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STRUCTURAL STUDIES OF THE ANTIGENIC POLYSACCHARIDE OF *Eubacterium saburreum*, STRAIN T17

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ABSTRACT

The antigenic polysaccharide produced by *Eubacterium saburreum*, strain T17, is a homoglycan composed of D-*glycero*-D-*galacto*-heptose (Hep) residues having the pentasaccharide repeating-unit →6)-[α-Hep*f*-(1→4)]-β-Hep*p*-(1→6)-[α-Hep*f*-(1→2), α-Hep*f*-(1→4)]-β-Hep*p*-(1→. The polysaccharide does not contain *O*-acetyl group.

INTRODUCTION

The antigenic polysaccharide produced by *Eubacterium saburreum*, strain L44 (ref. 1), was reported to be a linear polysaccharide composed of (1→6)-D-*glycero*-β-D-*galacto*-heptopyranosyl residues. This polysaccharide is the first known example of a bacterial homoglycan composed of heptose residues. The second example of a bacterial homoglycan composed of D-*glycero*-D-*galacto*-heptose (Hep) residues was the polysaccharide produced by another strain, T27 (ref. 2), of the same organism. The polysaccharide from strain T27 was reported to have the nonasaccharide repeating-unit →6)-[α-Hep*f*-(1→4)]-β-Hep*p*-(1→$_3$6)-[α-Hep*f*-(1→2), α-Hep*f*-(1→4)]-β-Hep*p*-(1→. The polysaccharides produced by both strains L44 and T27 contain a high proportion of *O*-acetyl groups.

Recently, it was found that the antigenic polysaccharide produced by *E. saburreum*, strain T17, is the third example of a bacterial homoglycan composed of D-*glycero*-D-*galacto*-heptose residues. The present report describes the structure of this new antigenic polysaccharide.

RESULTS AND DISCUSSION

Components of the polysaccharide. — Acid hydrolysis of the antigenic polysaccharide yielded two compounds which were isolated by column chromatography on a column (0.75 × 50 cm) of Dowex 1 (BO_4^{3-}) anion-exchange resin. The minor product, which was eluted first with 18mM potassium tetraborate, was identified as 1,6-anhydro-D-*glycero*-D-*galacto*-heptofuranose. The ^{1}H-n.m.r. spectrum of the

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per-*O*-acetylated sugar was identical with that of an authentic sample[3]. The mass spectrum of the per-*O*-trimethylsilyl derivative was in good agreement with the published pattern[2]. The sugar showed no reducing power and the optical rotation of its per-*O*-acetyl derivative was $[\alpha]_D^{20}$ +147° (*c* 0.8, chloroform)[3]. The second compound to be eluted from the column of Dowex 1 was the major product and was identified as D-*glycero*-D-*galacto*-heptose. The mass spectrum of its per-*O*-acetylalditol derivative showed that it was derived from a heptose. The ^{1}H-n.m.r. spectrum of the sugar was indistinguishable from that of an authentic sample, and its optical rotation, $[\alpha]_D^{20}$ +58° (*c* 1.1, water), was in good agreement with the published value[2]. These results indicate that D-*glycero*-D-*galacto*-heptose is the sole sugar component of this antigenic polysaccharide, and that 1,6-anhydro-D-*glycero*-D-*galacto*-heptofuranose was formed from the heptose residues during acid hydrolysis.

Glycoside linkages of heptose residues. — The ^{1}H-n.m.r. spectrum of the polysaccharide showed signals for two anomeric protons at δ 5.20 ($J_{1,2}$ small) and 4.65 ($J_{1,2}$ 7 Hz) with relative intensities of ~1.5:1.0. The polysaccharide did not contain *O*-acetyl group, as indicated by the ^{1}H-n.m.r. spectrum.

Methylation analysis of the polysaccharide, with analysis of the partially methylated sugars as alditol acetates, gave 2,3,5,6,7-penta-*O*-methyl-, 2,3,7-tri-*O*-methyl-, and 3,7-di-*O*-methyl-heptose, in the ratio of 3:1:1. This result demonstrates that, of the polysaccharide heptose residues in the repeating unit, three occur as furanosyl end-groups, one as pyranosyl branch-residue linked at O-4 and -6, and one as pyranosyl branch-residue linked at O-2, -4, and -6. The possibility that the two branch-residues are in furanosyl or septanosyl form was eliminated as previously described[2], namely, mild acid hydrolysis of the polysaccharide (0.5M sulfuric acid) and fractionation of the products gave oligosaccharides that were reduced with borodeuteride, methylated, and hydrolyzed. The products were reduced again with borohydride and acetylated to give (g.l.c.–m.s.) 1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methyl-heptitol, 6-*O*-acetyl-1,2,3,4,5,7-hexa-*O*-methyl-(1-^{2}H)heptitol, and 1,5,6-tri-*O*-acetyl-2,3,4,7-*O*-methylheptitol. The second compound was derived from reducing heptosyl residues, the third compound from internal-chain heptosyl residues of oligosaccharides, and the first compound from heptopyranosyl, and not from furanosyl or septanosyl nonreducing end-groups. These results suggest that the heptosyl residues of the main polysaccharide chain are present in the pyranosyl form.

Acid hydrolysis of the polysaccharide with 50mM sulfuric acid, followed by dialysis, yielded a polymer. Methylation analysis of it gave, as alditol acetates, 2,3,5,6,7-penta-*O*-methyl-, 2,3,4,7-tetra-*O*-methyl-, and 2,3,7-tri-*O*-methyl-heptose, in the ratio of 11:14:11. As shown in Table I, the proportion of the sugar residues of the polymer were calculated on the assumption that the total amount of heptopyranosyl residues would not change during mild acid hydrolysis, as 2,3,7-tri-*O*-methylheptose was not decreased. Therefore, the 11:14:11 ratio of the product of mild hydrolysis can be expressed as 0.88:1.12:0.88 relative to the original

TABLE I

ALDITOL ACETATES OBTAINED BY METHYLATION ANALYSIS OF THE POLYSACCHARIDE FROM *E. saburreum*, STRAIN T17, AND OF A PRODUCT OF HYDROLYSIS[a]

O-*Methyl alditol acetate of* D-glycero-D-galacto-*heptose*	*Original polysaccharide*	*Product of hydrolysis*
2,3,5,6,7-Penta-	3.00	0.88
2,3,4,7-Tetra-		1.12
2,3,7-Tri-	1.00	0.88
3,7-Di-	1.00	

[a]Molar ratios relative to original polysaccharide.

polysaccharide, indicating that ~70% of heptofuranosyl residues were removed by mild acid hydrolysis. The methylation data indicated that the nonreducing terminal heptofuranosyl groups are linked to O-4 of the 4,6-di-*O*-glycosylated heptopyranosyl residues, and to O-2 and -4 of the 2,4,6-tri-*O*-glycosylated heptopyranosyl residues.

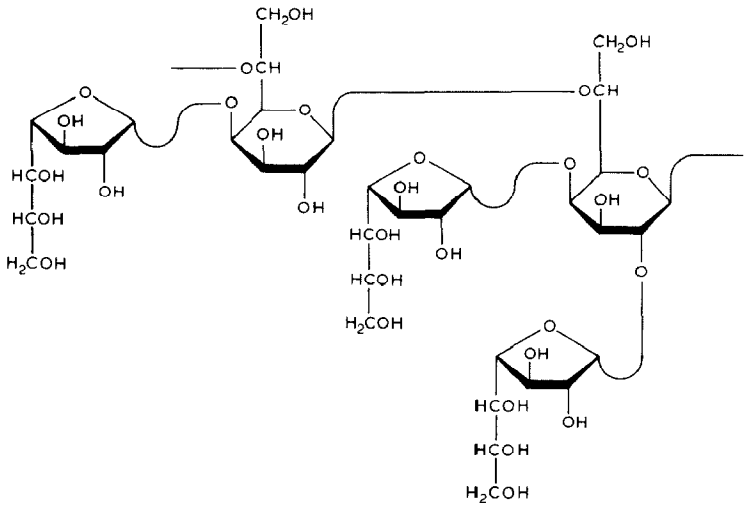
Periodate oxidation, reduction with borodeuteride, hydrolysis with acid, and a second reduction with borohydride yielded glycerol, pentitol, and heptitol, in the ratio of 3:1:1. (1,3-^{2}H)Glycerol was derived from terminal heptofuranosyl groups, and (1-^{2}H)pentitol from 4,6-linked heptopyranosyl residues. The heptitol, which was identified as D-*glycero*-D-*galacto*-heptitol, was derived from 2,4,6-linked D-*glycero*-D-*galacto*-heptopyranosyl residues. These data are in complete agreement with the postulated structure.

The optical rotation of the original polysaccharide, $[\alpha]_D^{20}$ +9.0° (*c* 1.0, water), decreased on mild acid hydrolysis, and the product, from which 71% of the heptofuranosyl residues had been removed, showed $[\alpha]_D^{20}$ −3.0° (*c* 1.0, water). Methyl D-*glycero*-*β*-D-*galacto*-heptopyranoside and methyl D-*glycero*-*β*-D-*galacto*-heptofuranoside have a rotation[4] of −5.1° and −111°, respectively. These results indicate that the D-*glycero*-D-*galacto*-heptopyranosyl residues are *β* linked, and the D-*glycero*-D-*galacto*-heptofuranosyl residues *α* linked.

The ^{1}H-n.m.r. spectrum of the polymer obtained by mild acid hydrolysis showed two signals for the anomeric protons at *δ* 4.65 ($J_{1,2}$ 7 Hz) and 5.20 ($J_{1,2}$ small) with relative intensities of ~1:0.6. These signals could be assigned to H-1 of the D-*glycero*-D-*galacto*-heptopyranosyl residues and to H-1 of the D-*glycero*-D-*galacto*-heptofuranosyl residues, respectively.

The ^{13}C-n.m.r. spectrum of the antigenic polysaccharide showed signals at *δ* 105.0 and 102.7. The latter signal was due to C-1 of the D-*glycero*-*β*-D-*galacto*-heptopyranosyl residues as shown by Hoffman *et al.*[5]. Thus, the former signal was assigned to C-1 of the D-*glycero*-*α*-D-*galacto*-heptofuranosyl residues on the basis of the data reported by Gorin and Mazurek[6].

From the aforementioned evidence, the structure of the repeating-unit of the antigenic polysaccharide produced by *E. saburreum*, strain T17, was established as **1**.



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EXPERIMENTAL

Culture conditions. — *Eubacterium saburreum*, strain T17, was cultured anaerobically in the same medium as previously described[7]. After incubation for 36 h at 37°, the cell were harvested by centrifugation, washed with saline solution, and freeze-dried.

Extraction and purification of antigenic polysaccharide. — The antigenic polysaccharide was extracted with formamide and digested with Pronase as previously described[7]. The digested sample was taken up in a small volume and applied to a column (1.5 × 150 cm) of Toyopearl HW-55 (super fine). The column had been equilibrated and was eluted with 20mM phosphate buffer, pH 7.4, containing 0.02% NaN_3. Serologically-active fractions were pooled, dialyzed against distilled water, and freeze-dried.

General analytical methods. — Optical rotations were determined with a Horiba Sepa-200 photoelectric polarimeter. G.l.c. was performed with a Hitachi 163 instrument with f.i.d., and g.l.c.–m.s. with a Hitachi M-60 instrument. The *O*-acetyl and *O*-methyl derivatives of sugars were analyzed by use of a G-Scot silicon OV-225 (capillary column, 0.28 mm × 20 m) at 180°. The trimethylsilyl derivatives were analyzed by use of a glass column (0.3 × 300 cm) packed with 3% OV-17, the temperature being raised from 150 to 250° at the rate of 5°/min. ^{1}H-N.m.r. spectra were recorded with a JEOL FX-200 spectrometer, Me_4Si being the internal standard. ^{13}C-N.m.r. spectra were recorded with a JEOL GX-400 spectrometer using external Me_4Si as reference.

Methylation analysis. — The polysaccharide, in a screw-capped bottle, was methylated by the Hakomori procedure[8]. The methylated polysaccharide in 90% formic acid was kept for 2 h at 100°, the solution concentrated to dryness, and the

residue hydrolyzed with 0.25M H_2SO_4 for 16 h at 100°. The hydrolyzate was neutralized with $BaCO_3$, and the sugars were reduced with $NaBD_4$, acetylated, and analyzed by g.l.c.–m.s. G.l.c. of the products showed three components having mobilities (*T* value) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-*O*-methyl-D-glucitol of 1.61, 3.82, and 6.86. The first component (*T* 1.61) was identified as 1,4-di-*O*-acetyl-2,3,5,6,7-penta-*O*-methylheptitol; m.s.: *m/z* 322(3.9), 278(16), 249(7), 162(19), 133(28), 118(55), 102(68), 101(69), 89(19), 45(75), and 43(100). The second component (*T* 3.82) was identified as 1,4,5,6-tetra-*O*-acetyl-2,3,7-tri-*O*-methylheptitol; m.s.: *m/z* 349(1), 305(12), 275(1), 231(7.5), 203(5), 162(15), 129(48), 118(65), 45(20), and 43(100). The third component was identified as 1,2,4,5,6-penta-*O*-acetyl-3,7-di-*O*-methylheptitol; m.s.: *m/z* 305(12), 231(7.5), 190(34), 130(78), 45(20), and 43(100).

Partial hydrolysis with acid. — The polysaccharide (10 mg) was hydrolyzed with 0.5M H_2SO_4 for 2.5 h at 100°. After neutralization, the hydrolyzate was applied to a column (1.5 × 100 cm) of Sephadex G-25 which had been equilibrated with distilled water. Elution with the same solvent gave fractions containing oligosaccharides which were pooled, reduced with $NaBD_4$, and methylated. The methylated oligosaccharides, which were isolated by partition between chloroform and water, were hydrolyzed, reduced with $NaBH_4$, acetylated, and analyzed by g.l.c.–m.s. The following components were detected: 1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methylheptitol, 6-*O*-acetyl-1,2,3,4,5,7-hexa-*O*-methyl-(1-^{2}H)heptitol, and 1,5,6-tri-*O*-acetyl-2,3,4,7-tetra-*O*-methylheptitol in the ratio of 5:3:4. Another portion of the polysaccharide was hydrolyzed with 50mM H_2SO_4 for 3.5 h at 80°. The polymer recovered by dialysis was subjected to methylation analysis.

Periodate oxidation. — The polysaccharide was dissolved in 0.1M Na acetate buffer, pH 3.9, and the solution treated with a final $NaIO_4$ concentration of 35mM for up to 10 days at 4°. The excess of periodate was decomposed with 1,2-ethanediol, and the oxidized material purified by dialysis against distilled water and reduced with $NaBD_4$. After further dialysis, the material was hydrolyzed, reduced with $NaBH_4$, and per-*O*-trimethylsilylated. G.l.c. analysis indicated three products of degradation having mobilities (*T* value) relative to per-*O*-trimethylsilylinositol of 0.17, 0.57, and 1.12. The first compound (*T* 0.17) was identified as (1,3-^{2}H)glycerol; m.s.: *m/z* 295 (1, $M^+ - 15$), 210 (10, $M^+ - 90$), 206(38), 147(47), 117(58), 104(32), and 73(100). The second product (*T* 5.7) was identified as (1-^{2}H)-pentitol; m.s.: *m/z* 423 (3, $M^+ - 90$), 410(1), 409(1), 320(18), 319(20), 308(26), 307(24), 218(35), 217(33), 206(32), 205(37), 147(49), 104(48), 103(63), and 73(100). The third product was identified as D-*glycero*-D-*galacto*-heptitol, *T* 1.12; m.s.: *m/z* 626 (1, $M^+ - 90$), 523(1), 431(4), 421(12), 409(12), 409(11), 319(61), 307(30), 217(38), 205(49), 147(45), 103(68), and 73(100).

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