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Note

Structural investigation of the capsular polysaccharide from *Lactobacillus kefiranofaciens* K_1

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Although some *Lactobacillus* strains are reported to produce extracellular polysaccharides[1–8], nothing is known of their structure. We recently isolated a capsular polysaccharide-producing *Lactobacillus kefiranofaciens* from kefir grains[9–11]. The polysaccharide extracted from kefir grains was named kefiran and its structure was elucidated by Kooiman[12]: it has a branched hexasaccharide repeating-unit. However, methylation analysis of the polysaccharide from *L. kefiranofaciens* suggested that it is very similar to kefiran but at the same time has an unknown structural part[11]. We have here attempted to elucidate the complete structure of the capsular polysaccharide from *L. kefiranofaciens* strain K_1. This is the first report concerning structural analysis of extracellular polysaccharide from *Lactobacillus* species.

When *L. kefiranofaciens* K_1 was grown for 3 days at 30° in IKPL medium[13], the culture became viscous due to the production of extracellular polysaccharide. India ink stain[14] showed that the polysaccharide is a capsular type. The polysaccharide fraction obtained from the culture supernatant has been shown by methylation analysis[11] to have the same structure as that from the capsule. In this paper, the supernatant was used for the structural study because of its facility in purification. The purified polysaccharide was obtained in a yield of 63 mg/L from the culture supernatant. It gave a single peak by gel filtration chromatography on an h.p.l.c. column (Asahipak GS-710) and had mol.wt. 1.0×10^6. The polysaccharide had $[\alpha]_D$ +54° (*c* 1.0, water), was composed of only D-glucose and D-galactose in the molar ratio of 0.9:1.1, and did not contain nitrogen.

To obtain information concerning the glycosyl sequence, the polysaccharide was treated with alpha-amylase, galactanase[15], Zymolyase-20T, and cellulase. Only cellulase hydrolyzed the polysaccharide, giving ~80% of D-glucose, D-galactose, and an oligosaccharide **1** in the molar ratio of 1.0:0.3:1.0. F.a.b.-m.s. of the oligosaccharide **1** gave

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m/z 851, corresponding to the M + Na^+ ion of a pentasaccharide. Complete acid hydrolysis of **1** gave D-glucose and D-galactose in the molar ratio of 2.0:3.1. Pentasaccharide **1** gave two disaccharides (**2** and **3**) and two trisaccharides (**4** and **5**) by partial acid hydrolysis. On the basis of the results of methylation analysis (Table I) and enzymic hydrolysis, disaccharide **2**; ($R_{Lactose}$ 1.03 in t.l.c.), disaccharide **3**; ($R_{Lactose}$ 1.00), trisaccharide **4**; ($R_{Lactose}$ 0.90), and trisaccharide **5** ($R_{Lactose}$ 0.85) were identified as follows: β-D-Glc-(1→2)-D-Gal, **2**; β-D-Gal-(1→4)-D-Glc (lactose), **3**; β-D-Glc-(1→2)-β-D-Gal-(1→4)-D-Gal, **4**; and D-Gal-(1→4)-D-Gal-(1→3)-D-Gal, **5**. Methylation analysis of the reduced **1** indicated that **1** is a pentasaccharide having non-reducing terminal D-glucose and reducing-terminal 4-substituted D-glucose, as shown in Table I. ^{13}C-N.m.r. data in the anomeric region of **1** and assignments are shown in Table II. On the basis of the foregoing results, the structure of pentasaccharide may be assigned as **1**.

β-D-Glc-(1→2)-β-D-Gal-(1→4)-α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Glc

1

Methylation analysis of the polysaccharide indicated that its repeating unit has one side chain branched at the 2-substituted D-galactose in **1** and terminated by D-glucose (Table I). The presence of 0.2 molar 6-substituted D-galactose suggested a random arrangement of it in the repeating unit. The ^{13}C-n.m.r. chemical shifts of the polysaccharide and the assignments are shown in Table II. By comparison with published chemical shifts and by empirical rules[19], it is suggested that the polysaccharide has five β and one α linkages. As linear pentasaccharide **1** contained three β-linked sugar residues and one α-linked sugar residue, it may be deduced that 4-substituted glucose at the reducing end of **1** has a β linkage and that non-reducing terminal glucose in the side chain of the polysaccharide has the β linkage. ^1H-N.m.r. spectroscopy showed one α-D-galactose, three (or two) β-D-galactose, and three (or four) β-D-galactose residues in the polysaccharide (Table III). It may be deduced that 6-substituted D-galactose has the β linkage. On the basis of the foregoing results, a hexa- or hepta-saccharide repeating-unit (**6**) is the most probable, in which a random arrangement of one or two sugar residues are attached to a regular pentasaccharide repeating-unit **1**.

β-D-Glc-{1→2(6)}-β-D-Gal-(1→4)-α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Glc

6(2)
↑
1
X

X = β-D-Gal or β-D-Glc-(1→6)-β-D-Gal

6

TABLE I

Methylation analysis of *L. kefiranofaciens* K_1 polysaccharide and pentasaccharide **1** obtained by its enzymic digestion, and oligosaccharides **2**, **3**, **4**, and **5**

Methylated sugar as alditol acetates	*Molar ratio*					
	Polysaccharide	*Oligosaccharide*				
		1	**2**	**3**	**4**	**5**
1,2,3,5,6-Glc[a]		0.85		1.00		
1,3,4,5,6-Gal			1.00			
1,2,3,5,6-Gal					1.00	
1,2,4,5,6-Gal						1.00
2,3,4,6-Glc	1.00	1.00	1.20		0.95	
2,3,4,6-Gal				1.07		1.15
2,3,6-Gal	1.31	0.95				1.18
2,3,6-Glc	1.03					
3,4,6-Gal + 2,4,6-Gal		1.81[b]				
3,4,6-Gal					1.12	
2,4,6-Gal	0.72					
2,3,4-Glc	1.19					
2,3,4-Gal	0.23					
3,4-Gal	0.77					

[a] 4-*O*-Acetyl-1,2,3,5,6-penta-*O*-methylglucitol, and so on. [b] A mixture of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol was shown by m.s.

TABLE II

^{13}C-Chemical shifts[a] of *L. kefiranofaciens* K_1 polysaccharide and pentasaccharide **1** obtained by its enzymic digestion

Linkage	*Polysaccharide*		*Pentasaccharide* **1**	
	Anomeric carbon	*Glycosidic carbon*	*Anomeric carbon*	*Glycosidic carbon*
β-D-Gal-(1→	103.13	—	—	
α-D-Gal-(1→3)-β-D-Gal	96.12	78.16	96.50(96.2)[d,ref. 16]	78.44(78.3)[ref.16]
β-D-Gal-(1→4)-α-D-Gal	103.25	79.43	103.60(103.4)[ref. 17]	79.72(79.2)[ref. 17]
β-D-Gal-(1→4)-β-D-Glc	103.49	79.37	103.71(103.7)[ref. 18]	79.41[e], 79.30[f] (79.4, 79.3)[ref. 17]
β-D-Glc-(1→6)-β-D-Glc	104.74	69.04	—	—
→2,6)-β-D-Gal-(1→	104.74	68.97[b],80.27[c]	—	—
β-D-Glc-(1→2)-β-D-Gal	—	—	104.15	81.04(81.0)[ref. 18]
α,β-D-Glc	—	—	96.54, 92.55	

[a] Chemical shifts (p.p.m.) from sodium 4,4-dimethyl-4-silapentanoate. [b] C-6. [c] C-2. [d] Values in parentheses are from the literature. [e] β anomer. [f] α anomer.

TABLE III

^{1}H-N.m.r. data for the anomeric protons of *L. kefiranofaciens* K_1 polysaccharide

Chemical shift (p.p.m.)[a]	$J_{1,2}$ *(Hz)*	*Assignment*
5.05	3.5	αGal
4.68	7.5	βGlc
4.66	7.4	βGlc
4.53	b[b]	βGlc or βGal
4.46	8.0	βGal
4.37	7.8	βGal
4.33	7.8	βGal

[a] Same as [a] in Table II. [b] Broad, unable to assign accurate coupling constant.

Structure **6** is consistent with the experimental data, and the results of enzymic digestion are interpreted as follows. At the first stage of the action of the cellulase preparation, the polysaccharide would be hydrolyzed to the hexa- or hepta-saccharide **6** by glucanase activity. As the *T. reesei* cellulase preparation used in our study hydrolyzed cellobiose but not sophorose by its β-glucosidase activity (data not shown), a β-D-Glc-(1→6)-D-Gal sequence at the branching point in **6** would be sensitive to it and would be hydrolyzed by it, whereas β-D-Glc-(1→2)-D-Gal would be resistant and remain intact. At the second stage, D-glucose would be liberated from **6**. As the cellulase preparation used in this study contained β-galactosidase activity (5.6 units/mg solid acted on *o*-nitrophenyl β-D-galactoside as substrate), a 6-substituted D-galactose residue would be also liberated at the final stage to produce pentasaccharide **1**.

In this report it is shown that the polysaccharide produced by *L. kefiranofaciens* has exactly same structure as that extracted from the kefir grains[20], which clearly shows that *L. kefiranofaciens* is a kefiran producer in the grains. The structure of **6** with a side chain of one sugar residue is consistent with that of kefiran proposed by Kooiman[12]. La Rivière *et al.*[21] reported the capsulated organism responsible for kefiran production in the kefir grains to be *Lactobacillus brevis* (now regarded as *L. kefir*). They reported that *L. brevis* lost irreversibly the ability to produce capsules even after one passage on MRS lactose agar[21]. A recent study by Kandler and Kunath[22] was unable to detect capsule formation by *L. kefir*. Thus La Rivière *et al.*[21] seemed to perform identification of the isolate from a mixture of *L. brevis* and *L. kefiranofaciens*.

EXPERIMENTAL

Bacterial strain and isolation of the polysaccharide. — *L. kefiranofaciens* strain K_1 was isolated from kefir grains and maintained in IKPL broth as described previously[9,10,13]. A three-day culture of *L. kefiranofaciens* K_1 was inoculated (5%) into IKPL broth and incubated for 3 d at 30° under anerobic conditions. The supernatant was obtained from the broth culture by centrifugation at 10 000*g* for 30 min. The poly-

saccharide was precipitated by addition of an equal volume of EtOH to the supernatant. The precipitated polysaccharide was dispersed in water and undissolved materials were removed by centrifugation at 12 000*g* for 30 min. The polysaccharide was reprecipitated from the clear supernatant by addition of an equal volume of EtOH. The precipitated polysaccharide was dialyzed against distilled water and lyophilized. The polysaccharide was further purified by cation-exchange chromatography on DEAE-Toyopearl (Tosoh Corporation, Tokyo, Japan) as follows. The polysaccharide (100 mg) was dissolved in 0.05M Tris-HCl buffer (pH 8.6) and applied to the column (2.2 × 50 cm). The column was eluted with 1 L of a linear gradient of 0–0.5M NaCl in the 0.05M Tris-HCl buffer (pH 8.6). The polysaccharide fraction eluted was collected, dialyzed against distilled water, and lyophilized.

General. — Enzymic digestion of the polysaccharide (100 mg) by *T.reesei* cellulase (70 units/mL in pH 5.0 acetate buffer, 37°, 6 days, Lot No. 77644M, Cooper Biochem. Inc., Malvern, U.S.A.) and purification of the resulting oligosaccharide were performed as described previously[20]. The polysaccharide (1 mg) was also treated with alpha-amylase (50 units/mL in pH 7.0 MacIlvaine buffer, 37°, 5 d, Boehringer–Mannheim GmbH, F.R.G.), galactanase from *Penicillium citrinum*[15] [endo-(1→4)-β-D-galactanase, 65 units/mL in pH 4.5 acetate buffer, 37°, 3 d], and Zymolyase-20T [(1→3)-β-D-glucan laminarapentaohydrolase from *Arthrobacter luteus*, 20 units/mL in pH 7.0 MacIlvaine buffer, 37°, 3 d, Seikagakukougyo Co., Ltd., Tokyo, Japan]. Toluene was added to prevent microbial growth.

Oligosaccharide **1** was partially hydrolysed in 0.5M trifluoroacetic acid for 3 h at 80°. Oligosaccharides **2**, **3**, **4**, and **5** in the hydrolyzate was obtained by preparative paper chromatography as described previously[23]. Enzymic digestion of oligosaccharides **2**, **3**, **4**, and **5** (0.1–0.5 mg) was performed with β-D-glucosidase (0.2 units/mL in pH 5.0 MacIlvaine buffer, 37°, 20 min, Boehringer–Mannheim GmbH) and β-D-galactosidase (0.5 units/mL in pH 7.0 MacIlvaine buffer, 37°, 20 min, Boehringer–Mannheim GmbH).

^{1}H- and ^{13}C-N.m.r. spectra were measured in D_2O with sodium 4,4-dimethyl-4-silapentanoate as the internal standard with a Jeol FX-400 instrument operated in the pulsed Fourier-transform mode at 70°.

Analytical methods. — The average molecular weight of the polysaccharide was determined by h.p.l.c. on an Asahipak GS-710 column (7.6 × 500 mm, Asahi Chemical Industry Co., Ltd., Tokyo, Japan) using 0.1M NaCl as solvent[24]. Optical rotation was measured on a Jasco DIP-4 digital polarimeter using 1-cm cells at 25°. Colorimetric determination of sugars were performed by the phenol–H_2SO_4 method[25]. Nitrogen was determined by elemental analysis. Methylation analysis of polysaccharide and oligosaccharide alditols was performed as described previously[20] except that permethylated polysaccharides and oligosaccharide alditols were hydrolyzed successively with 90% HCO_2H for 1 h at 100° and 2M CF_3CO_2H for 1 h at 120°. Other analytical methods were the same as those described previously[20].

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