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Structural study on an exocellular polysaccharide produced by *Lactobacillus helveticus* TY1-2

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Abstract

Lactobacillus helveticus TY1-2 produced an exocellular polysaccharide when it was cultured in reconstituted skim milk. This polysaccharide is a high molecular weight heteropolymer of D-glucopyranosyl, D-galactopyranosyl, and 2-acetamido-2-deoxy-D-glucopyranosyl residues in the molar ratio 3.0 : 2.8 : 0.9. The primary structure of the polysaccharide was shown by glycose analysis, methylation analysis, Smith degradation, and NMR spectroscopy to be composed of branched heptasaccharide repeating units having the following structure:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad {}_{0.8}\{\alpha\text{-D-Gal}p\} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 4 \\ \rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\alpha\text{-D-GlcNAc}p \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 3 \\ \qquad\beta\text{-D-Gal}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow \end{array}$$

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1. Introduction

It has been reported that many species of both Gram-negative and Gram-positive bacteria produce exocellular polysaccharides, which are capsular polysaccharides attached to the cell wall or viscous ones secreted into the extracellular environment [1]. Concerning the exocellular polysaccharides elaborated by *Lactobacillus* species, characterizations of slimy polysaccharides of *L. brevis* [2,3], *L. kefir* [4], *L. bulgaricus* [5,6], and *L. kefiranofaciens* [7] have been reported with respect to the improvement of body and texture of dairy products.

During the course of our investigation on chemical characterization of *Lactobacillus* exopolysaccharides we found that *L. helveticus* TY1-2 produced an exocellular polysaccharide with D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose when it was cultured in a liquid medium containing skim milk. For the exopolysaccharide produced by *L. helveticus*, Oda et al. [8] reported the isolation and physicochemical properties of a polysaccharide produced by *L. helveticus* var *jugurti*, in which they showed that it contained D-glucose and D-galactose, but no 2-acetamido-2-deoxy-D-glucose, as its component monosaccharides. Here we report the chemical structure of an exocellular polysaccharide produced by *L. helveticus* TY1-2.

2. Results and discussion

Isolation and molecular weight of the polysaccharide.—The crude exopolysaccharide was isolated as an ethanol precipitate from the culture supernatant solution of

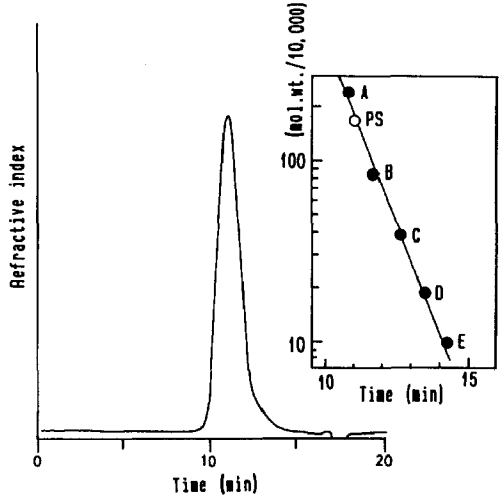


Fig. 1. HPLC profile of exopolysaccharide from *L. helveticus* TY1-2, and a calibration curve for the determination of its molecular weight: PS, polysaccharide from *L. helveticus* TY1-2; A, B, C, D, and E, standard pullulans (mol wts 246×10^4, 85.3×10^4, 38.0×10^4, 18.6×10^4, and 10.0×10^4, respectively).

Lactobacillus helveticus TY1-2. The purified polysaccharide was obtained as a protein free material after DEAE-cellulose column chromatography. About 200 mg of the purified polysaccharide was obtained from one liter of the culture supernatant solution. The purified polysaccharide was monodisperse by size-exclusion high performance liquid chromatography (HPLC, Fig. 1) and had $[\alpha]_D$ +50.3° (*c* 0.5, H_2O). Its molecular weight was estimated to be ~ 1 600 000 by comparison of its elution time to those of standard pullulans on HPLC (Fig. 1).

Sugar analysis.—The component sugars were quantitated by HPLC analysis of an hydrolyzate, which showed the glucose : galactose : 2-amino-2-deoxy-glucose molar ratio to be 3.0 : 2.8 : 0.9. All three sugars were shown to be D isomers by GLC analysis of their trimethylsilylated (−)-2-butyl glycosides [9,10].

The ^{13}C NMR spectrum of the polysaccharide in D_2O (Fig. 2) contained signals for methyl carbons of *N*-acetyl groups at 23.39 and 23.34 ppm (weak), signals for carbonyl carbons at 175.6 (weak) and 175.2 ppm, and signals for C-2 of amino sugars at 56.3 and 56.7 ppm (weak), indicating that the amino sugars are *N*-acetylated in the polysaccharide.

Methylation analysis.—The polysaccharide was methylated and the derived alditol acetates were analyzed by GLC–MS (Table 1, native polysaccharide). This methylation analysis, together with the NMR analysis, showed that the polysaccharide is composed of terminal D-galactopyranose, 3-linked D-glucopyranose, 4-linked D-glucopyranose, 6-linked D-glucopyranose, 6-linked 2-acetamido-2-deoxy-D-glucopyranose, 3,6-linked D-galactopyranose, and 4,6-linked 2-acetamido-2-deoxy-D-glucopyranose.

In order to obtain structural information about the side chains of the polysac-

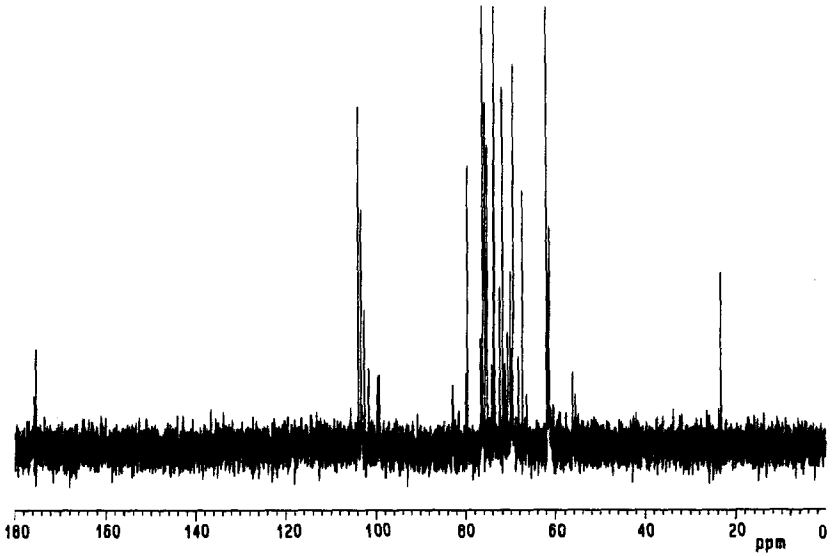


Fig. 2. ^{13}C NMR spectrum of the polysaccharide from *L. helveticus* TY1-2.

charide, it was hydrolyzed by β-D-galactopyranosidase of Jack bean, and the resulting digest was subjected to methylation analysis. After 96 h incubation with the enzyme, the quantitative determination of D-galactose in the mixture showed that ~48% of the total nonreducing terminal D-galactosyl residues was liberated. After enzyme digestion, the polysaccharide fraction was isolated and methylated. The methylated digest yielded the same partially methylated sugars as the methylated native polysaccharide, but the molar ratios of 2,3,4,6-tetra-*O*-methyl-D-galactitol and 2,3,6-tri-*O*-methyl-D-glucitol decreased, whereas the molar ratio of 2,3,4,6-tetra-*O*-methyl-D-glucitol increased (Table 1, β-galactosidase digested polysaccharide). Taking into account the molar ratio of the methyl sugars from the methylated native polysaccharide, this result suggested that the disaccharide units of β-D-Gal*p*-(1 → 4)-D-Glc*p* are involved in the side chains, and β-galactosidase resistant galactosyl terminals may be bound to the (4 → 6)-linked 2-acetamido-2-deoxy-D-glucosyl residues.

Smith degradation.—Acid hydrolysis of the IO_4 oxidized and $NaBH_4$ reduced polysaccharide gave glycerol, erythritol, glucose, galactose, and 2-acetamido-2-deoxyglucose in the molar ratio 3.8:1.4:1.6:1.2:0.8. This sugar ratio agreed well with that expected from methylation analysis (4.1:1.4:1.4:1.6:0.8).

In order to isolate oligosaccharide fragments from the polysaccharide, the IO_4 oxidized and $NaBH_4$ reduced polysaccharide was subjected to mild Smith degradation [11]. During the course of hydrolysis, the degradation products were monitored by size-exclusion HPLC every 4 h. The HPLC profiles showed that the oxidized–reduced polysaccharide disappeared at an early stage of hydrolysis to give low molecular weight oligosaccharides. Fig. 3 depicts an HPLC profile of the mild Smith degradation products after 20 h of hydrolysis. This result indicated that

Table 1
Methylation analysis of *L. helveticus* polysaccharide, β-galactosidase digested polysaccharide, and oligosaccharides

Methylated sugars	Molar ratios			
	Native polysaccharide	β-Galactosidase digested polysaccharide	Oligosaccharides [b]	
			A	*B*
2,3,4,6-Tetra-*O*-Me-Glc [a]	0.1	0.8	1.2	3.9
2,4,6-Tri-*O*-Me-Glc	1.1	1.1	ND [c]	1.0
2,3,6-Tri-*O*-Me-Glc	1.1	0.2	ND	4.0
2,3,4-Tri-*O*-Me-Glc	1.0	1.0	ND	ND
2,3,4,6-Tetra-*O*-Me-Gal	1.9	1.0	ND	1.0
2,4,6-Tri-*O*-Me-Gal	ND	ND	1.1	ND
2,4-Di-*O*-Me-Gal	1.2	1.2	ND	ND
3,4-Di-*O*-Me-GlcN(AcMe)	0.2	0.2	1.0	ND
3-*O*-Me-GlcN(AcMe)	0.8	0.8	ND	ND

[a] 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and so on.
[b] *A* was obtained by mild Smith degradation and *B* was a product obtained by acetolysis of the polysaccharide.
[c] ND, Note detected.

the IO_4 oxidation resistant repeating units of the polysaccharide are joined by IO_4 oxidation sensitive monosaccharide residues, such as 4- or 6-linked D-glucosyl residues. After the mild Smith degradation, the products were fractionated by chromatography on Bio-Gel P-2. The main constituent contained glucose, 2-acetamido-2-deoxyglucose, galactose, and glycerol in the molar ratio 1.2:1.0:1.1:1.2, and secondary ion mass spectrometry (SIMS) indicated a molecular mass of 619. Methylation analysis of the oligosaccharide (Table 1, A) indicated that the oligosaccharide contained terminal D-glucopyranose, 3-linked D-galactopyranose, and 6-linked 2-acetamido-2-deoxy-D-glucopyranose in the molar ratio 1:1:1.

The sequence and anomeric configurations of the residues in the oligosaccharide obtained by mild Smith degradation were established by ^1H NMR spectroscopy. The ^1H NMR spectrum of the oligosaccharide recorded at 24°C (Fig. 4) contained signals for two H-1β at δ 4.73 (d, $J_{1,2}$ 8.3 Hz) and 4.49 (d, $J_{1,2}$ 7.8 Hz), and a H-1α at δ 4.91 (unresolved). COSY data at 24°C obtained on the oligosaccharide allowed the unambiguous assigments of anomeric and ring proton signals. The residues in the oligosaccharide are designated *a* to *c* in order of decreasing chemical shift of their anomeric protons and the ring protons were traced via cross peaks starting from each H-1 signal (Fig. 4). In the COSY contour map, the H-1 signal of *c* gave crosspeaks with H-2 at 3.29 ppm. Based on the fact that signals for H-2 of β-glucopyranose occur at higher field than the corresponding shifts for galactose and 2-acetamido-2-deoxyglucose [12,13], residue *c* was identified as β-D-glucopyranose. Residue *b* was identified as β-D-galactopyranose from the chemical shift of H-4 (δ 4.19), which occurs at lower field than the corresponding shifts for the other monosaccharides [12,13]. Consequently, residue *a* was identified as 2-acetamido-2-deoxy-α-D-glucopyranose. The sequence of the three sugars

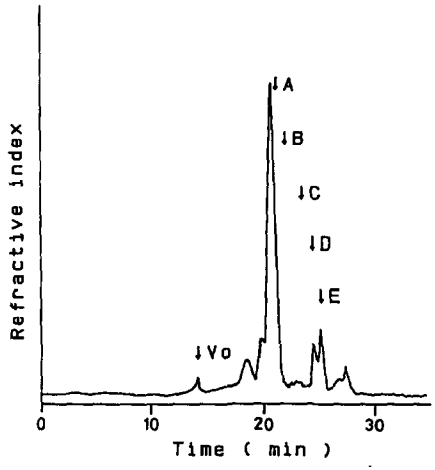


Fig. 3. HPLC profile of the mild Smith degradation products after 20 h of hydrolysis. Arrow at V_0 shows the void volume of the column, and arrows at A, B, C, D, and E indicate the elution positions of trisaccharide, disaccharide, monosaccharide, erythritol, and glycerol, respectively.

was determined by 1D NOE difference spectroscopy (data not shown). In this experiment, irradiation of H-1 of the terminal β-D-glucopyranose (*c*) produced an interresidue NOE at H-2 of *c* (δ 3.29) and an interglycosidic NOE at H-6 of *a* (δ 3.45, 3.48), indicating that the β-D-glucopyranosyl residue is bound to O-6 of the 6-linked 2-acetamindo-2-deoxy-α-D-glucopyranosyl residue. The above ^{1}H NMR data and the methylation analysis indicated that the oligosaccharide has a structure depicted in **1**.

β-D-Glc*p*-(1 → 6)-α-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → glycerol
c *a* *b*

1

Acetolysis.—The products obtained by acetolysis of the polysaccharide were *O*-deacetylated and fractionated by size-exclusion column chromatography. The main product purified by subsequent preparative silica-gel TLC was estimated to be a disaccharide from its R_f value. Methylation analysis of this product gave 2,3,4,6-tetra-*O*-methyl-D-glucitol, 2,3,4,6-tetra-*O*-methyl-D-galactitol, 2,4,6-tri-*O*-

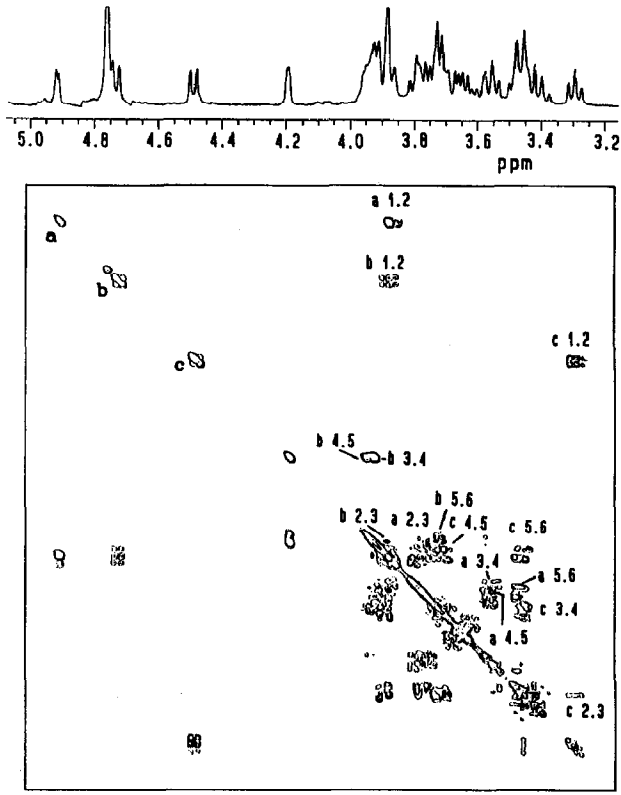


Fig. 4. COSY contour plot (3.2–5.0 ppm region) for the oligosaccharide obtained by mild Smith degradation.

methyl-D-glucitol and 2,3,6-tri-*O*-methyl-D-glucitol in the molar ratio 3.9:1.0:4.0:1.0 (Table 1, B). Taking into account its molecular size, this result indicated that the main product is a mixture of two disaccharides, *O*-D-glucosyl-(1 → 3)-D-glucose and *O*-D-galactosyl-(1 → 4)-D-glucose. Production of the former disaccharide confirmed the occurrence of the glucosidic sequence → 6)-Glc-(1 → 3)-Glc-1(→ in the backbone of the polysaccharide. The production of the latter disaccharide also supported the side-chain structure suggested by methylation analyses of the native and β-galactosidase digested polysaccharides.

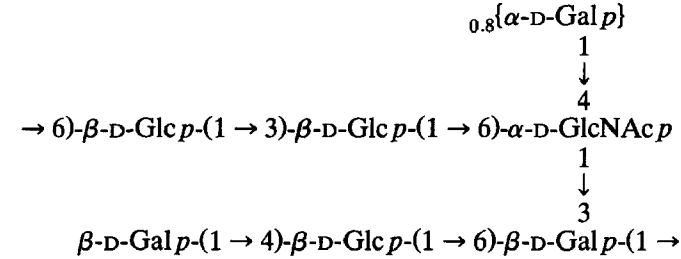
In view of the results of the methylation analyses, and the isolation and production of the oligosaccharides by the mild Smith degradation (**1**) and acetolysis, a structure for the repeating unit of the polysaccharide can be proposed (**2**),

$$\begin{array}{l} \qquad\qquad\quad R^1 \qquad\qquad R^2 \\ \qquad\qquad\quad \downarrow \qquad\qquad \downarrow \\ \qquad\qquad\quad 4 \qquad\qquad 6 \\ \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow 6)\text{-D-Glc}p\text{-}(1 \rightarrow \\ \qquad\qquad\quad 2 \end{array}$$

where R^1 = D-Gal*p*-(1 → and R^2 = β-D-Gal*p*-(1 → 4)-D-Glc*p*-(1 → .

^{1}H NMR spectroscopy.—In order to determine the anomeric configurations of the sugar residues, such as terminal D-galactopyranose (R^1 of **2**), 4-linked D-glucopyranose (in R^2 of **2**), and 6-linked D-glucopyranose (in the backbone of **2**), the ^{1}H resonances of the native polysaccharide were assigned in the COSY spectrum. As shown in Fig. 5, the ^{1}H NMR spectrum at 70°C of the polysaccharide contained signals for two H-1α at δ 5.35 (d, $J_{1,2}$ 3.5 Hz) and δ 4.97 (unresolved), and five H-1β at δ 4.78 (d, $J_{1,2}$ 7.3 Hz), δ 4.70 (d, $J_{1,2}$ 7.3 Hz), δ 4.57 (d, $J_{1,2}$ 7.9 Hz), δ 4.51 (d, $J_{1,2}$ 7.8 Hz) and δ 4.44 (d, $J_{1,2}$ 7.8 Hz), which confirmed that the polysaccharide is composed of heptasaccharide repeating units. The residues in the repeating unit were labelled *d* to *j* in order of decreasing chemical shift of their anomeric protons. Residues *e* and *f* were assigned to the (4,6)-linked 2-acetamido-2-deoxy-α-D-glucopyranose and the (3,6)-linked β-D-galactopyranose, respectively, by comparison of their H-1 chemical shifts (δ 4.97 and 4.78, respectively) and coupling constants with those of the H-1 resonances of the corresponding residues in the oligosaccharide obtained by the mild Smith degradation (see Fig. 4). The assignment of residue *d*, which has the α configuration, is based on the chemical shifts of the ring protons determined from the crosspeaks in the COSY spectrum at 70°C (Fig. 5). From the chemical shifts of H-4 (δ 3.91), which occurs at lower field than the corresponding shifts for glucosyl residues [12], residue *d* was identified as α-D-galactopyranose. This assignment is consistent with the fact that about one-half of the terminal D-galactosyl residues were resistant to β-D-galactopyranosidase. Consequently, the other residues (*g*–*j*) which have the β configuration are attributed to the 3-linked, 4-linked, and 6-linked glucopyranoses, and the terminal β-D-galactopyranose. In the ^{1}H NMR spectrum of the β-galactosidase digested polysaccharide (data not shown), the intensities of the signals at δ 4.57 (corresponding to H-1 of *i*) and δ 4.44 (corresponding to H-1 of *j*) diminished, which indicated that residue *i* and *j* are the 4-linked β-D-glucopyranose and the terminal β-D-galactopyranose.

Overall, the data permit the primary structure of the repeating units of the exocellular polysaccharide produced by *L. helveticus* TY1-2 to be formulated as follows:



The most characteristic feature of this polysaccharide, when compared to those of other Lactobacillus exopolysaccharides, is the presence of 2-acetamido-2-deoxy-D-glucopyranose as a component monosaccharide. As described in the Introduction,

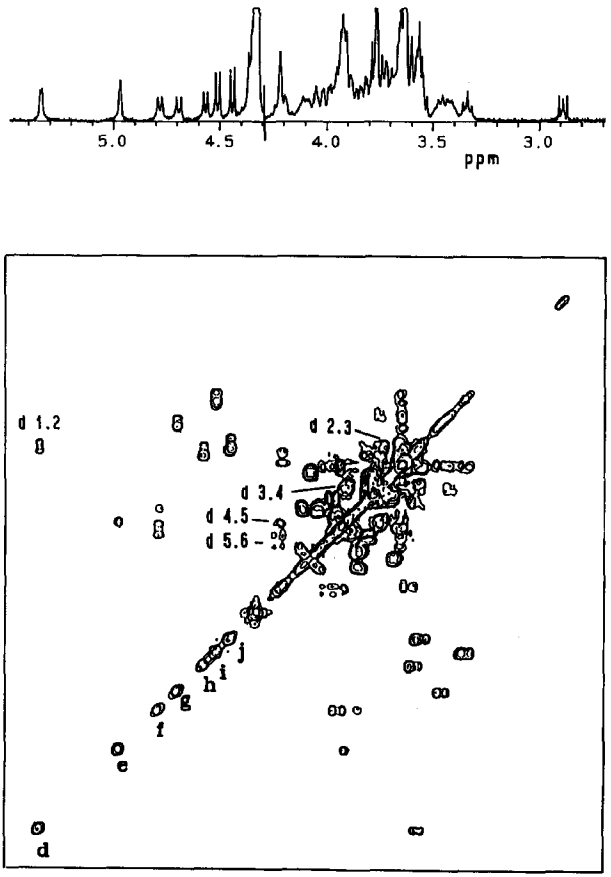


Fig. 5. COSY contour plot (2.7×5.4 ppm region) for the polysaccharide from *L. helveticus* TY1-2.

exopolysaccharides of *L. helveticus* var *jugurti* were reported to be composed of only glucose and galactose in a ratio of 2:1, and other *Lactobacillus* exopolysaccharides so far reported contain only glucose and galactose in different ratios. Toba et al. [4] summarized the results of methylation analyses of seven polysaccharides including those from Kefir grains, *Lactobacillus* species and *L. kefir*. A comparison of those results with that of *L. helveticus* TY1-2 shows that the glycosidic linkages of *L. kefir* KL3 are similar in a few respects, such as the occurrence of (4,6)-linked D-glucosyl residues and nonreducing terminal D-galactose.

In conclusion, this report has shown that the exopolysaccharide of *L. helveticus* TY1-2 has a unique structural feature in comparison with other *Lactobacillus* polysaccharides.

3. Experimental

General methods.—GLC was performed with a Shimadzu GC-14A apparatus fitted with a flame-ionization detector. A glass capillary column (df = 0.15 μm, 0.25 mm × 50 m) coated with OV-1 (GLscience, Co. Ltd., Japan) was used for separating partially methylated alditol acetates (program I) and trimethylsilylated (−)-2-butyl glycosides (program II). The temperature programs used were: I, 150°C for 10 min, then 2.5°C/min to 200°C; II, 150°C for 5 min, then 2°C/min to 200°C. GLC–MS spectra and secondary ion mass spectrometry (SIMS, thioglycerol was used as a matrix) were obtained with a Hitachi M2000 mass spectrometer. HPLC was performed with a Hitachi L6200 apparatus equipped with a Hitachi L-3300 RI differential refractometer as a detector. Specific rotations were determined with a Horiba SEPA200 polarimeter. ^{13}C NMR spectra (internal standard, 1,4-dioxane) were recorded with a Jeol GSX-280 spectrometer in D_2O at 70°C. ^{1}H NMR spectra (internal standard, 3-trimethylsilylpropanoate) were recorded with a Jeol α-400 spectrometer in D_2O at 70 or 24°C. COSY and 1D NOE experiments were performed using a Jeol α-400 standard program at 70 or 24°C. TLC was carried out on silica gel 60 aluminum sheets (Merck, Germany) and the solvent system was 2:1:1 EtOAc–HOAc–H_2O.

Organism.—*Lactobacillus helveticus* TY1-2 was used in this study. *L. helveticus* TY1-2 was stored at −85°C in a medium containing 5% skim milk powder and 5% monosodium L-glutamate monohydrate salt until used.

Isolation and purification of the polysaccharide.—A preculture of *L. helveticus* TY1-2 was inoculated into sterilized, reconstituted skim milk (100 g/L) and cultured at 32°C for 72 h. After removal of insoluble materials in the culture broth by centrifugation (6000 rpm for 15 min), the polysaccharide was precipitated by the addition of 95% EtOH to the supernatant (1:1, v/v) at 4°C. The polysaccharide precipitate was collected by centrifugation (10000 rpm, 20 min). This EtOH-precipitation procedure was repeated three times to give crude polysaccharide, which was dissolved in 10 mL of 25 mM Tris · HCl buffer (pH 8.5), and applied to a column (2.5 × 20 cm) of DEAE-cellulose DE-52 (Whatman Co. Ltd., Japan)

equilibrated with the same buffer. The polysaccharide eluted with the same buffer without being adsorbed on the column. Carbohydrate content in the fraction was determined by the phenol–H_2SO_4 method [14]. Fractions containing carbohydrate were collected, dialyzed against deionized water, and lyophilized.

Molecular weight determination.—The molecular weight of the polysaccharide was determined by size-exclusion HPLC at 30°C on an Asahipak GS-710 column (7.6 × 500 mm, Asahi Chemical Industry Co. Ltd., Japan) using 0.1 M $NaNO_3$ as an eluent with a flow rate of 1.0 mL/min. Several pullulan standards of different molecular weights (246×10^4, 85.3×10^4, 38.0×10^4, 18.6×10^4, and 10.0×10^4) were purchased from Showa Denko Co. Ltd., Japan.

Sugar analysis.—The polysaccharide was hydrolyzed with 2.5 M CF_3CO_2H at 100°C for 12 h, and the acid was removed by evaporation. Monosaccharides were analyzed by HPLC at 40°C on an Asahipak NH_2 P-50 column (4.6 × 250 mm, Asahi Chemical Industory Co. Ltd., Japan). The eluent consisted of 10 mM tetrapropylammonium hydroxide-acetic acid (pH 10) and CH_3CN (20:80, v/v) and its flow rate was 0.6 mL/min.

Methylation analysis.—The polysaccharide was completely methylated after three successive methylations by the Hakomori method [15]. The permethylated polysaccharide was treated with aq 90% formic acid (1 mL for 5 h at 100°C), the solvent was evaporated under reduced pressure, and the residue was treated with 2.5 M CF_3CO_2H for 10 h at 100°C. The acid was removed by evaporation, and the resulting hydrolyzate was reduced with $NaBH_4$ at 25°C for 14 h. The excess $NaBH_4$ was removed by the addition of 25% acetic acid and the solution was evaporated under reduced pressure to dryness. After removal of borate by co-evaporation with CH_3OH, the partially methylated glycitols were acetylated with 1:1 pyridine–Ac_2OAc_2 at 25°C overnight. The partially methylated glycitol acetates were subjected to GLC and GLC–MS.

β-Galactosidase digestion.—The polysaccharide (25 mg) was dissolved in 5 mL of 20 mM citrate buffer, pH 3.5, and the solution was incubated with Jack bean β-galactosidase (5 units, Sigma Chemical Co., USA) for 4 days at 30°C. The galactose released into the mixture was measured by an F-kit of lactose/galactose (Boehringer Mannheim Yamanouchi Co. Ltd., Japan). After the enzymic reaction, the solution was neutralized with 0.1 M NaOH and heated for 4 min at 100°C to inactivate the enzyme. The solution was centrifuged at 10000 rpm for 15 min to remove insoluble materials, the supernatant was dialyzed against deionized water, and the polysaccharide in the dialyzate was recovered by lyophilization.

Smith degradation.—The polysaccharide (152 mg) was dissolved in 50 mM $NaIO_4$ (150 mL) and the solution was stirred for 7 days at 5°C in the dark. Excess ethylene glycol was added to the solution and the mixture was dialyzed against running water. The oxidized polysaccharide was reduced with $NaBH_4$ at 25°C for 14 h. The excess of $NaBH_4$ was decomposed by the addition of 25% acetic acid and the solution was dialyzed against distilled water. The oxidized–reduced polysaccharide was recovered by lyophilization. For complete Smith degradation, the oxidized–reduced polysaccharide (10 mg) was hydrolyzed with 4 M CF_3CO_2H (2 mL) for 4 h at 100°C, and the acid was removed by evaporation under reduced

pressure. The resulting materials were analyzed by HPLC under the same conditions as those for the sugar analysis. For the mild Smith degradation, the oxidized–reduced polysaccharide (100 mg) was hydrolyzed with 0.1 M CF_3CO_2H (15 mL) at 25°C for 20 h. The acid was removed by repeated evaporations in the presence of methanol. The resulting material was dissolved in water and subjected to size-exclusion HPLC at 50°C on a column of Asahipak GS220 (7.6 × 500 mm, Asahi Chemical Industry Co. Ltd., Japan) using water as an eluent (flow rate, 0.6 mL/min). On the preparative scale, the mild Smith degradation products were fractionated at 40°C on a Bio-Gel P2 column (2.6 × 80 cm) using water as an eluent (flow rate, 12 mL/min). The main product was subjected to sugar analysis, methylation analysis, and ^1H NMR spectroscopy.

Acetolysis of the polysaccharide.—The polysaccharide (180 mg) was acetylated with 1:1 pyridine–Ac_2O (20 mL) at 80°C for 6 h. The resulting acetates were dissolved in 24:16:3 Ac_2O–AcOH–H_2SO_4 (21.5 mL). The mixture was kept at 25°C for 5 days and thereafter at 60°C for 75 min. The mixture was poured into ice–water and neutralized with $NaHCO_3$. The derived acetates were extracted with $CHCl_3$ and concentrated by evaporation. The product was *O*-deacetylated with 5.6% (v/v) CH_3ONa–CH_3OH (10 mL) at 25°C for 3 h. The resultant mixture was poured into ice–water and neutralized with M HCl and evaporated to dryness. The mixture of oligosaccharides was fractionated at 25°C on joined columns (2.6 × 80 cm × 2) of TSK gel TOYOPEARL HW-40S (Tosoh Co. Ltd., Japan) using water as eluent (20 mL/h). The carbohydrate content of each fraction was determined by the phenol–H_2SO_4 method [14]. Fractions containing the main product corresponding to disaccharide were combined and lyophilized. For further purification of the main product, this sample was subjected to preparative TLC.

Acknowledgments

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