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STRUCTURE OF AN EXOCELLULAR POLYSACCHARIDE PRODUCED BY *Streptococcus thermophilus**

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ABSTRACT

Streptococcus thermophilus strains grown on skimmed milk produced a viscosifying, exocellular, and water-soluble polysaccharide which contains D-glucose, D-galactose, and *N*-acetyl-D-galactosamine in the ratio of 1:2:1. Methylation analysis identified the glycosidic linkages in the tetrasaccharidic repeating-unit, and Smith degradation and nitrous deamination after *N*-deacetylation gave the sequence of monosaccharides in the repeating-unit. The anomeric configurations of the sugar residues were determined by oxidation of the peracetylated polysaccharide with chromium trioxide and by ^{1}H- and ^{13}C-n.m.r. spectroscopy. The following structure was assigned to the repeating unit of the polysaccharide, →3)-β-D-Gal*p*-(1→3)-[α-D-Gal*p*-(1→6)]-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1→.

INTRODUCTION

Many microorganisms produce exopolysaccharides which are located outside the cell wall, either attached to it in the form of capsules or secreted into the extracellular environment in the form of slime. Such polymers vary considerably in their chemical structure[1]. In the dairy industry, especially in France, the use of slime-producing bacteria in the starter culture to increase product-viscosity has been advocated by many researchers[2–7].

The polysaccharide produced by *Streptococcus thermophilus* or *Lactobacillus bulgaricus* would be commercially interesting, but detailed studies on these poly-

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saccharides are rare. Recent publications on *L. bulgaricus*[2-5] and *S. thermophilus*[3,6,7] gave carbohydrate compositions with large variations of the nature of the monosaccharides and of the molar carbohydrate composition of their exopolysaccharides. In addition, no data have been published on the primary structure of these exopolysaccharides from bacteria used in the dairy industry. We describe herein the purification, identification, and primary structure of an exocellular polysaccharide produced by a ropy strain of *S. thermophilus*.

RESULTS AND DISCUSSION

S. thermophilus bacteria were grown on sterilized, reconstituted, skimmed milk, and the exocellular polysaccharide was isolated, after Pronase digestion of milk protein, by ethanol precipitation and gel-permeation chromatography on Sephacryl S-1000. The treatment of 115 g of lyophilized fermented milk gave 42 mg of polysaccharide. The purified product obtained after Sephacryl S-1000 chromatography was shown to be homogeneous by gel-permeation chromatography on Sepharose 4B (mol. wt. 1.10^6).

Gas-liquid chromatography of per(trifluoroacetylated) methyl glycosides obtained after methanolysis and per(trifluoroacetylation) of the polysaccharide and of polyol acetates obtained after acid hydrolysis, reduction, and peracetylation indicated the presence of galactose, glucose, and 2-acetamido-2-deoxygalactose in the ratios of 1.95:1.0:0.89 (Table I).

After methanolysis of the permethylated polysaccharide, the partially methylated and acetylated methyl glycosides were analyzed by g.l.c.–m.s. Four methyl ethers were identified (Table II) as 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-galactoside, 2,4-di-*O*-methylglucoside, and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactoside. These results indicated that the exopolysaccharide from *S. thermophilus* is constituted of a tetrasaccharide repeating-unit having a (1→3)-linked backbone composed of one galactosyl, one glucosyl, and one 2-acetamido-2-

TABLE I

CARBOHYDRATE COMPOSITION[a] OF NATIVE POLYSACCHARIDE AND PERIODATE-RESISTANT PRODUCT FROM *S. thermophilus*

Monosaccharides	*Native polysaccharide*		*Periodate-resistant product*	
	Hydrolysis[b]	*Methanolysis*[c]	*Hydrolysis*[b]	*Methanolysis*[c]
Galactose	1.98	1.95	0.9	1.13
Glucose	1.0	1.0	1.0	1.0
2-Acetamido-2-deoxygalactose	0.73	0.89	0.81	0.73

[a]Molar proportions; glucose taken as 1.0. [b]4M Trifluoroacetic acid, 100°, 4 h. [c]0.5M HCl in methanol, 80°, 24 h.

TABLE II

G.L.C. ANALYSIS OF METHYL ETHERS OBTAINED FROM METHYLATED NATIVE POLYSACCHARIDE (A), PERIODATE-RESISTANT PRODUCT (B), AND OLIGOSACCHARIDE OBTAINED BY *N*-DEACETYLATION AND NITROUS ACID DEAMINATION (C) FROM *S. thermophilus*

Methylated sugars[a]	*Molar ratio*[b]		
	A	*B*	*C*
2,3,4,6-Tetra-*O*-Me-Gal	0.80		2.4
2,4,6-Tri-*O*-Me-Gal	1.0	1.0	
2,4,6-Tri-*O*-Me-Glc		0.6	
2,4-Di-*O*-Me-Glc	0.83	0.17	1.0
4,6-Di-*O*-Me-GalN(AcMe)	0.8	0.4	
2,5-Anhydro-1,4,6-tri-*O*-Me-Talol			0.8

[a]As methyl glycosides, except the talitol derivative. [b]Values are given relative to one unit of 2,4,6-tri-*O*-methylgalactoside (A and B), and one unit of 2,4-di-*O*-methylglucoside (C).

deoxygalactosyl residue and a side-chain of a (1→6)-linked nonreducing galactosyl group.

The insoluble, periodate-resistant product obtained by Smith degradation of the native exopolysaccharide yielded galactose, glucose, and 2-acetamido-2-deoxy-galactose in the molar ratio of 1.13:1.0:0.73. As expected from the results of the methylation analysis, the results indicated that only the side-chain galactosyl group

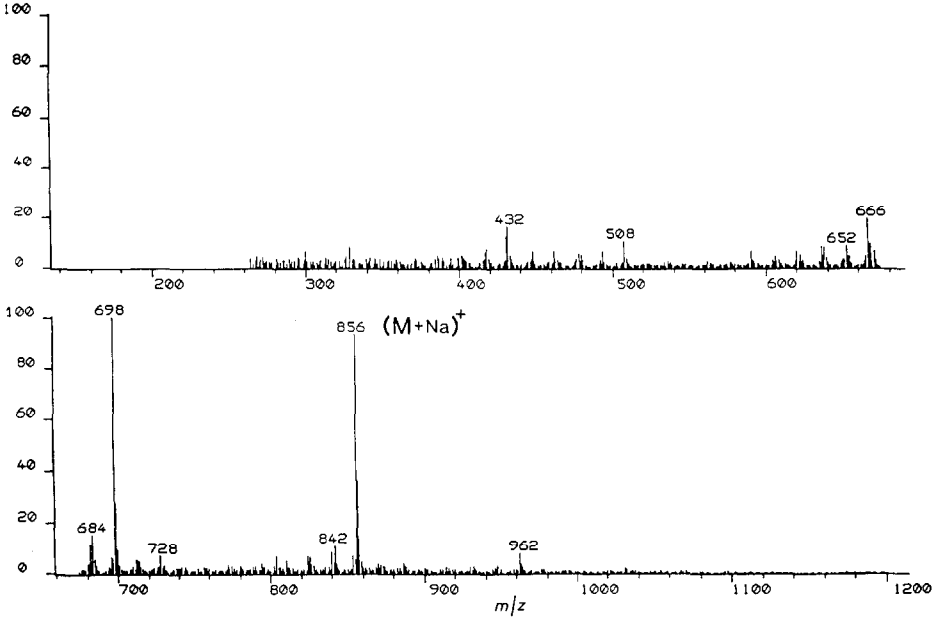
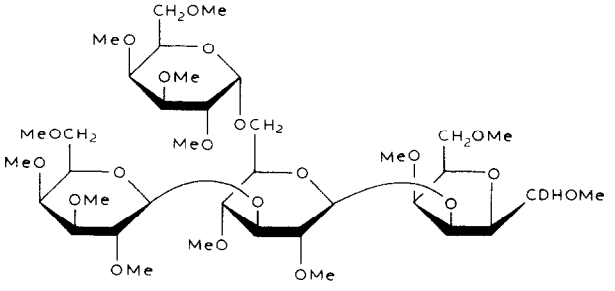


Fig. 1. F.a.b.–m.s. spectrum of permethylated oligosaccharide alditol **1** obtained by methylation of oligosaccharide alditol obtained by *N*-deacetylation–nitrous acid deamination degradation of *S. thermophilus* polysaccharide.

was degraded during the periodate oxidation. Methylation analysis of the periodate resistant product (Table II) yielded three major, methylated monosaccharides, *i.e.*, 2,4,6-tri-*O*-methylgalactoside, 2,4,6-tri-*O*-methylglucoside, and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactoside, showing a linear (1→3)-linked trisaccharide repeating-unit for the periodate-resistant polysaccharide, and confirming that the side-branch galactosyl group was linked to the glucose residue of the backbone by a (1→6) linkage.

In order to determine the sequence of monosaccharides in the tetrasaccharide repeating-unit, the 2-acetamido-2-deoxygalactosyl residue of the polysaccharide was *N*-deacetylated and the resulting product subjected to nitrous acid deamination, followed by reduction of the liberated oligosaccharide. The monosaccharide composition of the oligosaccharide isolated by Bio-Gel P-2 chromatography showed the transformation of the 2-acetamido-2-deoxygalactose into a 2,5-anhydrotalitol unit, and the presence of galactose and glucose. These results were confirmed by the analysis of the permethylated oligosaccharide (**1**) which showed (Table II) three methyl ethers, *i.e.*, 2,3,4,6-tetra-*O*-methylgalactoside, 2,4-di-*O*-methylglucoside, and 2,5-anhydro-1,4,6-tri-*O*-methyltalitol. The tetrasaccharide nature of this oligosaccharide was confirmed by f.a.b.-m.s. analysis of the permethylated oligosaccharide in the positive ion-mode which showed a molecular-ion at *m/z* 856 $(M + Na)^+$ (Fig. 1). These results are in accordance with the structure Gal-(1→3)-[Gal-(1→6)]-Glc-(1→3)-2,5-anhydroTal for the oligosaccharide obtained by *N*-deacetylation–nitrous deamination from the native polysaccharide.

To determine the anomeric configuration of the various sugar residues, the polysaccharide was oxidized with chromium trioxide. This degraded all D-glucosyl residues (Table III), indicating the β configuration, and half of the galactosyl residues, indicating that half these residues have the β configuration and half the α



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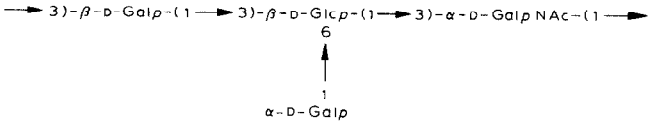

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TABLE III

OXIDATION OF *S. thermophilus* POLYSACCHARIDE WITH CHROMIUM TRIOXIDE

Time of oxidation (h)	*Monosaccharides*[a]		
	Glucose	*Galactose*	*2-Acetamido-2-deoxy-galactose*
0	1	1.98	0.73
1	0.24	0.99	1
2	0.14	0.80	1

[a]As alditol acetates.

configuration. The proportion of 2-acetamido-2-deoxygalactose did not decrease, thus indicating the α configuration. These results led to structure

$$\rightarrow 3)\text{-}\beta\text{-Gal-}(1\rightarrow 3)\text{-}[\alpha\text{-Gal-}(1\rightarrow 6)]\text{-}\beta\text{-Glc-}(1\rightarrow 3)\text{-}\alpha\text{-GalNAc-}(1\rightarrow$$

or

$$\rightarrow 3)\text{-}\alpha\text{-Gal-}(1\rightarrow 3)\text{-}[\beta\text{-Gal-}(1\rightarrow 6)]\text{-}\beta\text{-Glc-}(1\rightarrow 3)\text{-}\alpha\text{-GalNAc-}(1\rightarrow$$

for the repeating unit of the polysaccharide.

To choose between these two repeating units, the native polysaccharide and the periodate-resistant product were subjected to n.m.r. analysis. The ^{1}H-n.m.r. spectrum of the native polysaccharide showed two signals for H-1α, a singlet at δ 5.088 ($J_{1,2}$ 3.65 Hz) and one at δ 4.989 ($J_{1,2}$ 1.46 Hz), and two signals for H-1β, a doublet at δ 4.613 ($J_{1,2}$ 7.67 Hz) and one at δ 4.418 ($J_{1,2}$ 10 Hz). The ^{1}H-n.m.r. spectrum of the periodate-resistant product showed the disappearance of the resonance for H-1α at δ 4.989, indicating that the terminal, nonreducing galactosyl groups that had been eliminated by the Smith degradation had the α-D configuration. In the same way, a comparison between the ^{1}H-n.m.r. spectra of the native

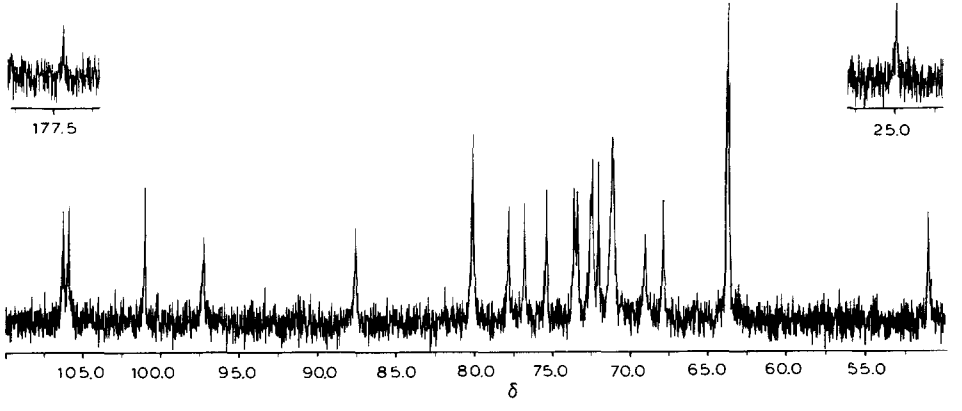

Fig. 2. ^{13}C-N.m.r. spectrum of exopolysaccharide from *S. thermophilus*.

and the periodate-resistant degradation product showed a shift of the H-1β doublet from δ 4.613 to 4.584, indicating that the attachment of the side-chain galactosyl group affected the anomeric proton of the β-D glucosyl residue, thus allowing the assignment of the chemical shift at δ 4.613 to 1-Hβ of the glucosyl residue. The signals of the two other anomeric protons were not modified by the Smith degradation, *i.e.*, H-1 δ 5.088 and 4.418 attributed to H-1 protons of the 2-acetamido-2-deoxy-α-D-galactosyl and β-D-galactosyl residues, respectively, of the backbone. These results are in accordance with structure **2** for the repeating unit of the polysaccharide from *S. thermophilus*.

The periodate-resistant product was not sufficiently soluble in deuterium oxide for a meaningful analysis of its ^{13}C-n.m.r. spectrum. The ^{13}C-n.m.r. spectrum of the native polysaccharide (Fig. 2) revealed four well-resolved signals with nearly equal intensities in the anomeric carbon region. Chemical shifts at δ 106.28 and 105.92 were attributed to C-1 of the β-Gal and β-Glc units of the backbone, respectively, on the basis of the chemical shift at δ 105.2 for C-1 of a 3-*O*-linked β-D-galactosyl residue[8] and of the chemical shift (δ 104.5) for C-1 of a 3,6-di-*O*-substituted D-glucosyl residue in β-D-glucans [9]. The chemical shift at δ 101.02 was assigned to C-1 of the side-chain D-galactosyl group on the basis of the chemical shift for C-1 of the D-galactosyl group in the trisaccharide, α-D-Gal*p*-(1→6)-[β-D-Man*p*-(1→4)]-α-D-Man[10].

The last anomeric-carbon chemical shift at δ 97.23 corresponds to C-1 of the 2-acetamido-2-deoxy-D-galactosyl residue. This value is close to that for C-1 of the D-galactosyl residue in the disaccharide β-D-Glc-(1→3)-α-D-GalOMe (δ 100.0)[11]. The presence of *N*-acetylhexosamine units in the polysaccharide from *S. thermophilus* could be confirmed by the signals at δ 176.88 and 24.92 corresponding to the carbon atoms of the 2-acetamido group.

The chemical shifts of the carbon atoms of the disubstituted D-glucose units in the main chain were assigned by comparison with the values described for 3,6-di-*O*-substituted D-glucosyl residues in β-D-glucans[9], especially at δ 87.6 for C-3 of the 3-*O*-substituted unit and at δ 69.06 for C-6 of the 6-*O*-substituted unit. The signal at δ 80.15 was assigned to C-3 of the 3-*O*-substituted β-D-galactosyl residue in the main chain by comparison with the value for C-3 of the internal D-galactosyl residue in the trisaccharide, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-α-D-Glc[8]. The signals at δ 51.10 and 63.70 were assigned to C-2 and C-6 of the 2-acetamido-2-deoxy-D-galactosyl residues, respectively[12].

EXPERIMENTAL

N.m.r. spectroscopy analysis. — ^{13}C-N.m.r. spectrum at 100 MHz were recorded with a Bruker AM-400 WB spectrometer, coupled with an Aspect 3000 calculator (Centre Commun. de Mesures, USTL-FA). The sample (50 mg.1 mL^{-1} of D_2O) was analyzed at 80° with the standard program POWGATE (1 H broadband with composite-pulse decoupling, D_1 0.1 s; PW = 90° = 6 μs; S_1 = S_2 = 1 w).

Spectral width was 26 000 Hz for 32K frequency-domain and time-domain data points. Chemical shifts are expressed relative to the signal of internal sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate (δ 0.0) with an accuracy of 0.1 p.p.m. ^{1}H-N.m.r. spectroscopy was performed with the same spectrometer on a sample of polysaccharide repeatedly treated with D_2O (99.95 atom %, ^{2}H, CEA) and intermediate lyophilization. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation according to Ernst[13].

Isolation and purification. — Ropy strains (CNCMI 733, CNCMI 734, CNCMI 735) of *S. thermophilus* were obtained from the particular collection of the Centre National de Cultures de Microorganismes (Institut Pasteur, Paris). A preculture was inoculated into sterilized, reconstituted skimmed milk (147 g.L^{-1}) and fermented at 43° until pH 5 was observed. The fermented milk was dialyzed for 3 days at 4° against distilled water, and the retentate was lyophilized to give a powder (115 g) that was dissolved in calcium acetate buffer (10mM, pH 8, 1 L). The casein was hydrolyzed with Pronase (2.3 g; Merck, Darmstadt) at an enzyme-to-substrate ratio of 1:50, pH 8, and 37° for 48 h in the presence of toluene; the pH was maintained at 8 by addition of M NaOH. This procedure was repeated three times, and then the proteins were precipitated by addition of 10% trichloroacetic acid.

After centrifugation, the supernatant solution was applied onto a column (42 × 3.2 cm) of Dowex 50-X8 (20–50 mesh; H^+), coupled with a column (42 × 3.2 cm) of Dowex 1-X8 (20–50 mesh; HCO_2^-). The neutral fraction was precipitated by addition of ethanol (3 vols.). The precipitate was collected by centrifugation (10 000 r.p.m., 4°), redissolved in water, and freeze-dried. The crude preparation (20 mg) was dissolved in 0.1M ammonium acetate buffer and applied to a column (50 × 1.6 cm) of Sephacryl S-1000 (LKB-Pharmacia, Stockholm), equilibrated and washed with the same buffer. Fractions were dialyzed against water and freeze-dried.

The molecular weight of the polysaccharide was determined by gel filtration on a column (92 × 1.6 cm) of Sepharose 4B (LKB Pharmacia), equilibrated in 0.05M phosphate buffer, pH 6.15. The column was calibrated with several dextran samples (Sigma Chem. Co., Saint Louis, MO; mol. wt. 5.10^6, 5.10^5, $2.3.10^5$, 4.10^4, and 1.10^4).

Carbohydrate analysis. — Qualitative and quantitative sugar analysis were carried out, after methanolysis (0.5M HCl in methanol, 24 h, 80°), by g.l.c. of the per(trifluoroacetylated) methyl glycosides[14] and, after hydrolysis (4M trifluoroacetic acid, 4 h, 100°) by g.l.c. of the alditol acetates[15].

Methylation analysis. — The polysaccharide was permethylated as described by Paz-Parente *et al.*[16]. The permethylated polysaccharide was subjected to methanolysis, and the partially methylated methyl glycosides were peracetylated with 1:5 (v/v) pyridine–acetic anhydride at room temperature overnight. The partially acetylated and methylated methyl glycosides were separated by g.l.c. and analyzed by g.l.c.–m.s under the conditions described by Fournet *et al.*[17].

Periodate oxidation[18]. — The polysaccharide (20 mg) was dissolved in aqueous 50mM $NaIO_4$ (10 mL) and the solution was kept for 168 h at room temperature in the dark. An excess of 1,2-ethanediol was then added to the solution and the mixture was dialyzed against water for 48 h. The retentate was treated with M KBH_4 at 20° for 14 h. After removal of the excess of KBH_4 by Dowex 50-X8 (20–50 mesh; H^+), the solution was dialyzed against distilled water. The retentate was hydrolyzed with 0.1M trifluoroacetic acid (5 mL) for 20 h at 20°. After elimination of trifluoroacetic acid by repeated evaporations in the presence of methanol, the resulting material was subjected to gel chromatography on a column (50 × 1.6 cm) of Fractogel TSK-HW40S (Merck, Darmstadt) using acetic acid 0.5% as eluent.

N-*Deacetylation and diazotation*. — For *N*-deacetylation, the polysaccharide (20 mg) was treated with a solution (5 mL) containing 2M NaOH and 2M KBH_4 at 100° for 6 h. After neutralization with 6M HCl, the solution was dialyzed. The retentate was subjected to chromatography on a column (50 × 1.6 cm) of Fractogel TSK-HW40S (Merck, Darmstadt) using 0.5% acetic acid in water as eluent. The *N*-deacetylated polysaccharide, dissolved in water (2 mL), was treated[19] with $NaNO_2$ (33 mg) and acetic acid (0.2 mL) at 4° for 20 h. After neutralization with Dowex 50-X8 (20–50 mesh; H^+), the resulting material was reduced with $NaBD_4$, followed by purification on Dowex 50-X8 (20–50 mesh; H^+) and gel filtration on a column (44 × 1.6 cm) of Bio-Gel *p*-2 (Bio-Rad) using 0.5% acetic acid in water as eluent.

Oxidation with chromium trioxide[20]. — The polysaccharide (5 mg) dissolved in formamide (2.5 mL) was acetylated with 1:5 (v/v) acetic anhydride–pyridine (2.5 mL). The peracetylated polysaccharide were dissolved in glacial acetic acid (3 mL), powdered Cr_2O_3 (45 mg) was added, and the mixture was stirred in an ultrasonic bath at 50°. Aliquots (1 mL) were removed at 0, 1, and 2 h, and immediately diluted with water. Each aliquot was partitioned between water and chloroform, and the chloroform extract was dried (Na_2SO_4) and evaporated to dryness. The residue was treated with 4M trifluoroacetic acid for 4 h at 100°. The alditol acetates were prepared in the usual way and analyzed by g.l.c.

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