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Structural studies of the antigenic polysaccharide of *Eubacterium saburreum*, strain T19

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ABSTRACT

The antigenic polysaccharide produced by *Eubacterium saburreum*, strain T19, contains unusual sugars, including D-*glycero*-D-*galacto*-heptose (Hep) and D-fucose (D-Fuc). A repeating unit of the polysaccharide is composed of a linear chain of D-*glycero*-D-*galacto*-heptopyranosyl tetrasaccharide as its backbone structure, i.e., -[→ 6)-β-Hep*p*-(1 → 3)-β-Hep*p*-(1-]$_2$ →, and a D-fucofuranosyl disaccharide as a branched group, i.e., α-D-Fuc*f*-(1 → 2)-α-D-Fuc*f*-(1 →, which is linked to O-4 of one (1 → 6)-linked D-*glycero*-D-*galacto*-heptopyranosyl residue. The polysaccharide also contains *O*-acetyl groups.

INTRODUCTION

Eubacterium saburreum is a nonsporing, Gram-positive, obligately anaerobic rod that inhabits the human oral cavity. This microorganism is particularly interesting because it produces a cell surface polysaccharide antigen of unique chemical composition. Investigations of the antigenic polysaccharides produced by *E. saburreum*, strains L44[1], T17[2], T18[3], T27[4], L49[5], O2[6], S29[7], T21[8], and T110[9] have shown that all contain a linear chain of β-D-*glycero*-D-*galacto*-heptopyranosyl residues as their backbone structure. According to the sugar composition, these polysaccharides containing D-*glycero*-D-*galacto*-heptopyranosyl residues can be classified into two chemotypes so far. The polysaccharides from strains of L44, T17, T18, and T27, which are all homoglycans composed of D-*glycero*-D-*galacto*-heptose units, are representatives of chemotype I. Those from strains of L49, O2, S29, T21, and T110, which are heteroglycans composed of D-*glycero*-D-*galacto*-heptose and 6-deoxy-D-*altro*-heptose, are representatives of chemotype II.

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Recently, a new strain, T19, of the same organism was isolated in our laboratory. It was found that the antigenic polysaccharide from the strain belongs to a new chemotype III and we report herein the chemical structure of the antigenic polysaccharide of *E. saburreum* T19.

RESULTS AND DISCUSSION

Acid hydrolysis of the antigenic polysaccharide yielded two monosaccharides, in the ratio of ~1:2, which were converted into alditols by reduction with $NaBD_4$ and then per-*O*-trimethylsilylated. The mass spectra of these compounds showed that one monosaccharide is a 6-deoxyhexose and the other a heptose. The monosaccharides, isolated by paper chromatography, were identified as D-fucose and D-*glycero*-D-*galacto*-heptose. The ^1H NMR spectra of the sugars were indistinguishable from those of authentic samples[6], and their optical rotations, $[\alpha]_D^{20}$ +76° (*c* 1.2, H_2O) for the 6-deoxyhexose and $[\alpha]_D^{20}$ +58° (*c* 1.0, H_2O) for the heptose, were in good agreement with published values[6]. Methylation analysis of the polysaccharide with analysis of the partially methylated sugars as alditol acetates, yielded 2,3,5-tri-, and 3,5-di-*O*-methyl-6-deoxyhexose, 2,4,6,7-tetra-, 2,3,4,7-tetra-, and 2,3,7-tri-*O*-methylheptose in the ratio of 0.85:1.15:1.90:1.13:1.00 (Table I). This result suggests that D-fucose occurs as furanosyl terminal groups and furanosyl chain residues linked through O-2, and heptose occurs as pyranosyl chain residues linked through O-3 and -6 respectively, and also heptose occurs as pyranosyl branching-residues linked at O-4,6. The results from the methylation analysis are in reasonably good agreement with the results of sugar analysis which showed that the ratio of D-fucose to D-*glycero*-D-*galacto*-heptose is 1:2.

Acid hydrolysis of the polysaccharide under mild conditions (50 mM H_2SO_4 at 80°C for 210 min) followed by dialysis, yielded a modified polysaccharide. The results from the methylation analysis of the modified polysaccharide are shown in Table I; the proportions of the sugar residues in the modified polysaccharide were

TABLE I

Alditol acetates obtained by methylation analysis of the antigenic polysaccharide from *Eubacterium saburreum*, Strain T19, and of the modified polysaccharide by mild acid hydrolysis

O-Methylalditol acetates of D-fucose and D-*glycero*-D-*galacto*-heptose	T_R [a]	Polysaccharide		Decrease or increase after mild-acid hydrolysis (B − A)
		Original (A)	Modified [b] (B)	
2,3,5-TriMeFuc	0.41	0.85	0.23	−0.62
3,5-DiMeFuc	0.52	1.15	0.28	−0.87
2,4,6,7-TetraMeHep	4.87	1.90	1.85	−0.05
2,3,4,7-TetraMeHep	5.11	1.13	1.83	+0.70
2,3,7-TriMeHep	6.99	1.00	0.34	−0.66

[a] Retention time, on OV-225 column, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b] Molar ratios relative to the original polysaccharide.

calculated on the assumption that the total amount of D-*glycero*-D-*galacto*-heptose would not change during mild acid hydrolysis, as no detectable amount of D-*glycero*-D-*galacto*-heptose could be demonstrated in the dialyzable fraction from the mild acid hydrolyzate. The major changes observed after mild hydrolysis are an increase in the amount of 2,3,4,7-tetra-*O*-methylheptose (+0.70), and a decrease in the amounts of 2,3,5-tri- (−0.62) and 3,5-di-*O*-methylfucose (−0.87), and 2,3,7-tri-*O*-methylheptose (−0.66). This observation indicates that the terminal fucofuranosyl groups are linked to O-2 of the 2-*O*-glycosylated fucofuranosyl residues and, furthermore, the 2-*O*-glycosylated fucofuranosyl residues are linked to 4,6-di-*O*-glycosylated heptopyranosyl residues.

The modified polysaccharide was subjected again to mild acid hydrolysis (50 mM H_2SO_4 at 100°C for 180 min) to give a polymeric product composed of D-*glycero*-D-*galacto*-heptose residues only after neutralization, and dialysis against distilled water. Methylation analysis showed that the hydrolyzate of the fully methylated polymeric product gave only 2,4,6,7-tetra- and 2,3,4,7-tetra-*O*-methylheptose, in the ratio of ~1:1. This result suggests that the backbone structure of the polysaccharide is composed of repeating units composed of D-*glycero*-D-*galacto*-heptose residues, and O-3- and O-6-linked-D-*glycero*-D-*galacto*-heptopyranosyl residues may alternate in the chain.

These methylation results indicate that the repeating unit of the original polysaccharide contains a linear tetrasaccharide backbone composed of (1 → 3)-, (1 → 6)-, and (1 → 4,6)-linked D-*glycero*-D-*galacto*-heptopyranosyl residues. To this backbone, the D-fucofuranosyl disaccharide side-chain is attached at O-4 of the 1,4,6-linked heptopyranosyl residue.

The molecular weight of the polysaccharide, M_w 60 000, was determined by gel filtration chromatography on a Toyopearl HW-55 column.

The optical rotation of the original polysaccharide, $[\alpha]_D^{20}$ +24° (*c* 1.2, H_2O), decreased on mild acid hydrolysis and the product, from which ~75% of the D-fucofuranosyl residues had been removed, showed $[\alpha]_D^{20}$ −12.5° (*c*, 0.8, H_2O). These results indicate that the D-*glycero*-D-*galacto*-heptopyranosyl residues are β linked and the D-fucofuranosyl residues α linked.

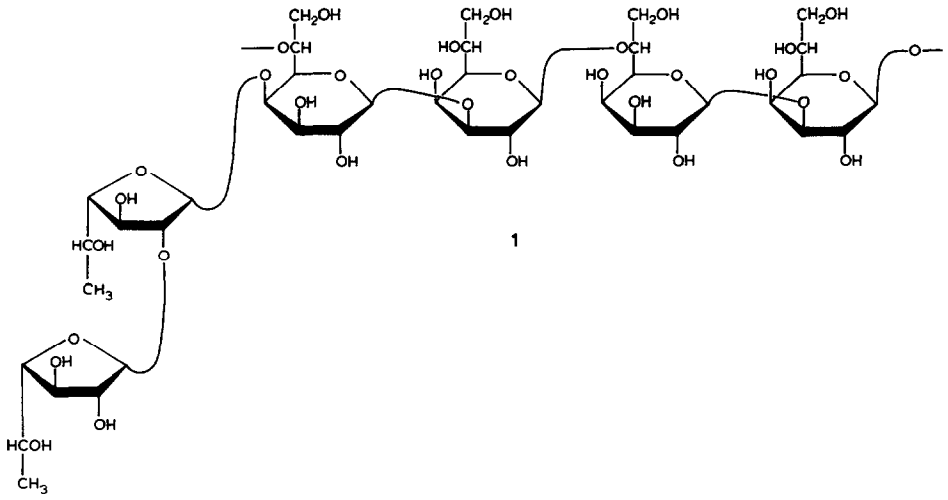
The ^1H NMR spectrum of the original polysaccharide shows signals for the methyl groups of D-fucofuranosyl residues at δ 1.29 (3 H, $J_{5,6a}$ 6.4 Hz) and 1.32 (3 H, $J_{5,6b}$ 6.4 Hz) for the *O*-acetyl groups at δ 2.20 (s, 4.5 H), and for the anomeric protons at δ 4.65 (1 H, $J_{1,2}$ 6.9 Hz,), 4.72 (1 H, $J_{1,2}$ 8.0 Hz), 4.75 (1 H, $J_{1,2}$ 6.9 Hz), 4.77 (1 H, $J_{1,2}$ 6.7 Hz), 5.17 (s, 1H), and 5.40 (s, 1H). The signals at δ 4.65 and 4.72 were assigned to H-1 of the β-(1 → 6)-linked D-*glycero*-D-*galacto*-heptopyranosyl residue, which is present in the antigenic polysaccharides from strains L44[1] (δ 4.67), T17[2] (δ 4.65), T27[4] (δ 4.67) and O2[10] (δ 4.62). The signals at δ 4.75 and 4.77 were assigned to H-1 of β-(1 → 3)-linked D-*glycero*-D-*galacto*-heptosyl residues. The two signals at δ 5.17 and 5.40 were weak for samples that had been subjected to mild acid hydrolysis and contained a low percentage of D-fucose; the signals were assigned to H-1 of α-D-fucofuranosyl residues.

The ^{13}C NMR spectrum of the deacetylated polysaccharide showed signals at δ 105.8, 101.9, and 101.5. The signal at δ 105.8 was assigned to C-1 of the α-D-fucofuranosyl residue on the basis of the data reported by Gorin and Mazurek[11]. The latter two signals were assigned to C-1 of D-*glycero*-D-*galacto*-heptopyranosyl residues, as shown in the spectra of polysaccharide T17[2]. The signals for the terminal methyl groups of the α-D-fucofuranosyl residue were observed at δ 19.8 and 19.1.

The polysaccharide contains *O*-acetyl groups, as shown by the ^1H NMR and IR spectra. They were located by the method of de Belder and Norrman[12], namely, the free hydroxyl groups were protected with methyl vinyl ethers and a trace of 4-toluenesulfonic acid, and then the polysaccharide was deacetylated, followed by methylation analysis. It is possible that migration of *O*-acetyl groups and hydrolysis of acetal groups occurred partly during this procedure. In order to avoid these problems, acetalation, deacetylation, and methylation were accomplished under nonbasic and low temperature conditions in the absence of water. The resulting, eight methylated components, in which the positions of methyl groups correspond to those of the *O*-acetyl groups of the original polysaccharide, were detected by GLC and identified by GLC–MS as per-*O*-acetyl-, 2-*O*-methyl-, and 3-*O*-methyl-fucose in the ratio of 17:2:1; and per-*O*-acetyl-, 3,7-di-*O*-methyl-, 7-*O*-methyl-, 2-*O*-methyl-, and 3-*O*-methylheptose in the ratio of ~ 63:4:28:2:3. These results indicate that the D-fucose residues are substituted by acetyl groups at O-2 (10%) and O-3 (5%), and that acetyl substituents are located at O-3,7 (4%), O-7 (28%), O-2 (2%), and O-3 (3%) of the heptose residues.

On the basis of the aforementioned results, the chemical structure for the repeating unit of the antigenic polysaccharide from *E. saburreum*, strain T19, was established as **1**. The polysaccharide also contains *O*-acetyl groups; namely, 10% of the D-fucose residues are acetylated at O-2 and 5% at O-3, and 4% of the D-*glycero*-D-*galacto*-heptose residues at O-3,7, 28% at O-7, 2% at O-2, and 3% at O-3.

The antigenic polysaccharides of *E. saburreum* strains L44, T17, T18, and T27 are homoglycans composed of D-*glycero*-D-*galacto*-heptose units (chemotype I); the heptose units of L44 and T27 are partly *O*-acetylated, whereas those of T17 and T18 are not. D-*glycero*-D-*galacto*-Heptose units, including partly *O*-acetylated units are also constituents of the backbone of the antigenic polysaccharides from *E. saburreum* L49, O2, S29, T21, and T110, but the heptose residues of all these polysaccharides are substituted by 6-deoxy-D-*altro*-heptofuranosyl groups (chemotype II). Although the antigenic polysaccharide of T19 has the same backbone structure as that of L49, the polysaccharide of T19 contains in addition a α-D-fucofuranosyl disaccharide as a branched group. On the other hand, Hofstad et al.[13] have reported that the polysaccharide antigen of *E. saburreum* strain L452 contains α-D-fucofuranosyl terminal groups. However, the polysaccharide of L452, which is a heteroglycan composed of D-fucofuranose, D-galactopyranose, and D-ribofuranose, does not contain D-*glycero*-D-*galacto*-heptose.



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On the basis of the sugar constituents, the antigenic polysaccharide of *E. saburreum* T19 is classified as a new chemotype III.

EXPERIMENTAL

Culture conditions.—*Eubacterium saburreum*, strain T19 was cultured anaerobically in the same medium as previously described[6]. After incubation for 36 h at 37°C, the cells were harvested by centrifugation, washed with saline solution, and freeze-dried.

Extraction and purification of the antigenic polysaccharide.—The antigen was extracted by formamide at 150°C for 15 min and digested with pronase as previously reported[6]. The digested antigen was applied to a column of Sepharose 6B (1.4 × 150 cm), previously equilibrated, and then eluted with 20 mM phosphate buffer, pH 7.4, containing 0.02% NaN_3. The elution volume of the polysaccharide was found to be 116 mL (vol_e/vol_o 1.87). The immunologically active fractions were pooled, dialyzed against water, and lyophilized. The lyophilized cells (1 g) yielded 40 mg of the antigenic polysaccharide.

General analytical methods.—Optical rotations were determined with a Horiba SEPA-200 photoelectric polarimeter. GLC was performed with a Hitachi 163 instrument with a flame-ionization detector, and GLC–MS with a Hitachi M-80 B instrument at an ionization potential of 70 eV. The *O*-trimethylsilyl derivatives of the alditols were analyzed by use of a glass column (0.3 × 300 cm), packed with 3% OV-17 on Shimalite, the temperature being raised from 150 to 250°C at a rate of 5°C/min. The *O*-methyl and *O*-acetyl derivatives of the alditols were analyzed by use of a glass column (0.2 × 200 cm) containing 5% OV-225 on Gaschrom Q at 160°C. PC was performed on Whatman No. 1 paper with 6:4:3 butanol–pyridine–

H_2O as irrigant. Determination of the mol w of the polysaccharide was performed on a column (1.4 × 290 cm) of Toyopearl HW-55 (superfine) equilibrated with 20 mM phosphate buffer, pH 7.2, containing 0.02% NaN_3; Dextran T10, T40, and T70 were used for the calibration curve. ^1H NMR spectra were recorded at 400 M Hz with a Jeol GX-400 spectrometer for D_2O solutions. ^{13}C NMR spectra were recorded with the same spectrometer at 100 MHz. Chemical shifts were expressed from the Me_4Si signal.

Identification of sugar components.—The antigenic polysaccharide (1 mg) was hydrolyzed with 1 M HCl (1 mL) for 5 h at 100°C. After cooling, the excess acid was removed by passage through a small column (1 × 10 cm) of Dowex 1 (AcO^-) anion-exchange resin, and effluent and water washings were evaporated to dryness. The hydrolyzate was reduced with $NaBD_4$, per-*O*-trimethylsilylated, and analyzed by GLC–MS. GLC of the derivative on a OV-17 column showed two components having relative retention times to per-*O*-trimethylsilylated inositol of 0.60 and 1.12, in the proportion 1:2. The first-eluted component was identified as D-fucose and the second was identified as D-*glycero*-D-*galacto*-heptose by their mass fragmentation patterns.

Methylation analysis.—The polysaccharide was methylated by the procedure previously described[6]. Briefly, the methylated polysaccharide was hydrolyzed in 90% formic acid at 100°C for 2 h and with 0.25 M H_2SO_4 for 16 h at 100°C. The hydrolyzate was reduced with $NaBD_4$, peracetylated and analyzed by GLC–MS. The results are shown in Table I.

Partial hydrolysis with acid.—Acid hydrolysis of the antigenic polysaccharide under mild conditions (50 mM H_2SO_4 at 80°C for 3.5 h), followed by dialysis, yielded the modified polysaccharide. Sugar analysis of this modified polysaccharide showed that 75% of the D-fucofuranosyl residues had been removed from the original polysaccharide. Furthermore, acid hydrolysis of the modified polysaccharide under further mild conditions (50 mM H_2SO_4 at 100°C for 180 min), followed by dialysis, yielded a polymer. Sugar analysis of the components, as their alditol acetates, confirmed that the polymer was composed of D-*glycero*-D-*galacto*-heptose residues only. The modified polysaccharide and the polymer were subjected to methylation analysis to identify the glycosidic linkages.

Location of O-acetyl groups.—The polysaccharide (15 mg) and 4-toluenesulfonic acid (5 mg) were mixed in Me_2SO (1.5 mL) in a screw-capped tube. Methyl vinyl ether (2 mL) was added and the mixture was incubated for 4 h at 15°C. The yellow-colored mixture was placed on a column (1.4 × 120 cm) of Sephadex LH 20, which was eluted with anhyd acetone. The separation was monitored by the 5% phenol–H_2SO_4 assay. The acetalated derivative of polysaccharide was eluted in the void volume of the column, free of reagent. It was deacetylated with 0.2% methanolic NaOMe, methylated, hydrolyzed, reduced with $NaBD_4$, acetylated, and analyzed by GLC–MS. GLC (OV-225 column 160°C) analysis showed the presence of 8 components having the following retention times relative to D-glucitol hexaacetate: 0.19, 0.23, 0.27, 1.19, 1.32, 1.58, 2.20, and 2.36.

They were identified as 1,3,4,5-penta-*O*-acetyl-2-*O*-methyl-, 1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl-, 1,2,3,4,5-penta-*O*-acetyl-(1-^{2}H)fucitol, and 1,2,4,5,6-penta-*O*-acetyl-3,7-di-*O*-methyl-, 1,2,3,4,5,6-hexa-*O*-acetyl-7-*O*-methyl-, 1,3,4,5,6,7-penta-*O*-acetyl-2-*O*-methyl-, 1,2,4,5,6,7-hexa-*O*-acetyl-3-*O*-methyl-, and 1,2,3,4,5,6,7-hepta-*O*-acetyl-(1-^{2}H)heptitol, respectively.

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