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Note

The structure of water-insoluble glucans of cariogenic *Streptococcus mutans*, formed in the absence and presence of dextranase

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Current clinical and biochemical studies have indicated that certain water-insoluble, dextran-like polysaccharides produced by oral *Streptococci*, such as strains of *Streptococcus mutans* and *S. sanguis*, may be responsible for the induction of dental caries and formation of plaque[1–4]. These insoluble polysaccharides were determined by chemical and enzymic studies to contain high proportions of (1→3)-α-D-glucosidic linkages, either as inter-residue or inter-chain linkages[5–9], which would contribute to the resistance of the glucans of cariogenic microorganisms to dextranase [(1→6)-α-D-glucan 6-glucanohydrolase]. Recently, Guggenheim *et al.*[4,5] showed that (1→3)-α-D-glucanase (mutanase) (isolated from fungal dextranase preparations) could degrade, to some extent, the insoluble D-glucan elaborated by *S. mutans* OMZ 176, and the enzyme appeared to reduce dental caries in rats.

During investigations on the structure and enzymic degradation of different types of dextrans, it was found that a non-inducible enzyme isolated from *Flavobacterium* sp. is capable of hydrolyzing the insoluble D-glucans of several strains of cariogenic *S. mutans*, with release of nigerose and α-D-(1→3)-linked oligosaccharides[10]. This observation has prompted the present investigation of the detailed structural features of such insoluble D-glucans, particularly the locations and sequences of the α-(1→3)-linked D-glucose residues in the enzymically synthesized glucans of *S. mutans* OMZ 176 formed in the absence and presence of dextranase.

RESULTS AND DISCUSSION

In order to exclude possible contamination by any cellular polysaccharide, the polysaccharides used in the present study were prepared from sucrose in a cell-

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free system. The glucosyltransferase fraction precipitated from the culture filtrate of *Streptococus mutans* OMZ 176 with ammonium sulfate added to 50% saturation was incubated with 10% sucrose at pH 6.8, and the insoluble polysaccharide was obtained by centrifugation. The native polysaccharide, designated glucan I, had α-D-glucosidic linkages as indicated by i.r. spectroscopy and its optical rotation (methylated derivative, $[\alpha]_D$ +222°). The glucan was water-insoluble but was soluble in dilute alkali, and showed adhesive characteristics on a glass plate. In addition to the foregoing insoluble glucan, a water-soluble polysaccharide was also obtained from the soluble fraction of the incubation mixture. It appeared to resemble an α-D-(1→6)-linked dextran, but its structure remains to be determined.

When the glucosyltransferase was incubated with sucrose in the presence of a highly active dextranase, a small, but significant, proportion of an water-insoluble, non-sticky polysaccharide, designated glucan II, was produced.

Both glucan I and II were methylated by the method of Hakomori[11] and the methylated sugar components were analyzed by g.l.c. as their alditol acetates. The identities and molar proportions of the methylated sugars are listed in Table I. It is evident from the methylation analysis that the native glucan (glucan I) is highly

TABLE I

HYDROLYSIS PRODUCTS FROM THE METHYLATED D-GLUCANS OF *Streptococcus mutans* OMZ 176

O-Methyl-D-glucose	*Linkage indicated*	*Molar percent*	
		Native glucan (glucan I)	*Dextranase-modified glucan (glucan II)*
2,3,4,6-Tetra-	Glc*p*-(1→	14.6	1.9
2,4,6-Tri-	→3)-Glc*p*-(1→	50.5	94.5
2,3,4-Tri-	→6)-Glc*p*-(1→	20.9	1.9
2,4-Di-	→3)-Glc*p*-(1→ 6 ↑	14.0	1.7

branched at O-3 and O-6 of the D-glucose residues, with an average repeating unit of 7 sugar residues, in which the linear-chain linkages consist of 70% α-(1→3)- and 30% (1→6)-D-glucosidic bonds. When glucan I was subjected to the Smith degradation[12], which involves periodate oxidation, borohydride reduction, and hydrolysis with 2M acid at 100°, D-glucose and glycerol were produced in an approximate ratio of 2:1, consistent with the methylation result. When the glucan polyalcohol was hydrolyzed with dilute acid at room temperature (controlled Smith degradation)[13], a degraded glucan still insoluble in water, but devoid of adhesive properties, was recovered in 63% yield from the parent glucan. A careful search of the water-soluble products in the mild-acid hydrolyzate of the glucan polyalcohol revealed only glycerol, which arises from the non-reducing terminals and (1→6)-inter-residue linkages. The absence of D-glucosylglycerol or glycerol glycosides of D-glucosaccharides, and the

high yield of the degraded glucan, indicated that all of the α-(1→3)-linked D-glucose residues surviving periodate oxidation were recovered as the insoluble D-glucan. The degraded glucan was methylated, and the hydrolyzate was examined, revealing a preponderant amount of 2,4,6-tri-*O*-methyl-D-glucose, with only traces of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose. The foregoing result is a strong indication that the consecutive α-(1→3)-linked D-glucose residues form relatively long chains, and are located in the backbone of a highly branched D-glucan. Thus, possible structures for the native D-glucan, and the polysaccharide derived by the Smith degradation, are illustrated as **1** and **2**, respectively.

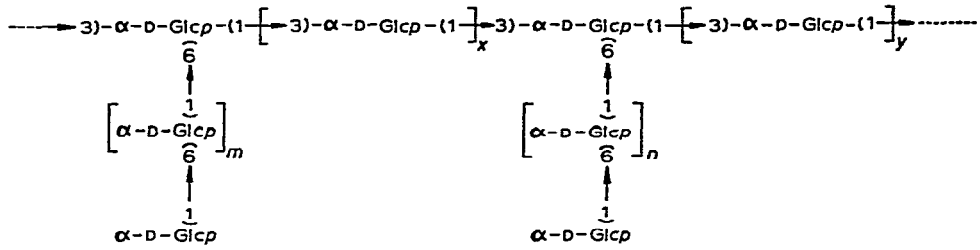
Although structure **1** can explain all of the experimental findings on the native D-glucan, alternative structural formulations, such as **3** and **4**, where all or some of the α-(1→3)-linked sugar residues are located in the side chains, might also be conceived. However, these structures contain relatively short (1→3)-linked chains in their branches ($x+y=7$ in formula **3**, $x+y<7$ in formula **4**), and would give on Smith degradation a mixture of the glycerol D-glucosides of (1→3)-linked oligosaccharides. Upon methylation and hydrolysis, structure **3** should yield seven molar proportions of 2,4,6-tri-*O*-methyl-glucose per two molar proportions of 2,3,4,6-tetra-*O*-methyl-glucose. Therefore, the fact that (*a*) there was no 1-*O*-α-D-glucosyl-glycerol or any other glycerol D-glucoside detected in the water-soluble products from the controlled (mild acid) Smith degradation, and (*b*) the insoluble product formed simultaneously (in high yield) was a polysaccharide and not a mixture of oligosaccharides (as revealed by methylation analysis), must rule out the possibility of structure **3** or **4**, and support structure **1** as the most feasible one.

Additional evidence for the absence of (1→3)-linked sugar residues at the terminal ends of the branches, as would occur in **4**, was provided by the use of a specific exo type of dextranase from *Achromobacter* sp.[14]. This enzyme hydrolyzes (1→6)-α-D-glucosidic linkages from the end of the chain, with liberation of isomaltose. When this enzyme acted on the native D-glucan, a small, but significant, proportion of isomaltose was produced (unpublished result)*.

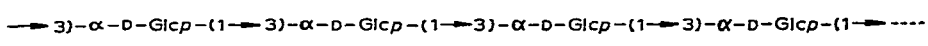
The presence of α-(1→3)-linkages in the main chain was further supported by structural studies on the dextranase-modified polysaccharide (glucan II). This glucan was methylated and then hydrolyzed, to yield mainly 2,4,6-tri-*O*-methyl-D-glucose, together with very small proportions of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose, as shown in Table I. This result indicates that the D-glucan synthesized in the presence of dextranase has only a slight degree of branching, namely, one (1→6)-linked branch (possibly two D-glucose residues long) for every 45–47 (1→3)-linked D-glucose residues.

Recent studies have shown that several types of α-D-glucans are elaborated by cariogenic *Streptococci*. The soluble glucan insolated from the broth of *S. mutans* E49 was shown to contain 20% of (1→3) linkages, located as the branch points[7], forming a "tree-like" structure[8]. The D-glucan of *S. mutans* Ingbritt A, synthesized

*The enzyme was provided by Dr. T. Sawai, Aichi Kyoiku University, Aichi, Japan.



1 ($x + y = 7$; $m + n = 3$, $m = 0 \sim 3$)



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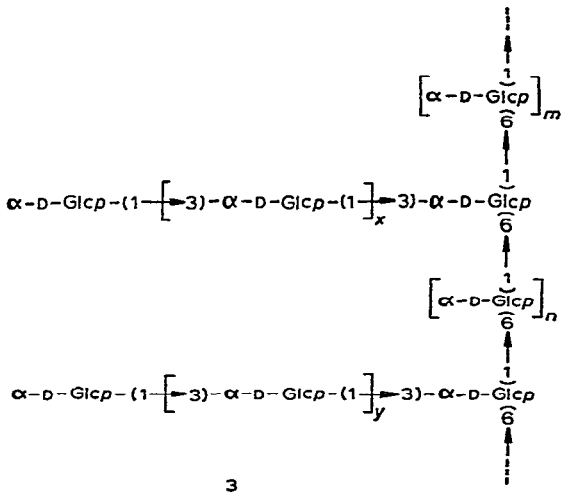
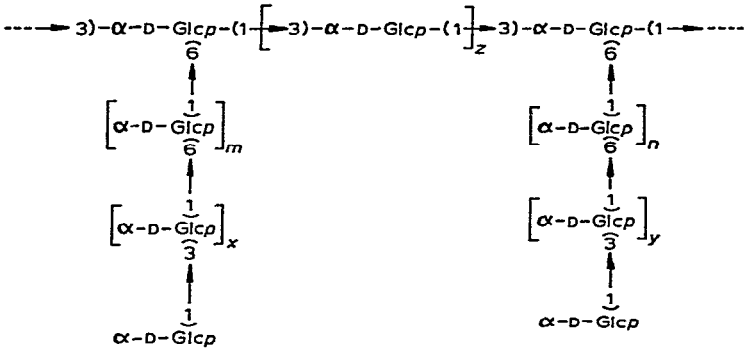


3

($x + y = 7$, $m + n = 3$, $m \geq 1$, $n \geq 1$)



4

($x + y + z = 7$, $m + n = 3$; $m \geq 1$, $n \geq 1$

by its glucosyltransferase, contained a high percentage (50%) of (1→3)-inter-residue linkages[9]. The D-glucan of *S. mutans* OMZ 176 also contained a high percentage of (1→3) linkages, as shown by the work of Guggenheim[5]. More recently, Ceska *et al.*[6] investigated several enzymically synthezized D-glucans of *S. sanguis*, and *S. mutans* OMZ 176 by the methylation technique, and showed the presence of different proportions of (1→6)- and (1→3)-linkages. Interestingly the structure of one of their D-glucan preparations (having a mol. wt. of 70,000) consisted preponderantly of (1→3) linkages, whose structure seems to be similar to that of our glucan II. Thus, the present results provide unambiuous evidence for α-(1→3)-linked backbone chain in the insoluble glucan of *S. mutans* OMZ 176, and it is clear that most (1→6)-linked D-glucose residues are located as the side chains, forming a ramified, comb-like structure. Taking into account its insolubility characteristics, the ramified native D-glucan appears to contain relatively long α-(1→3)-linked linear sequences in parts of the molecule. With regard to the length of these side chains, the fact that prolonged digestion of glucan I (**1**) by the (1→3)-α-D-glucanase of *Flavobacterium* sp. resulted in release of isomaltose, in addition to nigerose and other α-(1→3)-linked oligosaccharides (as will be reported in forthcoming paper), suggest that, in structure **1**, some branches are a single unit long.

It is not yet possible to say whether or not the main chain of the native D-glucan (**1**) consists entirely of α-(1→3)-linkages or contains also (1→6)-linkages, but the finding a small proportion of glycerol (molar ratio of glucose and glycerol, 25:1) in the hydrolyzate of the Smith-degraded glucan suggests a possibility that long chains of α-(1→3)-linked D-glucose residues are flanked by (1→6) linkages.

In the view of the physical properties of *Streptococcal* glucans, it is noteworthy tha the native glucan of *S. mutans* OMZ 176, having α-(1→6)-linked side-chains, is water-insoluble but does possess sticky and adhesive characteristics, whereas the D-glucan formed in the presence of dextranase is an essentially linear α-(1→3)-linked D-glucan. This dextranase-modified glucan is also water insoluble, but devoid of the original adhesive characteristics. Therefore, it can be deduced from the changes in physical properties of the native and modified glucans, that the insolubility property is attributable mainly to the linear, (1→3)-linked backbone chain, whereas the (1→6)-linked side-chains having a certain length are related to the adhesion of the D-glucan to the surface of teeth. In connection with this, it may be noted that the dextran of *Leuconostoc mesenteroides* NRRL 1355S contains a relatively high proportion of α-(1→3)-inter-residue and inter-chain linkages (Goldstein and Misaki, unpublished result). However, unlike the present D-glucan, it is readily soluble in water. Its solubility properties may be explained by the absence of consecutive, (1→3)-linked, D-glucose residues[15].

EXPERIMENTAL

Materials. — A culture of *Streptococcus mutans* OMZ 176 was kindly supplied by Dr. B. Guggenheim, Experimental Caries Research Laboratory, Dental Institute,

University of Zürich, Switzerland. The dextranase preparation of *Spicaria violaceae* IFO 6120 (ref. 16) was a gift from Dr. Y. Murayama, Department of Oral Medicine, Osaka University Dental School.

General methods. — Paper chromatography was usually performed on Toyoroshi No. 51A by the descending method with (A) 4:1:5, 1-butanol–acetic acid–water, and, (B) 6:4:3, 1-butanol–pyridine–water.

Gas–liquid chromatography (g.l.c.) was carried out with a Hitachi K-53 gas chromatograph, fitted with a flame-ionization detector and a stainless-steel column (0.4 × 200 cm). Methylated sugars were converted into their corresponding alditol acetates and separated on a column of 3% ECNSS-M on Gas Chrom Q at 180°. For g.l.c. of the Smith-degradation products, the acid hydrolyzate of the polysaccharide polyalcohol was reduced with sodium borohydride, and then acetylated by heating with 1:1 pyridine–acetic anhydride, for 1 h at 100°, and the products separated on the same column, at 60–190°, programed at a rate of 7.5° per min[17]. Nitrogen was used as the carrier gas, at a flow rate of 60 ml per min.

Preparation of enzymically synthesized glucans. — The α-D-glucosyltransferase preparation was obtained from the cultural filtrate of *Streptococcus mutans* OMZ 176, grown in Difco Brain Heart Infusion Broth (12 l), for 20 h at 37°. The enzyme fraction precipitated with ammonium sulfate at 0.5 saturation was dissolved in 0.05M phosphate buffer (100 ml, pH 6.8) and dialyzed against the same buffer. One half of the enzyme preparation so obtained was incubated with 0.05M phosphate buffer (pH 6.8) containing 10% of sucrose (total volume, 100 ml) at 37° for 48 h. The turbid incubation mixture was centrifuged (45,000 *g*, 30 m in), and the insoluble polysaccharide (glucan I), which tended to adhere on the surface of a glass plate, was washed thoroughly with water. After two treatments with crystalline trypsin to remove any contaminating protein, the insoluble D-glucan was lyophilized (yield, 2.136 g). To the supernatant solution of the incubation mixture was added ethanol (2 vols.), which yielded a water-soluble polysaccharide (772 mg).

In another experiment, a mixture of the remaining glucosyltransferase preparation and the dextranase of *Spicaria violaceae* (10,000 units)[16] was incubated in 10% sucrose solution (total volume, 100 ml), under the same conditions already described, after which time the insoluble D-glucan was separated from the incubation mixture, and purified in the same manner as for glucan I (yield, 43 mg). This polysaccharide, designated glucan II, was less adhesive compared to the native glucan (glucan I). There was no water-soluble polysaccharide in the supernatant of the incubation mixture.

Methylation analysis. — Glucan I (50 mg) was dissolved in dimethyl sulfoxide (3 ml) under ultrasonication (20 KHz.sec^{-1}, 5 min) at 25°, in a nitrogen atmosphere, and was then methylated by the method of Hakomori[11], with fresh methylsulfinyl carbanion (1 ml), and then methyl iodide (2 ml), as used previously[18]. After one more methylation, the reaction mixture was dialyzed against water and the fully methylated polysaccharide (as judged by i.r. spectroscopy) was extracted into

chloroform, and after drying (magnesium sulfate) the extract was evaporated to dryness (39 mg, $[\alpha]_D$ +222°, chloroform).

Glucan II (20 mg) was also methylated by the same procedure to give the fully methylated product (15 mg).

The methylated glucans (15 mg, each) were hydrolyzed by treatment with 72% sulfuric acid (0.1 ml) for 1 h at 5°, and for 6 h then at 100°, after having been diluted with water (7 vols.). The hydrolyzate was neutralized (barium carbonate), and the methylated sugar components were reduced, and converted into their corresponding alditol acetates by heating with a mixture (1:1, 0.2 ml) of pyridine and acetic anhydride for 1 h at 100°, and were then analyzed by g.l.c.[19].

Smith degradation of glucan I and methylation of the degraded polysaccharide. — The native glucan (glucan I, 195 mg) was oxidized with 0.08M sodium periodate (100 ml) at 20° in the dark. After complete oxidation (14 days; periodate consumption 0.75 mole, and formic acid production 0.35 mole per sugar residue), the insoluble oxidation-product was recovered by centrifugation. After washing with water, it was dissolved in 0.1M sodium hydroxide (8 ml), and reduced with sodium borohydride (150 mg) in the usual way. The excess borohydride was decomposed by the addition of acetic acid (final pH 6.5), and the reaction mixture containing insoluble, D-glucan polyalcohol was dialyzed at 5°, and the non-dialyzable fraction was lyophilized. A portion (20 mg) of the D-glucan polyalcohol was hydrolyzed with 2M sulfuric acid (0.2 ml) for 2.5 h at 100°. After neutralization barium carbonate, the hydrolyzate was examined by paper chromatography (solvents A and B), which showed spots corresponding to glycerol, glucose (major), and erythritol (trace). Quantitative analysis by g.l.c. revealed that the hydrolyzate contained glycerol (33.4%), glucose (65.5%), and probably erythritol (less than 1%).

The D-glucan polyalcohol derived from glucan I (100 mg) was subjected to mild hydrolysis with 0.05M sulfuric acid for 16 h at 25°. The hydrolyzate was centrifuged to separate the insoluble, degraded polysaccharide from the water-soluble products. The degraded D-glucan so obtained was washed with water (three times), and lyophilized (yield, 63 mg). The supernatant solution was neutralized and examined by paper chromatography; it showed a spot only for glycerol. A portion (10 mg) of the degraded D-glucan, collected as an insoluble product, was hydrolyzed with 2M sulfuric acid in the manner described previously, and the hydrolyzate was examined by g.l.c., which revealed the presence of glycerol and glucose in the approximate ratio of 1:25.

The Smith-degraded D-glucan (20 mg) was methylated by the method of Hakomori, and the methylated D-glucan was isolated from the chloroform extract of the reaction mixture. The product was hydrolyzed, and the methylated sugar components were analyzed by g.l.c., as their alditol acetates. The results showed that the hydrolyzate contained exclusively 2,4,6-tri-*O*-methyl-D-glucose, with only traces of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose (less than 0.5% of each).

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