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The structural analysis of a levan produced by *Streptococcus salivarius* SS2

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ABSTRACT

The structure of a water-soluble levan produced by *Streptococcus salivarius* SS2 has been determined by means of various chemical and instrumental methods. Methylation and periodate oxidation studies demonstrate that the levan is comprised of D-fructofuranosyl backbone residues linked β-(2→6) (about 70%) with β-(2→1) branches (about 30%). ^{13}C-N.m.r. spectral analysis of the polymer is consistent with the structure determined by chemical means.

INTRODUCTION

Levans are found in Nature in both plant and bacterial materials. Plant levans found in certain grasses have a low molecular weight and exhibit little branching[1,2]. The bacterial levans, on the other hand, have high molecular weights and are more extensively branched[1].

Structural determination of polysaccharides has traditionally been based on periodate oxidation studies as well as on methylation analyses. The Hakomori[3] method of methylation, using sodium hydride and methyl iodide in Me_2SO, is commonly used to methylate the polysaccharide. Reductive-cleavage of the methylated levan[4] gives rise to 2,5-anhydroalditol derivatives which keep the furanose ring form intact.

^{13}C-N.m.r. spectroscopy has proven to be useful for establishing branching type in fructofuranans[1,2,5–7]. Seymour and co-workers[8,9] have shown that glycosylation of a hydroxy-bearing carbon in a dextran glycopyranosyl residue generally results in a ~10 p.p.m. downfield shift for that resonance, while the corresponding shift changes are not as great (~2 p.p.m.) in fructofuranosyl residues[5]. These smaller substitution shifts do not, however, hinder the unambiguous differentation of a levan from an inulin by using main-chain chemical shifts[7]. This paper describes the uses of these techniques to establish the structure of the *Streptococcus salivarius* levan.

EXPERIMENTAL

Isolation of the levan. — The levan was precipitated by ethanol (2 vol.) from the spent culture broth of the *S. salivarius* SS2 grown in presence of sucrose (5%). The levan

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was reprecipitated twice from water by ethanol and was shown by chemical and chromatographic methods to contain only fructose[10]. The levan had the same chemical and physical properties as those of the levan produced by a crude enzyme preparation[10].

Periodate oxidation. — The method of Rankin and Jeanes[11] for periodate oxidation was used with the levan. Inulin was used as a standard in all analyses. The polysaccharides (2 $mg.mL^{-1}$) were mixed with sodium metaperiodate (10 $mg.mL^{-1}$) for 130 h at 25°. Aliquots were taken periodically and analyzed for periodate consumption by back-titration with iodine following addition of arsenite.

Methylation. — The levan and inulin (200 mg) were methylated using the Hakomori method[3]. The dimsyl carbanion was generated by adding NaH (250 mg) to Me_2SO (6 mL, vacuum distilled at 72° and stored over CaH_2). Methyl iodine (1 mL) was added, and the reaction stirred overnight at room temperature. The reaction mixture was extracted three times with chloroform. The combined chloroform layers were extracted with water, and the polymer was precipitated from the chloroform using petroleum ether (b.p. 30–60°). I.r. spectroscopy was used to test for completeness of methylation.

Reductive cleavage. — The methylated polysaccharides were subjected to reductive cleavage as described by Rolf and Gray[4]. The reducing agent was prepared using the following recipe: boron trifluoride etherate (310 μL), triethylsilane (400 μL), trifluoroacetic acid (64 μL), and dichloromethane (226 μL). The reducing agent (550 μL) mixture was added to the methylation products (1 mg) and was allowed to react for 24 h at 0°. Acetic anhydride (50 μL) was added and was allowed to react for 1 h[12] at room temperature. The acetylated, methylated anhydroalditols were dissolved in dichloromethane, the solution was extracted with water three times, and the organic layer was analyzed by g.l.c.–m.s.

Gas-liquid chromatography–mass spectroscopy (g.l.c.–m.s.). — A Hewlett-Packard 5790A series capillary gas chromatograph was used to analyse all methylated products. A methyl silicone (HP-5) capillary column (30 m × 0.25 mm i.d.) and a temperature program [90° (5 min), $2°.min^{-1}$, 150°] were used. The products were detected by a 5790A mass-selective detector by scanning over an m/z range of 40–300.

^{13}C-N.m.r. spectroscopy. — ^{13}C-Fourier-transform n.m.r. spectra were obtained at 50.3 MHz with a Varian XL-200 spectrometer. Typically, the levan (300 mg) was dissolved in D_2O (3 mL) with sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS, 90 mg, Aldrich Chemical Co.) as a reference in a 10-mm n.m.r. tube. All spectra were recorded at 35° and 85° with broad-band proton decoupling. Typically a 0.4 s acquisition time was used with various levels of Lorentzian to pseudo-Gaussian spin-echo filtration for resolution enhancement. All chemical shifts are expressed in p.p.m. relative to DSS. The temperature-dependent chemical shift values for DSS were calculated from the data of Hoffman and Davies[13]. Deuterium-induced differential isotope shift (d.i.s.) spectroscopy[14] was performed at 85° using a special Wilmad Glass equi-volume co-axial insert for a 10-mm tube.

RESULTS

Periodate oxidation. — The uptake of periodate by the water-soluble levan was complete after 24 h. Assuming that no over-oxidation had occurred, the levan consumed 0.97 mmol of periodate per anhydrofructose unit. The consumption of periodate indicated that the levan does not contain (2→3) or (2→4) linkages. This value is also consistent with the values reported by Rankin and Jeanes[11] for levans.

Analysis of the methylated levan. — The water-soluble levan was methylated overnight, and the absence of hydroxy groups was determined by the absence of the strong hydroxy band at 3000 cm^{-1} in the i.r. spectrum. The completely methylated polymer was subjected to the reductive-cleavage of Rolf and Gray[4] and the gas–liquid chromatogram of the acetylated, methylated anhydroalditols is shown in Fig. 1.

There are three sets of peaks in the chromatogram that correspond to the various methoxy derivatives of 2,5-anhydro-D-glucitol and 2,5-anhydro-D-mannitol. Rolf and Gray[15] determined that a D-fructose polymer linked (2→6) yielded a higher percentage of the 2,5-anhydro-D-glucitol product, while the (2→1) polymer and the terminal D-fructose yielded a higher percentage of 2,5-anhydro-D-mannitol. The identity of each set was determined by the relative peak abundance of the various fragments detected in the mass spectra. The fragmentation patterns of the methoxy derivatives were identical to the published data[15]. The m.s. fragmentation data is reported in Table I. The area of the sum of peaks 1 and 2 and the sum of peaks 5 and 6 had a ratio of about 1:1, indicating that there was a non-reducing end detected for each branch point. The summation of peaks 3 and 4 had a peak area that corresponded to 70% of the applied sample and

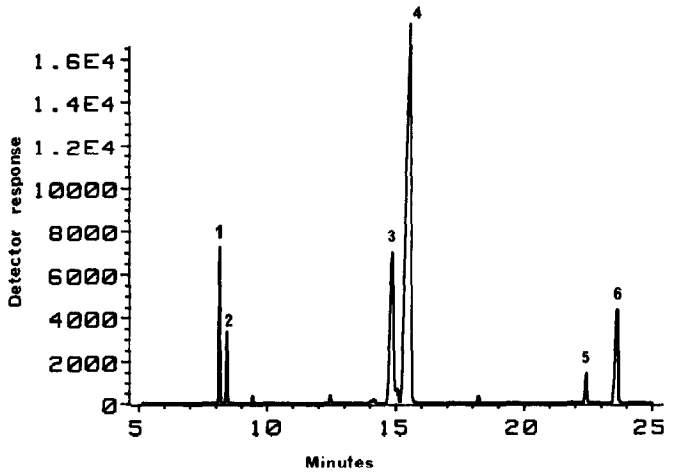

Fig. 1. The gas–liquid chromatogram of the products derived by the reductive-cleavage of the *Streptococcus salivarius*. **1**, 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol; **2**, 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-glucitol; **3**, 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-mannitol; **4**, 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol; **5**, 1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol; **6**, 1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-glucitol.

represented (2→6) linkages. The D-fructosyl residues that indicate the (2→1) branching; that is, peaks 5 and 6 comprise 15% of the residues.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of the *S. salivarius* SS2 levan were determined at 35° and 85°, and the chemical-shift values for the main-chain resonances at the two temperatures are given in Table II. These values confirm the identification of the polymer as being a levan since they are almost identical to the literature values. There is a small systematic difference between these data and reported chemical shifts[1,2,5–7] which may be due to the manner of referencing to DSS. Two sets of values are given here since there is some temperature dependency in the chemical shift data. We are presently investigating whether these dependences can be used, along with other techniques, in assigning minor resonances to certain structural features of the polymer. The d.i.s. values at 85° are also given in Table II. Substitution shifts in

TABLE I

Electron-impact mass spectral fragmentations of the 2,5-anhydroalditol derivatives produced by reductive cleavage and acetylation[a]

*2,5-Anhydro-1,3,4,6-tetra-*O*-methyl-*D*-glucitol (Peak 2)*
41(23) 42(5) 43(2) 45(100) 53(4) 55(8) 57(6) 59(17) 69(16) 71(40) 72(5) 73(6) 74(12) 75(10) 85(7) 87(18) 89(23) 99(15) 101(73) 102(6) 111(17) 115(10) 143(25) 175(8)

*6-*O*-Acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-*D*-glucitol (Peak 4)*
41(29) 42(12) 43(100) 45(76) 53(6) 55(10) 56(3) 57(5) 58(3) 59(10) 68(4) 69(16) 70(5) 71(60) 72(8) 73(10) 74(8) 75(9) 81(4) 83(16) 84(6) 85(13) 87(26) 88(4) 89(9) 97(5) 99(9) 101(43) 85(13) 87(26) 111(35) 115(21) 117(13) 126(14) 143(27) 156(3) 158(3)

*1,6-Di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-*D*-glucitol (Peak 6)*
41(16) 42(11) 43(100) 45(21) 69(13) 71(29) 83(10) 85(7) 87(40) 101(15) 111(19) 115(9) 117(16) 124(12) 216(9)

[a] The *m/z* is given first, followed by the relative abundance (%) in parentheses. The corresponding 2,5-anhydro-D-mannitol yielded almost identical fragmentation patterns.

TABLE II

^{13}C-N.m.r. data for *Streptococcus salivarius* strain SS2 levan.

Carbon atom	*Chemical shift*[a]		*D.i.s. Value*[d]
	34°[b]	*85*°[c]	
C-1	60.21	61.38	147
C-2	104.21	104.55	—[e]
C-3	76.57	77.67	157
C-4	75.36	76.16	157
C-5	80.31	80.68	48
C-6	63.43	63.85	0

[a] Chemical shift of main-chain D-fructofuranosyl unit. [b] Relative to DSS at −2.570 p.p.m. [c] Relative to DSS at −2.138 p.p.m. [d] In p.p.b. at 85°. [e] Not determined.

compounds containing the D-fructofuranosyl unit are smaller and less predictable than shifts in D-glucopyranose polymers. While the chemical-shift assignments for C-2, C-3, C-4, and C-5 based on model compounds, are in agreement with published data, there is a possibility that C-1 and C-6 could be interchanged. This possibility can be ruled out as can be seen from the d.i.s. values at 85° given in Table II. The upfield hydroxymethylene C-1 has a d.i.s. value of 147 p.p.b., indicating an exchangable proton within two bonds. The other hydroxymethylene type carbon has no d.i.s. split, indicating no exchangable proton within four bonds and, therefore, confirming that it is the C-6 that is involved in a main-chain glycosidic linkage.

DISCUSSION

The results of periodate oxidation, methylation–g.l.c.–m.s. analysis, and ^{13}C-n.m.r. analysis suggest the following structure for the levan of *S. salivarius* SS2:

$$-[-\beta\text{-D-fru}f\text{-}(2\rightarrow6)_n\text{-}\beta\text{-D-fru}f\text{-}(2\rightarrow6)\text{-}]_x$$

$$\begin{array}{c} 1 \\ \uparrow \\ 2 \\ (\beta\text{-D-fru}f)_d \\ 6 \\ \uparrow \\ 2 \\ (\beta\text{-D-fru}f) \end{array}$$

where β-D-fru*f* indicates β-D-fructofuranosyl residues, and n,d,x, indicate chain length of the sequences in the polymer. Even though only one branch point is shown, the polymer probably has a dendritic structure with about 15% of the fructosyl moieties being involved as (2→1) branches from the (2→6) chains yielding a globular-shaped molecule. Assignments of minor resonances due to branching points is currently under investigation using n.m.r. spectroscopy.

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