ELSEVIER

Carbohydrate Research 275 (1995) 319–332

CARBOHYDRATE RESEARCH

Structure of an exocellular polysaccharide of *Lactobacillus helveticus* TN-4, a spontaneous mutant strain of *Lactobacillus helveticus* TY1-2

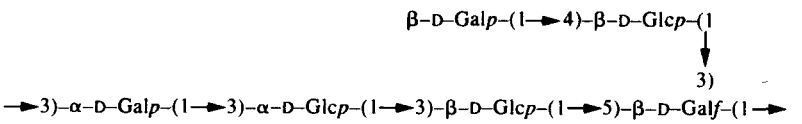
Yoshihiro Yamamoto [a,*], Tetsuo Nunome [b], Ryo Yamauchi [a], Koji Kato [a], Yoshiaki Sone [c]

[a] *The United Graduate School of Agricultural Science, Gifu University, Gifu 501-11, Japan*
[b] *Takeda Food Products, Ltd., 3–20 Imoji, Itami, Hyougo 664, Japan*
[c] *Faculty of Human Life Science, Osaka City University, Sugimoto-cho, Sumiyoshi-ku, Osaka 558, Japan*

Received 25 August 1994; accepted in revised form 25 January 1995

Abstract

Lactobacillus helveticus strain TN-4, a spontaneous mutant strain of *Lactobacillus helveticus* TY1-2, produced an exocellular polysaccharide from reconstituted skim milk. On the basis of the results of methylation analysis, enzymatic digestion, mild Smith degradation, mild acid hydrolysis, acetolysis, and 1D and 2D ^{1}H-NMR spectroscopy, it was concluded that the polysaccharide has a D-galactofuranose containing hexasaccharide repeating unit with the following structure:



Keywords: Exocellular polysaccharide; *Lactobacillus helveticus* TN-4; *Lactobacillus helveticus* TY1-2

1. Introduction

Lactobacillus species may produce slimy exocellular polysaccharides into their culture broth. Among them, *L. brevis* [1,2], *L. kefir* [3], *L. kefiranofaciens* [4], and *L.*

* Corresponding author. Present address: Takeda Food Products Ltd., 3-20 Imoji, Itami, Hyogo 663, Japan.

SSDI 0008-6215(95)00077-1

delbruckii subsp. *bulgaricus* [5–7] have been investigated on the production and characterization of the exopolysaccharide in light of its role on the rheological behaviour and texture of dairy products.

During the course of our investigation on the production and chemical characterization of an exocellular polysaccharide of *Lactobacillus helveticus* TY1-2 [8], we have found a spontaneous mutant which produces an exocellular polysaccharide of a different viscosity property than strain TY1-2. The mutant strain was found in the stock culture of *L. helveticus* TY1-2 which had been maintained at −19°C for one year in a medium containing 1.5% yeast-extract, 1% lactoalbumin hydrolyzate and 2% glucose, while no such a mutant was found in the stock culture stored at −85°C in a medium containing 5% skim milk powder and 5% monosodium L-glutamate monohydrate salt. Preliminary sugar analysis of the polysaccharide produced by the mutant strain showed that it consists of D-glucose and D-galactose in an equal molar ratio. This sugar composition differs from that of *L. helveticus* TY1-2 polysaccharide, which is composed of D-glucose, D-galactose and 2-acetamido-2-deoxy-D-glucose in the molar ratio 3.0 : 2.8 : 0.9 [8]. Therefore, we have isolated the mutant strain which was designated as *L. helveticus* TN-4, and studied the chemical structure of the polysaccharide in order to compare its structural features with those of the polysaccharide produced by strain TY1-2.

2. Results and discussion

Isolation, composition, and linkage analysis.—The crude exopolysaccharicle was isolated as an ethanol precipitate from the culture supernatant solution of *Lactobacillus helveticus* TN-4, and purified to a protein free material after DEAE-cellulose column chromatography. About 180 mg of the purified polysaccharide were obtained from one liter of the culture supernatant solution. The purified polysaccharide had $[\alpha]_D$ +60.7° (*c* 0.3, H_20) and its molecular weight was estimated to be ~1 800 000 by HLPC on a pullulan-calibrated column of Asahipak GS-710. Hydrolysis of the polysaccharide followed by HPLC examination of the hydrolyzate revealed the presence of glucose (Glc) and galactose (Gal) in approximately equimolar proportions. GLC analysis of their trimethylsilylated (−)-2-butyl glycosides [9] showed that the both sugars are of the D configuration. The polysaccharide was methylated by the Hakomori method [10] and the derived partially methylated alditol acetates were analyzed by GLC–MS (Table 1, PS). This methylation analysis showed that the polysaccharide is composed of terminal D-Gal*p*, 3-linked D-Glc*p*, 4-linked D-Glc*p*, 3-linked D-Gal*p* and 3,5-linked D-Gal*f* (or 3,4-linked D-Gal*p*) residues.

NMR spectroscopy.—The 500-MHz ^{1}H-NMR spectrum of the native polysaccharide in D_2O at 70°C (Fig. 1, projected along f_2 axis in HOHAHA spectrum) contained six H-1 signals at δ 5.369 (d, $J_{1,2}$ 3.2 Hz, residue *a*), δ 5.334 (d, $J_{1.2}$ 3.6 Hz, residue *b*), δ 5.255 (bs, residue *c*), δ 4.668 (d, $J_{1,2}$ 6.8 Hz, residue *d*), δ 4.650 (d, $J_{1,2}$ 6.8 Hz, residue *e*), δ 4.459 (d, $J_{1,2}$ 8.1 Hz, residue *f*) with nearly equal integrated intensities, indicative of a hexasaccharide repeating unit. Assignments of the non-anomeric protons of these residues were made on the basis of cross-peaks observed in the HOHAHA

Table 1
Methylation analysis of *L. helveticus* TN-4 native polysaccharide (PS), enzyme (β-galactosidase) digested polysaccharide (PS-ed), mild Smith degraded polysaccharide (PS-sd), mild acid hydrolysis product (Fraction A) and reduced acetolysis product III (Reduced III)

Methylated sugars	Molar ratios				
	PS	PS-ed	PS-sd	Fraction A	Reduced III
2,3,4,6-Tetra-*O*-Me-Glc [a)]	ND [b)]	0.8	ND	0.3	6.2
2,4,6-Tri-*O*-Me-Glc	2.0	2.0	2.2	2.0	7.1
2,3,6-Tri-*O*-Me-Glc	1.0	0.2	ND	1.1	ND
2,3,4,6-Tetra-*O*-Me-Gal	1.0	0.2	ND	1.9	1.0
2,4,6-Tri-*O*-Me-Gal	1.0	1.0	1.0	0.2	ND
2,6-Di-*O*-Me-Gal	1.0	1.0	0.2	1.0	ND
2,3,6-Tri-*O*-Me-Gal	ND	ND	0.8	ND	ND
1,2,4,5,6-Penta-*O*-Me-Glc	ND	ND	ND	ND	1.0
1,2,3,4,6-Penta-*O*-Me-Gal	ND	ND	ND	ND	6.2

[a] 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and so on. [b] ND, not detected.

spectrum as far as H-4 or H-5 (Fig. 1). On the H-1 tracks of residues *a*, *b* and *f* in the HOHAHA spectrum, cross-peaks were detected with H-2,3,4, and on the H-1 tracks of residues *c*, *d* and *e* with H-2,3. Assignments for H-4 and H-5 of residues *d* and *e* were made on the basis of cross-peaks observed on their H-2 tracks. The signals at δ 4.210 and δ 3.834 were assigned to H-4 and H-5 of residue *c*, respectively, based on the cross peakes observed on its H-3 track. Assignment for H-5 of residue *a* was based on the cross-peak detected on the corresponding H-4 track. The ^{1}H-chemical shifts for ^{1}H resonances of the constituent sugar residues are summarized in Table 2 (native polysaccharide). The ^{13}C-NMR spectrum recorded at 70°C (data not shown) contained, inter alia, signals for C-1 at δ 109.65, δ 103.83, 103.11, δ 102.88, δ 100.14 (2C), which also indicated that the polysaccharide has hexasaccharide repeating units. In addition, the appearance of C-1 signal at peculiar low-field (at δ 109.65) suggested the occurrence of β-D-Gal*f* in the polysaccharide based on the Ritchie's report where the C-1 signal of methyl-β-D-Gal*f* and methyl-α-D-Gal*f* appeared at δ 109.9 and δ 103.8, respectively [11]. This inference that β-D-Gal*f* is an inherent constituent of the polysaccharide was confirmed by the ^{1}H-NMR spectroscopy of the polysaccharide (see Fig. 1 and Table 2). Concerning the ^{1}H-chemical shifts of residue *c*, the signals of H-2 (δ 4.336) and H-3 (δ 4.475) appeared at relatively low field, and H-1 signal (δ 5.255) appeared as a broad singlet. These observations are characteristic of β-D-Gal*f* residue as described by Parra et al. [12] and by Takayanagi et al. [13], respectively. For the latter observation, Takayanagi et al. have reported that the value of $J_{1,2}$ for methyl β-D-Gal*f* (2 Hz) was smaller than that of α-anomer ($J_{1,2}$ 3.7 Hz). Therefore, the residue *c* could be identified as β-D-Gal*f*. These ^{1}H- and ^{13}C-NMR spectroscopy together with the methylation analysis indicated that the polysaccharide consists of one 3,5-linked β-D-Gal*f* residue (a branching point), two α-D- and three β-D-pyranosyl residues.

β-Galactosidase digestion.—In order to obtain the structural information about the side chains of the polysaccharide, it was hydrolyzed by β-D-galactopyranosidase of jack bean in the same manner as the polysaccharide of *L. helveticus* TY1-2 [8], and the

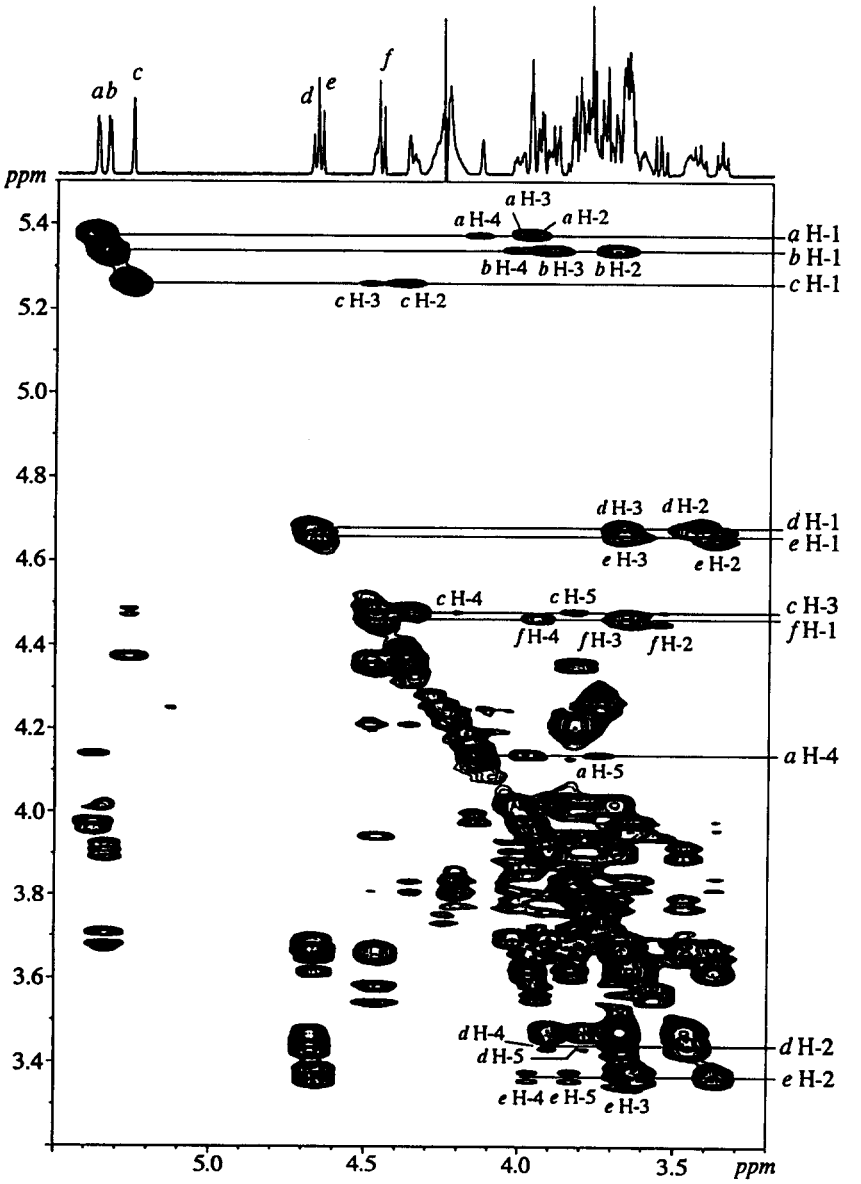


Fig. 1. The 500 MHz ^{1}H-NMR specrtum and assignments in the 2D HOHAHA spectrum of the native polysaccharide produced by *L. helveticus* TN-4.

resulting digest was subjected to methylation and ^{1}H-NMR analyses. After 96 h incubation with the enzyme, the quantitative determination of D-galactose in the mixture showed that ~80% of the non-reducing terminal D-Gal residues were liberated. The result of methylation analysis of the digest is shown in Table 1 (PS-ed). When this result is compared to that of native polysaccharide, it is clear that the methylated digest gave

Table 2
^{1}H-NMR chemical shifts (δ) of the native polysaccharide and of the hexasaccharidealditol obtained by mild acid hydrolysis followed by reduction with $NaBH_4$

Residue	Native polysaccharide					Hexasaccharide-alditol				
	H-1	H-2	H-3	H-4	H-5	H-1	H-2	H-3	H-4	H-5
a	5.369	3.968	3.970	4.232	3.734	5.386	3.912	3.835	3.994	4.259
b	5.334	3.697	3.900	4.013		5.361	3.660	3.912	4.051	3.802
c	5.255	4.336	4.475	4.210	3.834					
d	4.668	3.434	3.665	3.894	3.784	4.696	3.426	3.656	3.896	3.747
e	4.650	3.361	3.652	3.958	3.832	4.585	3.370	3.656	3.955	3.843
f	4.459	3.555	3.656	3.921		4.454	3.538	3.656	3.921	

2,3,4,6-tetra-*O*-methyl-D-glucitol in addition to the same partially methylated sugars as the methylated native polysaccharide, and the molar ratio of 2,3,4,6-tetra-*O*-methyl-D-galactitol and that of 2,3,6-tri-*O*-methyl-D-glucitol decreased from 1.0 (native) to 0.2 (digest). A comparison of the 400 MHz ^{1}H-NMR spectrum at 70°C of the digest (data not shown) with that of the native polysaccharide showed that the intensities of the signals at δ 4.65 (H-1 of residue *e*) and δ 4.46 (H-1 of residue *f*) diminished, while a new signal appeared at δ 4.63 (could be attributed to H-1 of terminal β-D-Glc*p*). Taking into account the methylation analysis and ^{1}H-NMR spectroscopy for the native polysaccharide, these results indicated that the disaccharide fragment, β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → , is involved in the side chains of the polysaccharide, and residues *e* and *f* could be assigned to 4-linked β-D-Glc*p* and the terminal β-D-Gal*p*, respectively (see mild acid hydrolysis section).

Concerning the residual terminal D-Gal*p* residues after 96 h incubation with the successive addition of β-D-galactosidase, the presence of concomitant terminal α-D-Gal*p* residues could be ruled out because the intensity of the H-1 signal of terminal β-D-Gal*p* residues diminished but the distinguishable signal still remained in the ^{1}H-NMR spectrum of the polysaccharide after the enzymatic digestion. In addition, the structural analysis of the hexasaccharide, which was isolated through the mild acid hydrolysis of the polysaccharide, showed that the occurrence of α-D-Gal*p* residues should be restricted to the main chain of the polysaccharide (see mild acid hydrolysis section).

Mild Smith degradation.—Mild Smith degradation, including periodate oxidation of the polysaccharide followed by reduction and mild acid hydrolysis with 0.2 M CF_3CO_2H at 30°C for 24 h, gave a water-insoluble polysaccharide in the non-dialyzable fraction. Methylation analysis of this product (Table 1, PS-sd) indicated that it contains 3-linked-D-Glc*p*, 3-linked D-Gal*p*, 5-linked D-Gal*f* and 3,5-linked D-Gal*f* in the molar ratio 2.2:1.0:0.8:0.2. This result indicated that the repeating unit of the main chain of this polysaccharide consists of two 3-linked D-Glc*p*, one 3-linked D-Gal*p* and one 5-linked β-D-Gal*f*, and confirmed that the side chain contains no periodate-oxidation resistant sugar residues, such as 3-linked glycoses. Consequently, it was proved that disaccharide of β-D-Gal*p*(1 → 4)-β-D-Glc*p* is attached to O-3 of the 5-linked β-D-Gal*f* residue. The occurrence of 3,5-linked D-Gal*f* (as a branching sugar) with no terminal sugar residue in the mild Smith degradation product may be due to the incomplete acid hydrolysis of the

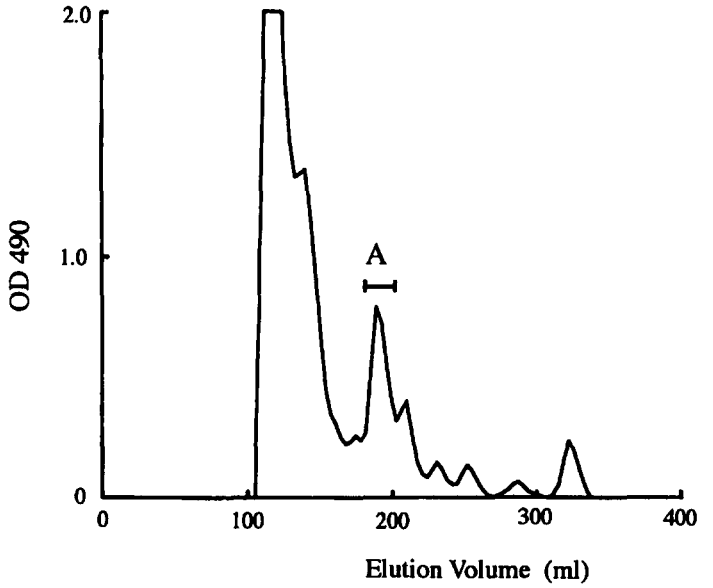


Fig. 2. Elution profile of the mild acid hydrolysis products on Bio-Gel P-2 column.

O-glycosyl bond between the "stub", which has been derived by the periodate oxidation of 4-linked D-Glc*p* in the side chain, and O-3 of the 5-linked D-Gal*f* residue [14].

Mild acid hydolysis.—In order to determine the sequence and anomeric configurations of the monosaccharide residues in the repeating unit of the polysaccharide, it was subjected to mild acid hydrolysis with 0.5 M CF_3CO_2H for 1 h at 90°C, and the resulting oligosaccharides were fractionated by gel permeation chromatography on Bio-Gel P-2 (Fig. 2). The main oligosaccharide fraction (Fraction A) was analyzed by methylation and NMR spectroscopy.

Acid hydrolysis of Fraction A and $NaBH_4$-reduced Fraction A gave D-Gal and D-Glc in the molar ratio 1.0:1.0, and D-Glc, D-Gal and galactitol (Gal-ol) in the molar ratio 3.3:2.3:1.0, respectively, indicative that the reducing end of the component oligosaccharide is occupied by a D-galactose residue. Methylation analysis of Fraction A gave terminal D-Gal*p*, 3-linked D-Glc*p*, 4-linked D-Glc*p*, and 3,5-linked D-Gal*f* in the molar ratio 1.9:2.0:1.1:1.0, with two minor partially methylated alditols (Table 1, Fraction A). The 500 MHz ^{1}H-NMR spectrum of reduced-Fraction A at 25°C contained five major H-1 signals of nearly equal intensity at δ 5.386 (d, $J_{1,2}$ 4.0 Hz, residue *a*), δ 5.361 (d, $J_{1,2}$ 4.0 Hz, residue *b*), δ 4.696 (d, $J_{1,2}$ 7.5 Hz, residue *d*), δ 4.585 (d, $J_{1,2}$ 7.5 Hz, residue *e*) and δ 4.454 (d, $J_{1,2}$ 8.0 Hz, residue *f*) and several minor H-1 signals (Fig. 3, residues were labelled according to the order of those in Fig. 1). This ^{1}H-NMR spectrum together with the results of monosaccharide and methylation analyses indicated that the Fraction A contained a branched hexasaccharide terminated as its reducing end by a Gal*f* residue. Assigments of the non-anomeric protons of residues *a*, *b*, *d*, *e*, and *f* in the hexasaccharide-alditol were made on the basis of cross-peaks observed in the TNTOCSY spectrum of the reduced Fraction A as far as H-4 or H-5 (Fig. 4). On the

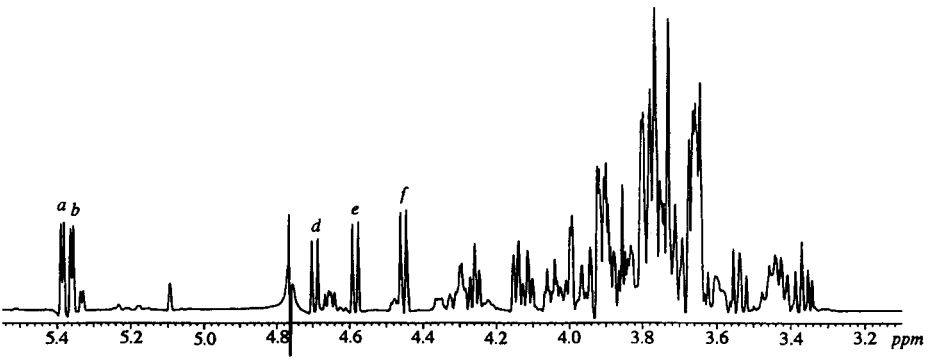


Fig. 3. The 500 MHz ^{1}H-NMR spectrum of the reduced-fraction A.

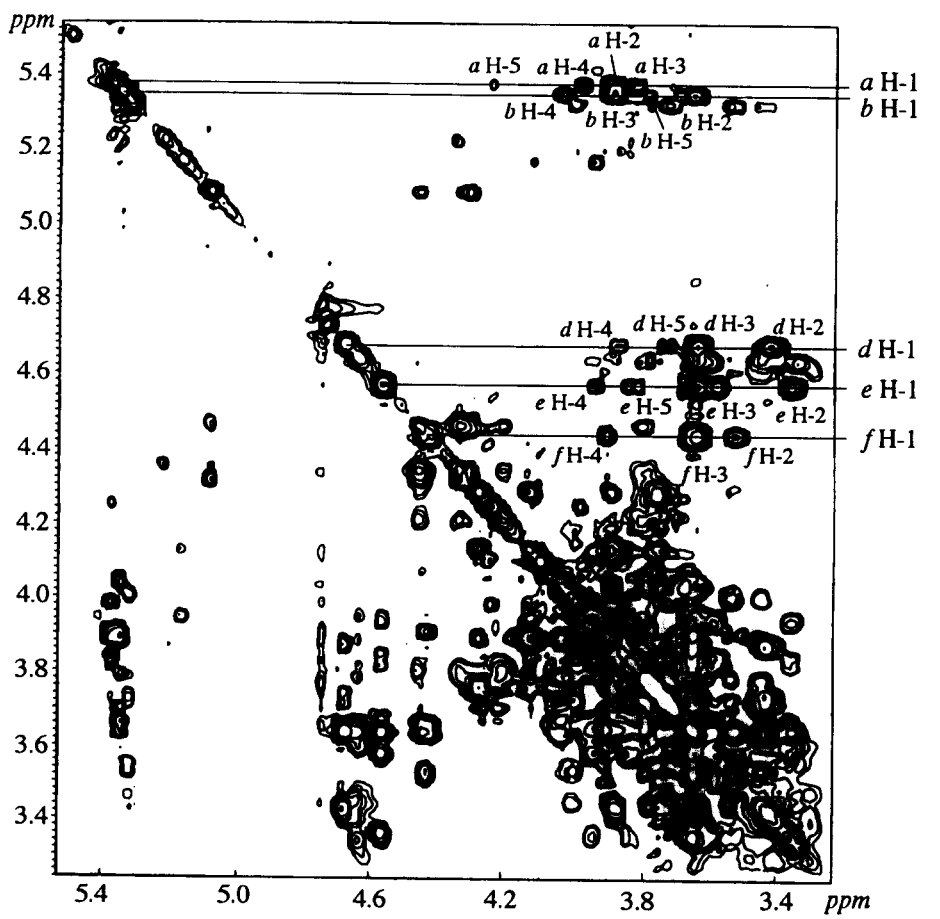


Fig. 4. The 500 MHz 2D TNTOCSY spectrum of the reduced-fraction A.

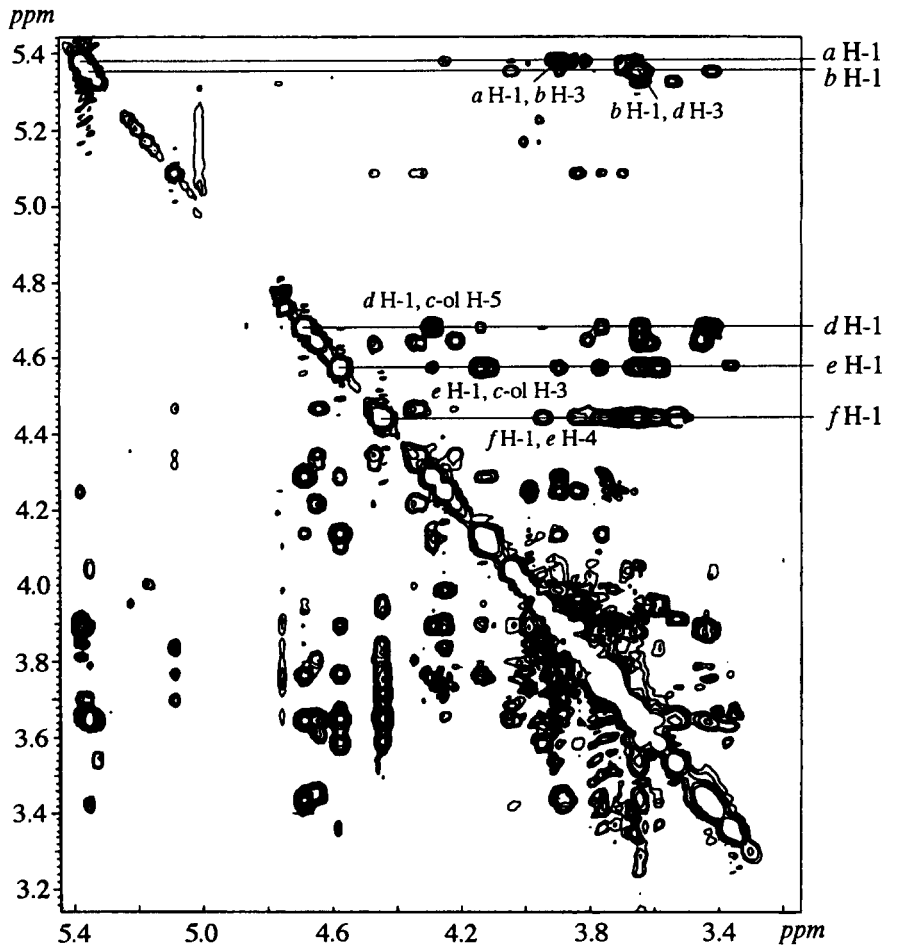
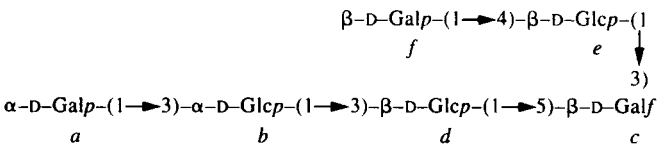


Fig. 5. The 500 MHz 2D TNROESY spectrum of the reduced-A.

H-1 tracks of residues *a*, *b*, *d*, *e*, and *f* in the TNTOCSY spectrum, cross-peaks were detected with H-2,3,4,5, and with H-2,3,4, respectively. The chemical shifts of the monosaccharide residues in the hexasaccharide are listed in Table 2.

The TNROESY spectrum of reduced-Fraction A at 25°C allowed determination of the sequential order of the component monosaccharide residues of the hexasaccharide (Fig. 5). The inter-residue connectivities *a* H-1,*b* H-3, *b* H-1,*d* H-3, and *f* H-1,*e* H-4 were observed for strong cross-peaks on the corresponding H-1 tracks in the TNROESY spectrum, which established the sequences and linkages as $a(1 \rightarrow 3)b(1 \rightarrow 3)d$ and $f(1 \rightarrow 4)e$. Taking account of the data hitherto obtained by methylation analysis of the Fraction A, by mild Smith degradation and by β-galactosidase digestion of the polysaccharide, residues *a*, *b*, *d*, *e*, and *f* were identified as terminal α-D-Gal*p*, 3-linked α-D-Glc*p*, 3-linked β-D-Glc*p*, 4-linked β-D-Glc*p* and terminal β-D-Gal*p*, respectively. In addition, the cross-peaks observed at δ 4.298 on the H-1 track of residue *d* and δ 4.147 on the H-1 track of residue *e* in the TNROESY spectrum were attributed to the

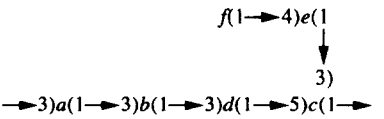
inter-residue connectivity between H-5 and H-3 of Gal-ol residue (residue C-ol), respectively. These data described above permit the structure of the hexasaccharide to be formulated as follows (**1**):



(1)

Acetolysis.—In order to obtain the additional structural information of the polysaccharide, it was subjected to acetolysis as the same manner as *L. helveticus* TY1-2 polysaccharide [8]. The products obtained by acetolysis of the native polysaccharide were de-*O*-acetylated then separeted by column chromatography on Bio-Gel P-2. The constituent saccharides in the major fractions II, III, and IV were estimated to be di-, tri- and tetra-saccharide, respectively, on the basis of their R_f values on silica gel TLC. The results of methylation analysis and ^{1}H-NMR spectroscopy of the oligosaccharide fractions were not simple, suggesting that they contained more than one oligosaccharide with different sugar composition and sequence. Among the oligosaccharide fractions obtained, fraction III gave relatively simple results of methylation and NMR analyses, therefore fraction III was reduced with $NaBD_4$ and the resulting oligosaccharide alditols were subjected to methylation analysis. GLC–MS of the hydrolyzate of the methylated $NaBD_4$-reduced fraction III (Table 1, reduced III) gave terminal D-Glc*p*, terminal D-Gal*p*, 3-linked D-Glc*p*, 3-linked glucitol, and 5-linked galactitol in the molar ratio approximately 6.2:1.0:7.1:1.0:6.2. This result suggested that the fraction III consists of two trisaccharides, D-Glc*p*-(1 → 3)-D-Glc*p*-(1 → 5)-D-Gal*f* and D-Gal*p*-(1 → 3)-D-Glc*p*-(1 → 3)-DGlc*p*. The production of these trisaccharides on acetolysis was consistent with the proposed structure (**1**) of the hexasaccharide obtained by mild acid hydrolysis.

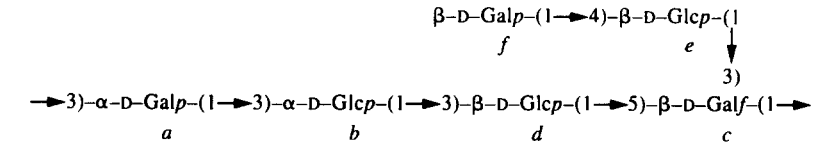
500 MHz 2D ROESY spectroscopy.—ROESY spectrum of the native polysaccharide at 70°C allowed direct, unambiguous determination of the glycosyl sequence and linkage types (Fig. 6). The ROESY spectrum showed inter-residue NOEs for *a* H-1, *b* H-3, *b* H-1,*d* H-3, *d* H-1,*c* H-5, *c* H-1,*a* H-3, *f* H-1,*e* H-4, and *e* H-1,*c* H-3, which indicated the sequence and linkages of the component monosaccharides depicted in (**2**).



(2)

This ROESY spectroscopy of the native polysaccharide established the sequence and the type of linkages for native polysaccharide, in agreement with the results of structural analyses hitherto obtained.

Overall, the data permit the primary structure of the repeating units of the polysaccharide produced by *L. helveticus* TN-4 (designated EPS-B) to be formulated as follows (**3**):



(3)

As mentioned in the Introduction, the most remarkable structural difference between the polysaccharide of *L. helveticus* TY1-2 (designated EPS-A, structure in Fig. 7) and that of EPS-B is the presence or the absence of 2-acetamido-2-deoxy-D-glucose as a component monosaccharide. In addition, when comparing the two proposed structures, EPS-B is quite different from EPS-A in the linkages and anomeric configurations of sugar residues involved in the backbone of the chain, except for the occurrence of 3-linked β-D-Glc*p* residues. EPS-B has, however, a structural similarity with that of EPS-A in possessing a disaccharide side-chain sequence, β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → , which differs from EPS-A by its attachment to C-3 of 5-linked β-D-Gal*f* residues instead of C-6 of 3-linked β-D-Gal*p* residues in the case of EPS-A.

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, 0.25mm × 50 m) coated with OV-1 (GLscience, Co. Ltd., Japan) was used for separation of 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 and II, 150°C for 5 min, then 2°C /min to 200°C. GLC–MS spectra 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 SEPA 200 polarimeter. TLC was carried out on Silica Gel 60 aluminum sheets (Merck, Germany) and the solvent system was 2:1:1 (v/v) $CH_3CO_2C_2H_5$–CH_3CO_2H–H_2O.

NMR spectroscopy.—^{13}C-NMR spectroscopy (internal standard; 1,4-dioxane) was performed with a JEOL GSX-280 spectrometer for D_2O solutions at 70°C. For the ^{1}H-NMR spectra, polysaccharide or oligosaccharide samples were repeatedly exchanged in D_2O with intermediate lyophilizations. 500-MHz ^{1}H-NMR, 2D HOHAHA and 2D ROESY spectra (internal standard; 3-trimethylsilylpropanoate) of the native polysaccharide were recorded using a Bruker AMX-500 spectrometer with standard Bruker programs for D_2O solutions at 70°C. 500 MHz ^{1}H-NMR, 2D TNTOCSY and TNROESY spectra (internal standard; 3-trimethylsilylpropanoate) of a mild acid hydrolysis product

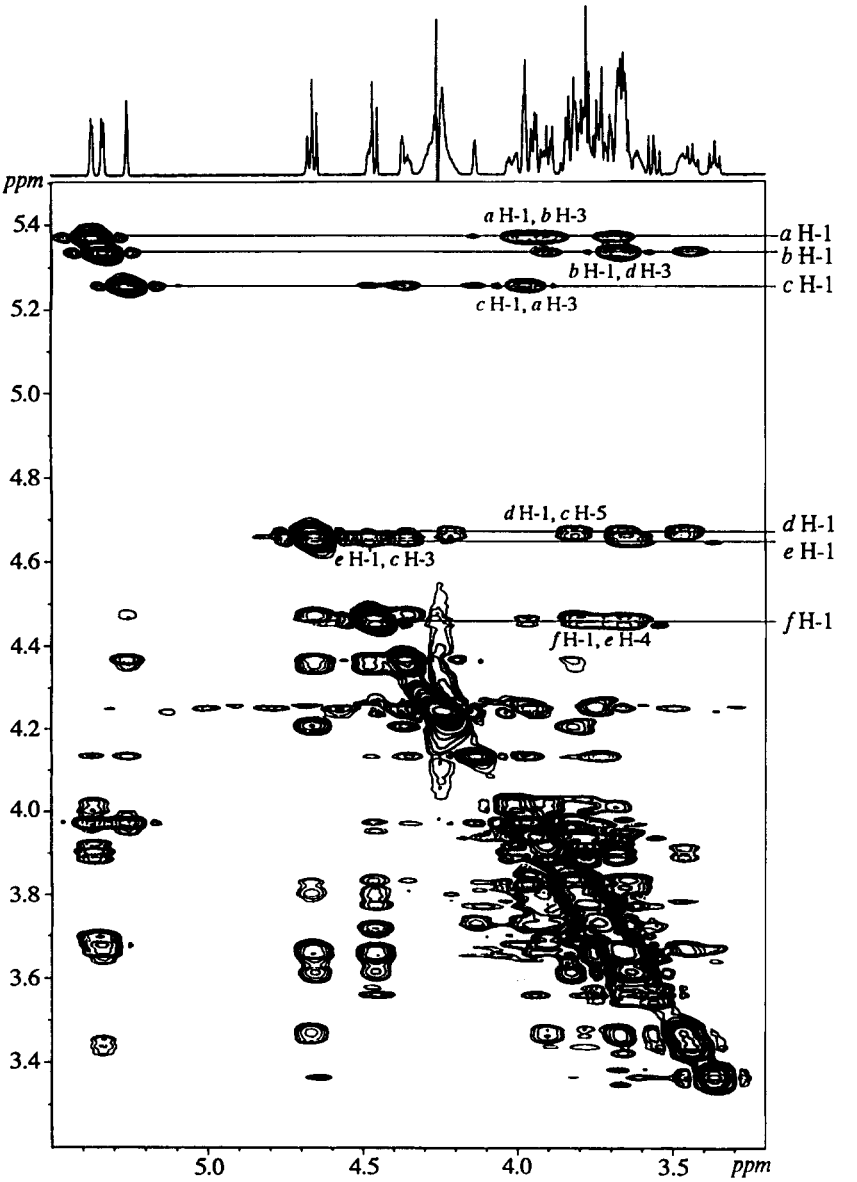


Fig. 6. The 500 MHz 2D ROESY spectrum of the the native polysaccharide produced by *L. helveticus* TN-4.

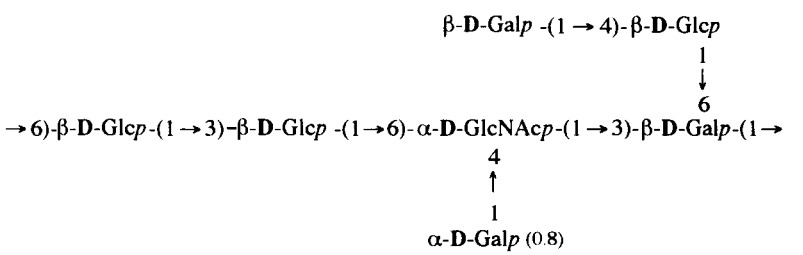


Fig. 7. The structure of the polysaccharide of *L. helveticus* TY1-2 [8].

were recorded using a Varian Unity-500 spectrometer with its standard programs for D_2O solutions at 25°C. The 400 MHz ^{1}H-NMR spectrum of β-galactosidase-digested polysaccharide (internal standard; 3-trimethylsilyl-propanoate) was recorded with a JEOL α-400 spectrometer for a solution in D_2O at 70°C.

Organism.—*L. helveticus* TN-4 which is a mutant strain of *L. helveticus* TYl-2 was used in this study. This mutant had been isolated from the stock culture of TY1-2 strain stored at −19°C for 1 year in a medium containing 1.5% yeast extract (Difco Laboratories, Detroit, MI), 1% lactoalbumin hydrolysate (Difco Laboratories, Detroit, MI) and 2% glucose. Isolated *L. helveticus* TN-4 has been stored at −85°C in a medium containing 5% skim milk powder and 5% monosodium L-glutamate monohydrate salt until use.

Isolation and purification of polysaccharide.—A preculture of *L. helveticus* TN-4 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), polysaccharide was precipitated by the addition of 95% EtOH to the supernatant (1:1, v/v) at 4°C. Polysaccharide precipitated was collected by centrifugation (10,000 rpm, 20 min). This EtOH-precipitation procedure was repeated three times to produce crude polysaccharide. Then, it was dissolved in 10 mL of 25 mM Tris · HCl buffer (pH 8.5), and the solution was applied onto a column (2.5 × 20 cm) of DEAE-cellulose DE-52 (Whatman Co. Ltd., Japan) equilibrated with the same buffer. Polysaccharide was 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 [15]. Fractions containing carbohydrate were collected, dialyzed against deionized water, and then lyophilized.

Measurement of molecular weight.—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 flow-rate of 1.0 mL/min. Several pullulan standards with 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. The absence of amino sugars in this polysaccharide was confirmed by HPLC at 40°C on an Asahipak NH_2P-50 column (4.6 × 250 mm, Asahi Chemical Industry 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. Neutral sugars were analyzed by HPLC at 60°C on a Wakopak WB-T131-E column (7.8 × 300 mm, Wako Pure Chemical Industries, Ltd., Japan). The eluent consisted of 0.1 mM NaOH and its flow-rate was 0.5 mL/min.

Methylation analysis.—The carbohydrate samples were completely methylated after three successive methylations by Hakomori's method [10]. The permethylated carbohydrate was treated with aq 90% formic acid (1 mL for 2 h at 100°C), the solution 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. An excess of $NaBH_4$ was decomposed by the addition of Amberlite IR-120 (H^+) resin 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 pyridine–$(CH_3CO)_2O$ (1:1, v/v) 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. This 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 reaction mixture was measured by 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 then heated for 4 min at 100°C to inactivate the enzyme. The solution was centrifuged at 10,000 rpm for 15 min to remove insoluble materials, and the supernatant was dialyzed against deionized water, and the polysaccharide in the dialyzate was recovered by lyophilization. The resulting digested polysaccharide was subjected to methylation and ^{1}H-NMR analyses.

Mild Smith degradation.—The polysaccharide (100 mg) was dissolved in 50 mM $NaIO_4$ (100 mL) and the solution was stirred for 6 days at 5°C in the dark. Excess ethylene glycol was added to the solution and 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. The oxidized-reduced polysaccharide (40 mg) was hydrolyzed with 0.2 M CF_3CO_2H (15 mL) at 30°C for 24 h. The acid was removed by repeated evaporations in the precence of methanol. The resulting materials were subjected to methylation analysis.

Mild acid hydrolysis.—The polysaccharide (100 mg) was dissolved in 0.5 M CF_3CO_2H and the solution was heated for 1 h at 90°C. After hydrolysis, the acid was removed by repeated evaporations in the presence of methanol. The resulting hydrolyzate was fractionated at 24°C on Bio-Gel P2 column (2.6 × 80 cm) using water as an eluent (28 mL/h). The carbohydrate content of each fraction was determined by the phenol–H_2SO_4 method. The fraction A was subjected to monosaccharide and methylation analyses. For ^{1}H-NMR experiments, the fraction A was reduced with $NaBH_4$ at room temperature for 14 h. An excess of $NaBH_4$ was decomposed by the addition of Amberlite IR-120 (H^+) resin and the solution was evaporated under reduced pressure to dryness. After removal of borate by co-evaporation with CH_3OH, the sample was dissolved in D_2O and then lyophilized.

Acetolysis.—The polysaccharide (100 mg) was acetylated with pyridine–Ac_2O (1:1, v/v, 20 mL) at 60°C for 24 h. The resulting acetates were dissolved in $(CH_3CO)_2O$–CH_3CO_2H–H_2SO_4 (12:8:1.5, v/v, 21.5 mL). The reaction mixture was kept at 25°C for 7 days. Then the reaction 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 de-*O*-acetylated with 5.6% (v/v) CH_3ONa–CH_3OH (10 mL) at 25°C for 3 h. The resulting mixture was poured into ice–water and neutralized with M HCl and evaporated to dryness. The mixture of oligosaccharides was fractionated at 40°C on a Bio-Gel P2 column (2.6 × 80 cm) using water as an eluent (12 mL/h). The carbohydrate content of each fraction was determined by the phenol–H_2SO_4 method.

The fraction III was reduced with $NaBD_4$ at room temperature for 14 h. An excess of $NaBD_4$ was decomposed by the addition of Amberlite IR-120 (H^+) resin and the solution was evaporated under reduced pressure to dryness. After removal of borate by co-evaporation with CH_3OH, the sample was subjected to methylation analysis.

Acknowledgements

The authors thank the NMR application section of Bruker Japan (Tsukuba) and Varian Japan (Tokyo) for recording the 500 MHz 2D NMR spectra.

References

[1] P. Kooiman, *Carbohydr. Res.*, 7 (1968) 200–211.
[2] M. Pidoux, J. M. Brillouet, and B. Quemener, *Biotech. Lett.*, 10 (1988) 415–420.
[3] T. Toba, K. Arihara, and S. Adachi, *Milchwissenschaft*, 42 (1987) 565–568.
[4] T. Mukai, T.Toba, T. Itoh, and S. Adachi, *Carbohydr. Res.*, 204 (1990) 227–232.
[5] M.C. Manca de Nadra, A.M. Straser de Saad, A.A. Pesce de Ruiz Holgado, and G. OLiver, *Milchwissenschaft*, 40 (1985) 409–411.
[6] J. Cerning, C. Bouillanne, M.J. Desmazeaud, and M. Landon, *Biotech. Lett.*, 8 (1986) 625–628.
[7] M. Gruter, B. R. Leeflang, J. Kuiper, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 239 (1993) 209–226.
[8] Y. Yamamoto, S. Murosaki, R. Yamauchi, K. Kato, and Y. Sone, *Carbohydr. Res.*, 261 (1994) 67–78.
[9] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1979) 349–357.
[10] S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
[11] R.G.S. Ritchie, N. Cyr, B. Korsch, H.J. Koch, and A.S. Perin, *Can. J. Chem.*, 53 (1975) 1424–1433.
[12] E. Parra, J.J. Barbero, M. Bernabe, J. A. Leal, A. Prieto, and B. G. Miranda, *Carbohydr. Res.*, 257 (1994) 239–248.
[13] T. Takayanagi, A. Kimura, S. Chiba, and K. Ajisaka, *Carbohydr. Res.*, 256 (1994) 149–158.
[14] E. Kishida, Y. Sone, and A. Misaki, *Carbohydr. Polym.*, 17 (1992) 89–95.
[15] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.