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Note

Structure of a viscous exopolysaccharide produced by *Lactobacillus helveticus* K16

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Abstract

A viscous extracellular polysaccharide produced by *Lactobacillus helveticus* K16 has been investigated. Sugar and methylation analysis, ^{1}H and ^{13}C NMR spectroscopy revealed that the polysaccharide is composed of a hexasaccharide repeating unit. The sequence of sugar residues was determined by use of two-dimensional nuclear Overhauser effect spectroscopy and heteronuclear multiple bond connectivity experiments. The structure of the repeating unit of the exopolysaccharide from *L. helveticus* K16 is as follows:

β-D-Gal*p*
1
↓
4
β-D-Glc*p*-(1→2)-β-D-Glc*p*
1
↓
6
→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→

Keywords: *Lactobacillus helveticus*; Exopolysaccharide; Viscous; NMR

Lactic acid bacteria synthesize polysaccharides as cell wall components and storage polymers, and in many species, as extracellular macromolecules [1]. The production of the exopolysaccharides (EPS) by the *Lactobacillus helveticus* species has been reported, and the primary molecular structures of several EPS have been determined. The EPS produced by *L. helveticus* 776 [2] and TN-4 [3] are composed of hexasaccharide repeating units of D-galactose and D-glucose in molar ratios 1:2 and 1:1, respectively. However, the repeating unit of the EPS of the strain TY1-2 is a heptamer, containing *N*-acetyl-D-glucosamine in addition to D-galactose and D-glucose [4]. The EPS of strain Lh59 [5] has an identical structure to the one of strain TN-4.

We have shown previously that the EPS produced by *L. helveticus* Äki4 [6] and Lb161 [7] consist of hexa- and heptasaccharide repeating units, the main chain being branched

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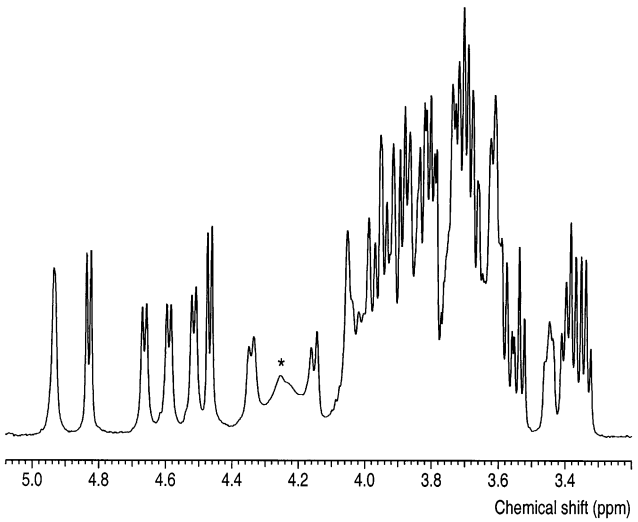


Fig. 1. The ^{1}H NMR spectrum at 600 MHz of the exopolysaccharide produced by *L. helveticus* K16. HOD resonance is marked by an asterisk.

with one and two side chains, respectively. The EPS of the former was not viscous, but the latter was viscous [8]. Since the rheological properties of polysaccharides are closely related to their primary molecular structures, it is of interest to study the relation between structures and functionalities of the EPS of the lactic acid bacterial strains. In this study, we report the structure of an EPS produced by *L. helveticus* K16, which was more viscous than that of strain Lb161.

L. helveticus K16 was grown in skim milk, and the EPS was isolated as a 50% ethanol precipitate from the culture medium. A hydrolysate of the EPS contained glucose and galactose in the ratio 2:1. The absolute configuration analysis showed that the sugars had the D-configuration. Methylation analysis of the polysaccharide revealed, in comparison to authentic standards, 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, 3,6-di-*O*-methylglucose, 2,3-di-*O*-methylglucose, 2,3,4,6 - tetra - *O* - methylgalactose and 2,3,6-tri-*O*-methylgalactose in the ratio 11:12:11:10:14:12. From the ^{1}H NMR spectrum (Fig. 1) it was possible to identify six resonances in the region for anomeric protons at δ 4.93, 4.83, 4.66, 4.59, 4.52 and 4.47. The ^{1}H detected ^{13}C decoupled $^{1}H,^{13}C$ HSQC spectrum contained, inter alia, six resonances in the region for anomeric signals. The above sugar and methylation analyses together with NMR spectra show that the polysaccharide is composed of a hexasaccharide repeating unit. From the methylation analysis and NMR spectra, it is evident that the sugars are pyranoid.

The chemical shifts for the protons and carbons together with coupling constants for anomeric protons and carbons are given in Table 1. The assignments of the spin system for each sugar residue were performed using homo- and heteronuclear two-dimensional techniques. The chemical shifts of the H-2 to H-6 signals for residues **A** and **B** could be

Table 1
Chemical shift (ppm) of the ^{1}H and ^{13}C NMR signals of the exopolysaccharide produced by *L. helveticus* K16

Sugar residue	^{1}H–^{13}C					
	1	2	3	4	5	6
→4,6)-α-D-Glc*p*-(1→	4.93 (3.7) [a]	3.61	3.88	3.82	4.34	4.02, 4.15
A	100.7 [171] [b]	72.4	72.1	80.2	70.5	68.2
β-D-Glc*p*-(1→	4.83 (7.9)	3.33	3.53	3.36	3.44	3.73, 3.92
B	103.7 [164]	74.9	76.8	70.9	77.1	62.1
→2,4)-β-D-Glc*p*-(1→	4.66 (7.8)	3.69	3.89	3.71	3.60	3.82, 3.97
C	101.9 [163]	80.7	75.8	79.3	75.5	61.1
→4)-β-D-Glc*p*-(1→	4.59 (7.9)	3.39	3.67	3.69	3.64	~3.80
D	103.5 [164]	73.8	75.2	79.6	75.7	~61.8
→4)-β-D-Gal*p*-(1→	4.52 (7.7)	3.60	3.74	4.05	3.79	3.85, 3.92
E	104.3 [163]	71.8	73.1	78.2	76.2	61.1
β-D-Gal*p*-(1→	4.47 (7.8)	3.57	3.66	3.95	3.72	~3.80
F	103.8 [163]	71.8	73.6	69.5	76.2	~61.8

[a] $J_{H\text{-}1,H\text{-}2}$ values are given in Hz in parenthesis.
[b] $J_{C\text{-}1,H\text{-}1}$ values are given in Hz in brackets.

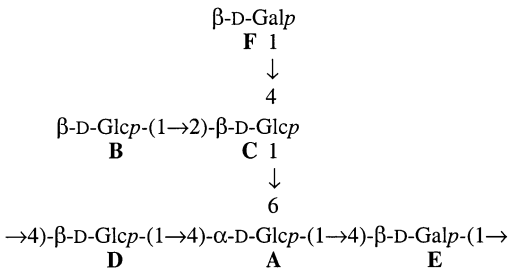
Table 2
Interglycosidic correlations from the anomeric protons observed in $^1H,^1H$ NOESY and $^1H,^{13}C$ HMBC spectra of the exopolysaccharide produced by *L. helveticus* K16

Sugar residue		Anomeric proton	NOE to	HMBC to	Connectivity
		δ_H or δ_C			
→4,6)-α-D-Glc*p*-(1→	**A**	4.93	4.05		**A**, H-1 to **E**, H-4
			3.85/3.92		**A**, H-1 to **E**, H-6
				78.2	**A**, H-1 to **E**, C-4
β-D-Glc*p*-(1→	**B**	4.83	3.69		**B**, H-1 to **C**, H-2
				80.7	**B**, H-1 to **C**, C-2
→2,4)-β-D-Glc*p*-(1→	**C**	4.66	4.02/4.15		**C**, H-1 to **A**, H-6
				68.2	**C**, H-1 to **A**, C-6
→4)-β-D-Glc*p*-(1→	**D**	4.59	3.82		**D**, H-1 to **A**, H-4
				80.2	**D**, H-1 to **A**, C-4
→4)-β-D-Gal*p*-(1→	**E**	4.52		79.6	**E**, H-1 to **D**, C-4
β-D-Gal*p*-(1→	**F**	4.47		79.3	**F**, H-1 to **C**, C-4

assigned using a homonuclear COSY and $^1H,^1H$ TOCSY experiments. The H-2, H-3 and H-4 of residues **C**–**F** were also assigned with those experiments. Intra-residue NOE correlations gave the chemical shifts of H-5 for residues **C**–**F** and the H-6 signals for residue **E**. Using $^1H,^1H$ TOCSY experiments, the H-6 resonances for residues **C** and **D** were assigned. From the $^1H,^{13}C$ HSQC spectrum the assignments of the carbon chemical shifts corresponding to the protons for residue **A**–**F** could be performed. Further assignments to corroborate these results were deduced from intra-residue correlations employing an HSQC–TOCSY experiment. In particular, the partial spectral overlap of H-4/C-4 in residues **C** and **D** (Table 1) were clarified by the following correlations, namely, H-3 in **C** to its C-2, C-3 and C-4 and H-5 in **D** to its C-4 and C-5.

The identification of a spin system with a specific sugar residue and substitution pattern as determined from the methylation analysis was done as follows. For residue **A** the value of $J_{H\text{-}1,H\text{-}2}$ was 3.7 Hz showing α configuration and for residues **B**–**F** the $J_{H\text{-}1,H\text{-}2}$ values were around 8 Hz showing β configuration. In the TOCSY experiment, the magnetisation transferred only to H-4 for residues **E** and **F** due to small couplings between H-4 and H-5, and residues **E** and **F** could therefore be assigned to the Gal*p* residues. The remaining residues **A**–**D** could then be assigned to the Glc*p* residues in accordance with sugar and methylation analysis. The downfield chemical shifts for the C-4 (δ 80.2) and C-6 (δ 68.2) of residue **A** compared with α-D-Glc*p* [9], demonstrated that this residue was 4,6-disubstituted in accordance with methylation analysis. The chemical shifts of residues **B** and **F** compared with those for terminal β-D-Glc*p* and β-D-Gal*p*, respectively, show that these residues are terminal. Residue **C** was assigned to the 2,4-disubstituted glucose supported by the downfield chemical shift of the C-2 signal (δ 80.7) and the C-4 signal (δ 79.3) compared with β-D-Glc*p*. Residues **D** and **E** having downfield chemical shifts for C-4, δ 79.6 and δ 78.2, respectively, demonstrated that they were four-substituted glucose and galactose in accordance with methylation analysis.

In order to determine the sequence of the sugar residues, $^1H,^{13}C$ HMBC and $^1H,^1H$ NOESY experiments were performed. In the HMBC experiment there are, in addition to correlations within a sugar residue, inter-residue correlations from the anomeric protons as well as the anomeric carbons, over the glycosidic linkage to carbons and protons on the adjacent residue. Inter-residue HMBC and NOE correlations are compiled in Table 2. From the results, it is concluded that the neutral exopolysaccharide from *L. helveticus* K16 is composed of a hexasaccharide repeating unit with the following structure:



Structures of some viscous EPS produced by lactic acid bacterial strains have been reported previously. The viscous EPS produced by *L. sake* 0-1 consists of repeating units of glucose, rhamnose and a phosphate group [10]. Several slime-forming *Lactococcus lactis* ssp. *cremoris* strains produced EPS of similar or identical structures, containing galactose, glucose and a phophodiester group [11–13]. The neutral EPS produced by *Streptococcus thermophilus* Rs and Sts are composed of the same heptasaccharide repeating unit of galactose and rhamnose, but they differ in viscosity of their milk cultures probably due to the difference in molecular mass of the polysaccharide [14]. In this study, the difference in viscosity (η) was also noticed among the EPS produced by the strains of *L. helveticus* Äki4 ($\eta \sim 1$ mm^2/s), Lb161 ($\eta = 8.8$ mm^2/s) and K16 ($\eta = 69.2$ mm^2/s) as measured with an EPS concentration of 0.5% (w/v) at 25 °C in aqueous solutions. The relatively high viscosity of the EPS of *L. helveticus* K16 may result from its unusual branching pattern of the polysaccharide structure shown above. In order to understand the structure–viscosity relationships of the polysaccharides, structural analysis in combination with rheological studies, is essential.

1. Experimental

Growth of the organism.—*L. helveticus* K16 was obtained from Valio's Culture Collection, Valio Ltd., Research and Development Service, Helsinki, Finland. The organism was maintained at −80 °C in glass beads, and it was subcultured twice in MRS broth [15] at 37 °C before use. The growth medium used for the production of EPS consisted of 10% skim milk powder (Valio) in water, heat treated at 121 °C for 15 min. The growth of the organism was carried out at 37 °C for 24 h with a 1% inoculum.

Isolation of the exopolysaccharide.—After bacterial growth, trichloroacetic acid (E. Merck, Darmstadt, Germany) was added to the culture (0.7 L) to a final concentration of 4% (w/v), and stirred for 2 h at rt. Cells and precipitated proteins were removed by centrifugation (35 min, 22,000*g*, 4 °C). The supernatant was collected and filtered through an AcroCap filter (0.2 μm, Gelman Sciences, MI, USA). To precipitate polysaccharide, an equal volume of cold EtOH was added gradually to the filtered supernatant. The precipitated EPS material was obtained by centrifugation, and it was washed and dissolved in water obtained from an Alpha-Q Reagent Grade Water Purification System (Millipore Co., Milford, MA, USA). The aq sol. of the EPS was filtered again through an Acrocap filter, and 0.11 g/L EPS was obtained after lyophilization on a DURA-DRY freeze-dryer (FTS Systems Inc., Stone Ridgeny, NY, USA). Part of the material (10 mg) was extensively dialysed against water overnight at 4 °C and the EPS soln was again lyophilized.

GLC/GLC–MS.—Alditol acetates and partially methylated alditol acetates were separated on an HP-5 fused silica column (0.20 mm × 25 m) using a temperature program of 180 °C for 1 min followed by 3 °C/min to 210 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett–Packard model 5890 series II gas chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equipped with a flame ionisation detector. GLC–MS analysis was performed on a Hewlett–Packard model 5970 mass spectrometer equipped with an HP-5MS fused silica column (0.20 mm × 25 m). A temperature program of 170 °C for 3 min followed by 3 °C/min to 250 °C was used with Helium as carrier gas.

NMR spectroscopy.—NMR spectra of a polysaccharide solution in D_2O (10 mg in 0.7 mL) were recorded at neutral pD and 80 °C using a Varian Inova 600 MHz instruments. Chemical shifts are reported in ppm relative to sodium 3 - trimethylsilyl - [2,2,3,3 - $^2H_4$]-propanoate (TSP), δ_H 0.00 or dioxan, δ_C 67.4 as external references. Data processing was performed using standard Varian VNMR

software. $^{1}H,^{1}H$ correlated spectroscopy (COSY) [16], total correlation spectroscopy (TOCSY) [17], gradient selected heteronuclear single quantum coherence (gHSQC) [18], gradient selected heteronuclear multiple-bond correlation (gHMBC) [18] and $^{1}H,^{13}C$ HSQC–TOCSY [19,20] experiments were used to assign signals and performed according to standard pulse sequences. For inter-residue correlations, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY) [21] experiment with a mixing time of 100 ms, and HMBC experiments with 60 and 90 ms delays for the evolution of long range couplings were used.

Sugar analysis.—The samples were hydrolysed with 0.5 M trifluoroacetic acid at 100 °C overnight. After reduction with $NaBH_4$ and acetylation, the samples were analysed by GLC. The absolute configuration of the sugars present in the EPS of *L. helveticus* K16 was determined by derivation of the sugars as their acetylated dithioacetals [22,23].

Methylation analysis.—The analysis was performed according to Hakomori [24] using sodium methylsulfinylmethanide and iodomethane in Me_2SO. The methylated compounds were recovered by use of Sep-Pak C_{18} cartridges (Millipore) [25]. The purified methylated sample was then hydrolysed (2 M trifluoroacetic acid, 120 °C, 2 h), reduced and acetylated. The partially methylated alditol acetates were analysed by GLC–MS.

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