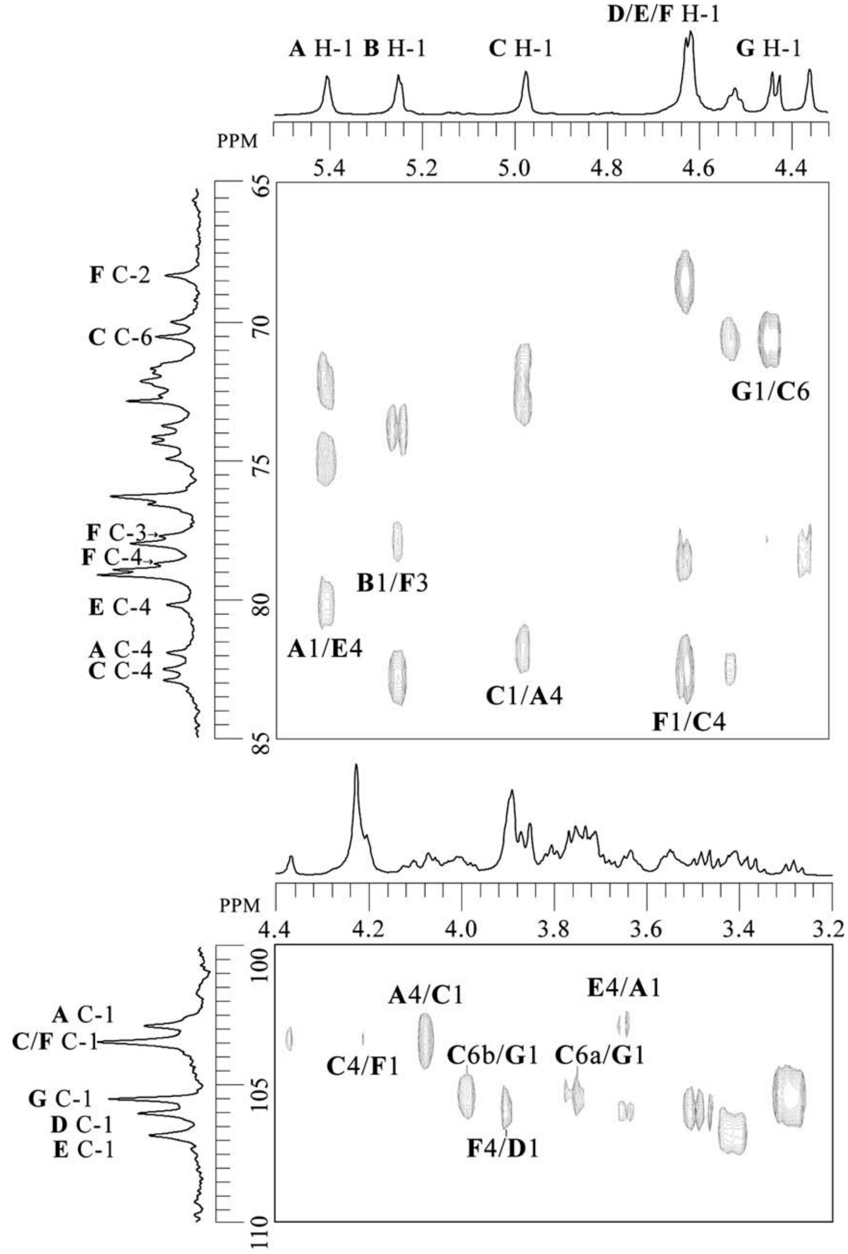
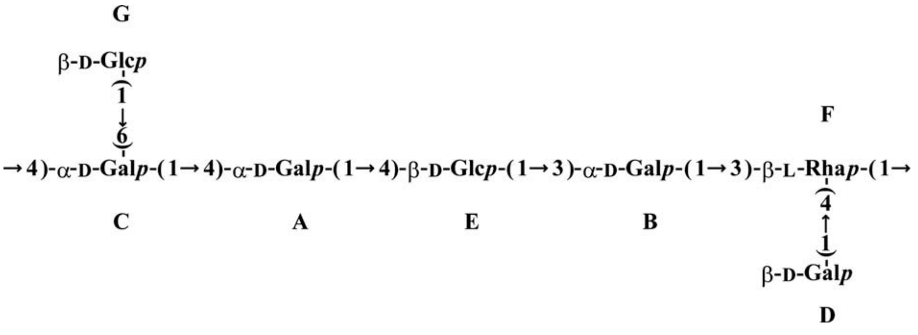


Fig. 5. 2D ^{1}H–^{13}C HMBC spectra of the partially acid-hydrolyzed EPS produced by *B. longum* JBL05, recorded in D_2O at 80 °C. **A**1/**E**4 represents the crosspeak of **A** H-1 and **E** C-4 that corresponds to glycosidic linkage.

Residues **A** and **E** were identified as 1,4-D-galactose and 1,4-D-glucose, respectively, based on the glycosidic linkage **C**(1 → 4)**A** and **A**(1 → 4)**E**, and chemical shifts, in particular by the values of H-4 (Faber et al., 2001; Harding et al., 2005; Maeda, Zhu, Suzuki, Suzuki, & Kitamura, 2004; Nakajima et al., 1992; van Casteren et al., 2000). The side-chain residues, β-D-glucose and β-D-galactose, were identified as residues **G** and **D**, respectively, by the chemical shifts that were obtained using the same procedure used for



$$\overset{\begin{array}{c}\mathbf{G}\\ \beta\text{-D-Glc}p\\ 1\\ \downarrow\\ 6\end{array}}{\underset{\mathbf{C}}{\rightarrow 4)\text{-}\alpha\text{-D-Gal}p}}\text{-}(1\rightarrow 4)\text{-}\underset{\mathbf{A}}{\alpha\text{-D-Gal}p}\text{-}(1\rightarrow 4)\text{-}\underset{\mathbf{E}}{\beta\text{-D-Glc}p}\text{-}(1\rightarrow 3)\text{-}\underset{\mathbf{B}}{\alpha\text{-D-Gal}p}\text{-}(1\rightarrow 3)\text{-}\overset{\mathbf{F}}{\underset{\begin{array}{c}4\\ \uparrow\\ 1\\ \beta\text{-D-Gal}p\\ \mathbf{D}\end{array}}{\beta\text{-L-Rha}p}}\text{-}(1\rightarrow$$

Fig. 6. Repeating unit of the EPS produced by *B. longum* JBL05.

residues **A** and **E** (Faber et al., 1998; Harding et al., 2005; Maeda et al., 2004; van Casteren et al., 2000). Finally, residues **B** and **C** were identified as 1,3-D-galactose and 1,4,6-D-galactose based on the results of HMBC.

According to previous studies of other EPSs, pyruvic acid is linked with α-D-galactose or β-D-mannose residues at positions C-4 and -6 of the side chain, or with the β-D-galactose residue at positions C-3 and -4 of the side chain (Edebrink, Jansson, & Widmalm, 1993; Rinaudo, Milas, Lambert, & Vincendon, 1983; Van Calsteren, Pau-Roblot, Begin, & Roy, 2002). The links may be similar to those of other EPSs that also contain pyruvic acid (Garegg et al., 1980), but should be a subject for further study.

We are now studying the immunological activity of this polysaccharide and have found immune system modulating activity in Peyer's patches. In addition, it can be used as a food texture modifier and we are further studying its solution properties.

In summary, we determined the chemical structure of the EPS produced by *B. longum* JBL05, and found that it contains heptasaccharide repeating units, as shown in Fig. 6.

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