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THE STRUCTURE OF AN EXTRACELLULAR, WATER-SOLUBLE POLYSACCHARIDE ELABORATED BY THE CARIOGENIC *Streptococcus mutans* GS-5*

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ABSTRACT

The extracellular, water-soluble polysaccharide elaborated by *Streptococcus mutans* GS-5 contains (1→6)- and (1→3,6)-linked α-D-glucopyranosyl residues. Its average repeating-unit contains 6 D-glucosyl residues and it is comb-like in structure. The majority of branches consist of only a few D-glucosyl residues, if not one D-glucosyl group.

INTRODUCTION

Several strains of streptococci which colonise the human mouth produce extracellular fructans and glucans when grown in a sucrose-containing medium. The fructans examined[2] are water-soluble. The water-soluble glucans seem to have lower, relative molecular masses than those that are water-insoluble[3]. Some *Streptococcus* spp. possess two D-glucosyltransferases, which separately synthesise water-soluble and water-insoluble glucans[4]. The former are "dextran-like" glucans, whereas the latter are unbranched α-D-glucans possessing essentially only (1→3) linkages. Water-insoluble α-D-glucans are also synthesised[4] by cell-free extracts of *Streptococcus* spp., and their complex structure seems to be the results of a concerted action of the two D-glucosyltransferases.

The water-soluble glucan elaborated by *S. mutans* GS-5 has been reported[5a] to contain 67 and 17% of (1→6)- and (1→3,6)-linked D-glucosyl residues, respectively, but further structural details were not reported. On the basis of ^{1}H-n.m.r. data[6] for extracellular glucans of 11 strains of *S. mutans*, it was concluded that "nearly all the glucans" possess adjacent (1→3)-linked D-glucosyl residues. However, structures **1** and **2** could not be distinguished. All of the D-glucosyl residues in the water-soluble glucan of *S. mutans* GS-5 that are linked through O-3 are indeed branching

*Studies on Dextrans and Dextranases, Part XII. For Part XI, see ref. 1.
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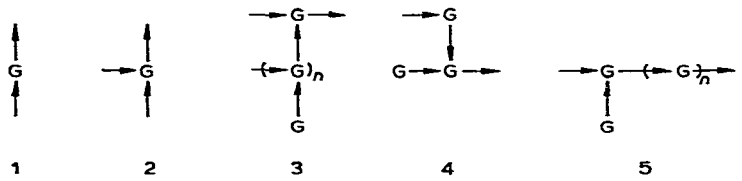

Fig. 1. Segments of dextran molecules in the vicinity of branching units: G, α-D-glucopyranosyl residue; →, (1→6) linkage; ↑, (1→3) linkage; ↓, (1→2) linkage.

units[5a] (*cf.* below), *i.e.*, (1→3,6)-linked. If the conclusions of ref. 6 apply also to this glucan, it would follow that it possesses segments such as **3** ($n \geqslant 1$), an uncommon structural feature of dextrans. We therefore report further chemical evidence for the structure of the water-soluble polysaccharide elaborated by *S. mutans* GS-5.

RESULTS AND DISCUSSION

Two polysaccharide fractions (*A* and *B*) were isolated from the culture medium by precipitation from 40 and 70% ethanol, respectively. As before[7], the precipitation procedure effected a partial separation of the glucan (*B*) from fructan or glucofructan components (*A*). The fructose content (2.5%) of polysaccharide *B* was judged to be sufficiently small not to interfere significantly in the subsequent studies.

The evidence presented here showed that polysaccharide *B* is essentially an α-D-glucan (*cf.* D-glucose content, optical rotatory power, and acetolysis results). Confirmatory evidence was obtained by degradation of polysaccharide *B* by the dextranase of *Penicillium lilacinum*[8] (I.M.I. 79197; NRRL 896), the products of which had properties similar to those of the products obtained[9] from the dextran produced by *Leuconostoc mesenteroides* NRRL B-1375. That dextran is known[10] to have a comb-like structure in which α-D-glucosyl residues are attached as branches by (1→3) linkages to the chain of (1→6)-linked α-D-glucosyl residues. These results also suggested that the essential structural features of polysaccharide *B* and the dextran of *L. mesenteroides* NRRL B-1375 are similar.

Chromatography of polysaccharide *B* on Sephadex G-200 (Fig. 2) showed it to be a polydisperse material, a significant proportion of which had $M_r \geqslant 40{,}000$. Although it is claimed[11] that dextrans are degraded by heating in dimethyl sulphoxide, such treatment did not degrade polysaccharide *B*, or affect its molecular aggregation as evidenced by the essentially identical elution patterns from Sephadex G-200 of the native and dimethyl sulphoxide-treated polysaccharide *B*.

Linkage analysis of polysaccharide *B* by methylation, followed by the procedure of Lindberg and co-workers[12], revealed (Table I) that it possessed (1→6)- and (1→3,6)-linked D-glucosyl residues and that the average repeating-unit contained approximately 6 D-glucosyl residues (*i.e.*, one terminal, four linked through C-1 and C-6, and one linked through C-1, C-3, and C-6). The same composition was found for the fractions of higher (*B*-i) and lower molecular weight (*B*-ii) obtained by chromato-

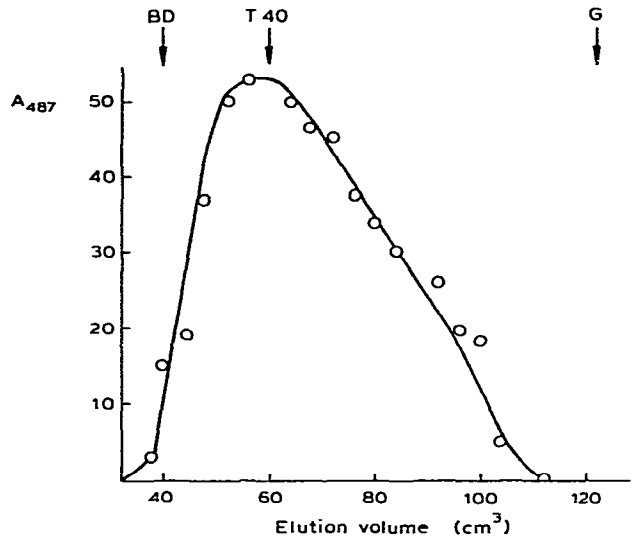

Fig. 2. Chromatography of polysaccharide *B* on Sephadex G-200: BD, Blue Dextran 2000; T40, Dextran T40; G, D-glucose. Arrows indicate elution volumes of BD, T40, and G.

TABLE I

LINKAGE ANALYSIS OF POLYSACCHARIDES

Polysaccharide	*Time of hydrolysis[a] (h) of methylated polysaccharide*	*Hexitol derivative (mole fraction)*		
		1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl	*1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl*	*1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl*
B	6	0.29	0.71	trace
	7.5	0.17	0.67	0.16
	8.5	0.18	0.65	0.17
	10	0.18	0.66	0.16
	12	0.18	0.65	0.17
	24	0.17	0.62	0.21
B (average)[b]		0.18	0.66	0.17
B-i	10	0.18	0.67	0.16
B-ii	10	0.17	0.67	0.16
B-iii	10	<0.02	>0.98	0

[a]With 90% HCO_2H. [b]Average values for 7.5, 8.5, 10, and 12 h.

graphy on Sephadex G-200, emphasising the polydispersity of polysaccharide *B*. Characterisation of nigerose as a product of partial acetolysis showed that the glucosyl residues linked to C-3 also had the α-D configuration.

The product of the Smith-degradation[13] procedure (*i.e.*, partial fragmentation of periodate-oxidised polysaccharide) contained components expected from a glucan possessing (1→6)- and (1→3,6)-linked D-glucosyl residues, namely glycerol, D-glucose,

and 1-*O*-α-D-glucopyranosylglycerol. Higher glucosylglycerols originating from a sequence of branching residues were, if produced at all, not present in quantities within the limits of detection.

Acid hydrolysis of dextran[14] (and other glucans[15]) is reported to proceed primarily by removal of small fragments (*i.e.*, D-glucose and small oligosaccharides) from non-reducing chain-ends. In consequence, the partial hydrolysis of the dextran of *Leuconostoc mesenteroides* NRRL B-1299 (**4**, partial structure) with acid resulted

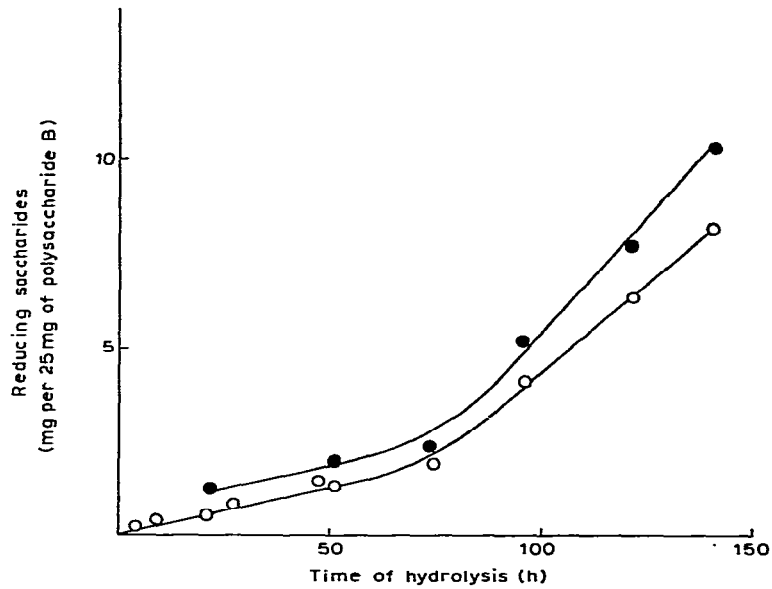

Fig. 3. Liberation of reducing saccharides from polysaccharide *B* in 0.1M sulphuric acid at 60°: —○—, D-glucose determined with D-glucose oxidase reagent; —●—, glucose equivalent of total reducing-saccharides. The amount of polysaccharide used is not corrected for ash and nitrogen content.

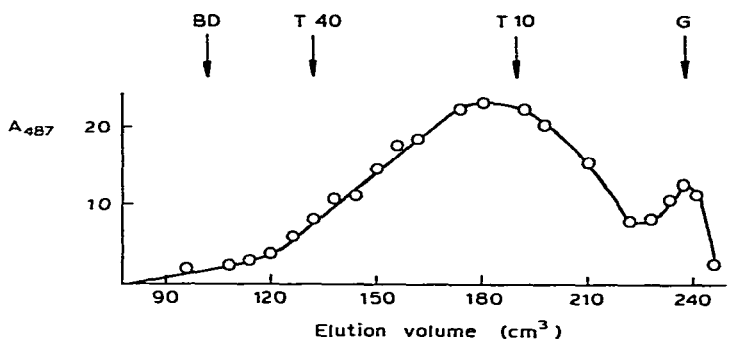

Fig. 4. Chromatography of polysaccharide *B*-iii (partially acid-hydrolysed) on Sephadex G-200: BD, Blue Dextran 2000; T40, Dextran T40; T10, Dextran T10; G, D-glucose. Arrows indicate elution volumes of BD, T40, T10, and G.

in the removal of the non-reducing D-glucosyl group attached to the branching unit by a (1→6) linkage[1]. We have adopted a similar procedure, in order to assign a structure to the segment in the vicinity of the branching unit in polysaccharide *B*.

Fig. 3 shows that the early stages of the hydrolysis of polysaccharide *B* in 0.1M sulphuric acid at 60° also proceed primarily by removal of D-glucose; after ~75 h, when ~7% of the polysaccharide has been thus hydrolysed, more-random degradation becomes more prominent. Chromatography of the 75-h hydrolysate on Sephadex G-200 (Fig. 4) revealed that the major portion of the polymeric material still had $M_r > 10{,}000$. Linkage analysis of the material (polysaccharide *B*-iii) obtained after removal of dialysable saccharides revealed that it contained, in addition to terminal D-glucosyl groups, only (1→6)-linked D-glucosyl residues, meaning that all (1→3) linkages of the native polysaccharide *B* had been hydrolysed. These results make it unlikely that the polysaccharide *B* had a laminated or arborescent structure, or that the branching units are arranged in sequence (*i.e.*, **3**). Hydrolysis of all (1→3) linkages in such structures would have produced mainly oligosaccharides.

We now conclude that the majority of the branches in polysaccharide *B* consists of only a few D-glucosyl residues, if not of a single group (**5**; $n = 4$, average). It is well known that the (1→3) linkage in glucans is more susceptible to acid hydrolysis than the (1→6) linkage[5b]. The removal of non-reducing chain-ends by acid-catalysed hydrolysis was thus further facilitated by the greater susceptibility to acid hydrolysis of the (1→3) linkage. The structure of this water-soluble α-D-glucan of the cariogenic *S. mutans* GS-5 is therefore similar to those of *L. mesenteroides* NRRL B-512[16] and NRRL B-1375[10]. Although other cariogenic micro-organisms are reported to produce "dextran-like" polysaccharides[4,5b,17,18], insufficient evidence is available to discern a common type of branched structure for these polysaccharides.

EXPERIMENTAL

Paper chromatography. — The solvents used were (*a*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*b*) 1-butanol–pyridine–water (6:4:3); and (*c*) ethyl acetate–acetic acid–water (9:2:2). Compounds were detected with silver nitrate in acetone–ethanolic sodium hydroxide.

Paper electrophoresis. — The electrolytes used have been described[7].

G.l.c.–mass spectrometry. — A Perkin–Elmer F11 gas chromatograph, operating at 190° and containing a glass column (2 m × 1 mm) packed with 3% of OV225 on Gas-Chrom Q (100–120 mesh), was used. The helium carrier-gas was removed from the effluent by passage through a Biemann separator. The effluent was then passed into a Hitachi RMS-4 mass spectrometer operating at 80 eV and 50-μA target-current.

Preparation of polysaccharides. — (*a*) The lyophilysed *Streptococcus mutans* GS-5 micro-organism was reactivated at 37° for 24 h in a medium containing Todd–Hewitt broth concentrate (Oxoid, 10 tablets/100 cm^3) and D-glucose (1%), and maintained in a medium containing Brain Heart Infusion concentrate (Oxoid, 5

tablets/100 cm^3), Thioglycolate (Difco, 2.4%), and D-glucose (0.5%). A suspension (5 cm^3) of an 18-h culture was used to inoculate medium (300 cm^3) contained in a dialysis sac which was immersed in medium (3 dm^3). The medium contained Trypton (Bacto, 1%), Yeast Extract Powder (Oxoid, 0.5%), dipotassium hydrogenphosphate (0.3%), and sucrose (5%). The conditions of incubation as well as the isolation and purification of the polysaccharide materials were as described before[7]. The materials precipitated from 40 and 70% ethanol were, as before[7], designated polysaccharides *A* and *B*, respectively. On average, 150 g of sucrose gave 0.42 and 1.15 g of polysaccharides *A* and *B*, respectively. Polysaccharides *A* and *B* had, respectively, D-glucose content (determined with D-glucose oxidase–peroxide reagent, Boehringer Biochemicals), 47.0 and 93.4; fructose content[7,19], 29.0 and 2.5; protein content, 24.9 and 1.5; ash, 3.25 and 0.85%; $[\alpha]_D^{20}$ +128° (*c* 0.16, M sodium hydroxide) and +169° (*c* 1, M sodium hydroxide).

(*b*) Polysaccharide *B* (30 mg) was chromatographed on Sephadex G-200, using a Pharmacia K15/90 column that had been calibrated with Blue Dextran 2000, Dextran T40, and D-glucose. Elution was with 1% sodium chloride (2-cm^3 fractions). Aliquots (0.2 cm^3) were used for determination of carbohydrate content by the phenol–sulphuric acid method[20]. The results are shown in Fig. 2.

(*c*) Polysaccharide *B* (50 mg) in dimethyl sulphoxide (5 cm^3) was heated at 70° for 1 h and then left at ambient temperature for 24 h. The solution was dialysed (3 days against tap water, and 3 days against distilled water). The product was chromatographed on Sephadex G-200 as described above.

(*d*) Polysaccharide *B* (500 mg) was chromatographed on Sephadex G-200, using a Pharmacia K26/100 column, and collected (5-cm^3 fractions) in fractions 35–95. Fractions 35–65 and 66–95 were combined and dialysed (3 days, tap water; 3 days, distilled water), to give, after freeze-drying, 250 mg of polysaccharide *B*-i (fructose content[7,19], 2.9%) and 152 mg of polysaccharide *B*-ii (fructose content[7,19], 0.8%), respectively. Polysaccharides *B*-i and *B*-ii were subjected to linkage analysis (Table I).

Acid hydrolysis of polysaccharide B. — (*a*) The polysaccharide (25 mg) was hydrolysed with 0.1M sulphuric acid (2 cm^3) for 1 h at 70°. P.c. of the hydrolysate revealed, as the main component, a material which had properties identical with those of fructose. A trace component had properties identical with those of glucose.

(*b*) Hydrolysis with M sulphuric acid at 100° for 8 h gave, as the main product, a material which had p.c. properties identical with those of glucose.

(*c*) Polysaccharide *B* (25 mg, exhaustively dried) in 0.1M sulphuric acid (0.5 cm^3) contained in a capped flask was heated at 60°. Aliquots (10 mm^3) were removed at time intervals and added to water (100 mm^3). The D-glucose content of the solutions was determined with the D-glucose oxidase–peroxide reagent (Boehringer Biochemicals), and the reducing-saccharide content was determined by using the Nelson copper reagent[21] with D-glucose as standard. The results are shown in Fig. 3.

The hydrolysis was repeated, but terminated after 75 h by neutralisation with barium carbonate.

A portion of the product, polysaccharide *B*-iii (~15 mg), was chromatographed on Sephadex G-200, using a Pharmacia K16/100 column calibrated with Blue Dextran 2000, Dextran T40, Dextran T10, and D-glucose. Elution was performed as described above. The results are shown in Fig. 4.

The remainder of the hydrolysate was dialysed, freeze-dried, and subjected to linkage analysis (Table I).

Acetolysis of polysaccharide B. — Acetolysis of the polysaccharide (150 mg) and work-up of products were performed essentially as described earlier[10]. The products obtained had properties (p.c., paper electrophoresis, d.p.) corresponding to those of glucose, nigerose, isomaltose, isomaltotriose, and isomaltotetraose. The product with properties corresponding to nigerose gave an octa-acetate, m.p. 152°; octa-*O*-acetyl-β-nigerose has[22] m.p. 151–152°.

Enzymic degradation of polysaccharide B. — The rate of liberation of reducing saccharides by the action of the dextranase of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) was determined as described previously[23]. The maximal amount of reducing saccharides, equivalent to 1.2 mg of glucose, was released from 10 mg of polysaccharide *B* by incubation for 7 h. The products had properties (p.c., d.p.) similar to those of the oligosaccharides produced[9] from dextran elaborated by *Leuconostoc mesenteroides* NRRL-B1375.

Methylation of polysaccharides. — Polysaccharides were methylated using the Hakomori reagent. The procedure adopted for polysaccharide *B* was as reported by Lewicki *et al.*[17], whereas that used for polysaccharides *B*-i, *B*-ii, and *B*-iii was as described earlier[24].

Characterisation and determination of O-*acetyl*-O-*methylhexitols obtained from methylated polysaccharides.* — (*a*) The methylated polysaccharides (~10 mg) were converted into *O*-acetyl-*O*-methylhexitols as described by Björndal *et al.*[12]; control experiments with methylated polysaccharide *B* showed that the mole fraction of the products remained constant when the hydrolysis in 90% formic acid was performed for 7.5–12 h (see Table I). The products were analysed by g.l.c.–m.s. Retention times and peak areas were determined separately with a Pye 104 gas chromatograph[24].

Fragmentation of periodate-oxidised polysaccharide B. — Polysaccharide *B* (1 g) was subjected to the procedure described earlier[10]. P.c. of the product revealed components having migration rates identical with those of glycerol, D-glucose, and 1-*O*-α-D-glucopyranosylglycerol.

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