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STRUCTURAL ELUCIDATION OF A WATER-INSOLUBLE GLUCAN PRODUCED BY A CARIOGENIC ORAL *Streptococcus*

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ABSTRACT

The structure of a water-insoluble polysaccharide produced by the D-glucosyltransferase of *Streptococcus mutans* 6715 has been elucidated through periodate oxidation, Smith degradation, dextranase digestion, concanavalin A binding studies, and methylation combined with g.l.c.–m.s. analysis. These studies show that the D-glucan is comprised of 67% α-(1→3) linkages in a contiguous backbone with the remaining 33% as α-(1→6) linkages, possibly as linear residues extending from α-(1→6) branch points. Of the residues, 14% are branch points and the ratio of linear α-(1→3) residues in the backbone to α-(1→6) residues in the side chain was found to be 5:2. Dextranase digestion and Smith degradation both gave rise to a high-molecular-weight fraction that is only α-(1→3) linked.

INTRODUCTION

Certain oral *Streptococci* produce water-insoluble glucans that are directly related to the cariogenicity of the organism[1]. Most water-insoluble glucans contain a high proportion of α-(1→3) linkages in a linear sequence[2,3]. In addition, α-(1→6) linkages are usually present and form side chains in the polysaccharide. The hypothesis advanced by Robyt and Corrigan[4] proposes that there are two types of D-glucosyltransferases that can transfer glucosyl moieties from sucrose to an acceptor, giving rise to glucans containing both linear and branched regions.

Structural determination of glycans commonly uses periodate oxidation–Smith degradation in conjunction with methylation [the Hakomori procedure[5] uses the methylsulfinyl ("dimsyl") carbanion in Me_2SO]; the methylated polysaccharide is then analyzed by g.l.c.–m.s. following methanolysis and acetylation[6], or via the corresponding alditol acetates[7]. The procedure for carbohydrate derivatization of Rolf and Gray[8] involves reductive cleavage of the methylated carbohydrates to give 1,5-anhydroalditol derivatives. This route has the advantage of eliminating anomeric peaks from the gas chromatogram and also keeps the ring form intact. Other methods of structural study employed herein involve digestion by dextranase and concanavalin A binding studies. An endodextranase produced by *Chaetomium gracile* has been shown to hydrolyze α-(1→6) linkages in polysaccharides produced

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by *Streptococcus mutans* species[9]. Concanavalin A has an affinity for terminal α-D-mannopyranosyl and α-D-glucopyranosyl residues, so that it can also be used to determine the degree of branching and the anomeric orientation in the polysaccharide. This paper utilizes the aforementioned techniques to ascertain the structure of a water-insoluble polysaccharide produced by the cariogenic bacterium, *Streptococcus mutans* 6715.

EXPERIMENTAL

Culture preparation. — *Strep. mutans* 6715 was obtained from F. P. Mertz of Eli Lilly and Co. (Indianapolis, IN). Cultures were lyophilized and stored at −20°. Culture conditions employed for growth of the bacterium were similar to that of Figures and Edwards[10]. Invertase was added to the Trypticase Soy Broth preparation in order to free the medium of traces of sucrose. The medium was then filtered through a PM-10 ultrafiltration membrane using an Amicon pressure-cell to remove any polysaccharides. The medium was supplemened with fructose and Tween-80 (T-80) at final concentrations of 1 and 0.05%, respectively.

Enzyme isolation. — After a growth period of 18–20 h, the cells were removed by centrifugation (10,000 *g*, 15 min). Potassium chloride and sodium azide were dissolved in the spent medium to give final concentrations of M and 0.02%, respectively, and the pH was adjusted to 6.0 with NaOH. The spent medium was concentrated and then diafiltered with a phosphate–KCl–T-80 buffer through the use of an Amicon pressure-cell. Final clarification of the enzyme solution was accomplished by filtration through a bacterial membrane filter (0.45 μm). The enzyme was assayed for total protein concentration by the method of Lowry[11] and for enzymic activity as previously described[10].

Glucan production and isolation. — The enzyme solution was incubated with sucrose (50mM, pH 6.0) for 8 h. After several hours, a white, flocculent glucan began to precipitate from the incubation mixture. The glucan was removed by centrifugation (10,000 *g*, 15 min) and then dissolved in KOH (M). The polymer (~5%) was precipitated by the addition of acetic acid. This procedure was repeated twice and the glucan was dried in a vacuum desiccator.

Total acid hydrolysis of the glucan. — Hydrolysis was accomplished with sulfuric acid (1.5M, 100°, 10 h). The hydrolyzate was made neutral by the addition of solid barium carbonate. The mixture was kept for several hours and the solids were removed by filtration. Total carbohydrate concentration was measured by the phenol–sulfuric acid assay of Dubois *et al.*[12] and the D-glucose was assayed by glucose oxidase–peroxidase[13]. Prior to chromatographic examination, the filtrate was then passed through a column of mixed-bed ion-exchange resins for desalting. Products from the hydrolysis were identified on paper chromatograms developed for 48 h with 5:1:4 (v/v) 1-butanol–acetic acid–water. An ammoniacal silver nitrate spray (0.2%) was used to locate the carbohydrate spots. Thin-layer chromatography was conducted on cellulose with 3:5:7:5 (v/v) formic acid–butanone–*tert*-

butyl alcohol–water as solvent. The plates were developed with a diphenylamine–phosphoric acid–acetone spray reagent and heated for 5 min at 100°.

F.t.–i.r. spectroscopy. — Infrared spectroscopy of the glucan (50 mg) with KBr (200 mg) was performed by diffused reflectance on an Analect fx-6160 F.t.–i.r. instrument.

Periodate oxidation. — The method of Rankin and Jeanes[14] was used with the water-insoluble glucan. *Leuconostoc mesenteroides* B512 dextran was used as the experimental standard for all analyses. The polysaccharides (2 mg/mL) were mixed with sodium metaperiodate (10 mg/mL) at 25° and continuously shaken for ~150 h. Periodically, aliquots were removed and analyzed for periodate uptake, formic acid production, and unoxidized polymer. The periodate uptake was measured by the method of Fleury and Lange[15], and formic acid production was determined by titration with NaOH (0.01M) using phenolphthalein as the indicator.

Smith degradation. — Ethylene glycol (50 μL) was added to aliquots (20 mL) of the periodate-oxidation mixture and these were incubated for 3 h. Sodium borohydride (40 mg) was added, followed after 4 h by a slight excess of sulfuric acid (5mM). The solution was then exhaustively dialyzed against distilled water. This sample was then divided into two fractions. One fraction was subjected to total acid hydrolysis and analyzed for glucose. The other was subjected to partial acid hydrolysis (0.25M HCl, 30 h, 50°) with the soluble products being subjected to column chromatography on Sephadex G-15. (The hydrolysis was terminated when glucose was detected in the hydrolyzate.) The void volume (v_0) was measured with Blue Dextran and the inclusion volume (v_i) with copper sulfate. Fractions were assayed for total carbohydrate with the phenol–sulfuric acid test.

Methylation. — The experimental standard dextran from *Leuconostoc* and the *Streptococcus* water-insoluble polysaccharide was methylated by the method of Hakomori. Dimsyl carbanion was generated by adding hexane-extracted NaH (0.27 g) to Me_2SO (5.4 mL, vacuum distilled at 68° and stored over CaH_2 under nitrogen). After warming the Me_2SO mixture for 20–30 min at 35–40°, the carbohydrate (100 mg) was added and allowed to react for 6 h at 25°. Methyl iodide (0.75 mL), diluted 2-fold with Me_2SO was added at 0° during 20 min and allowed to react overnight. The excess of CH_3I was then driven off under N_2 at 50°. Additional NaH (0.10 g) was added at 0° followed by CH_3I, and allowed to react overnight. The reaction was terminated by addition of water and the mixture was then extracted with chloroform. The chloroform extracts were combined and then exhaustively dialyzed against water. The dried (sodium sulfate) chloroform layer was evaporated. The carbohydrates could also be precipitated from chloroform by petroleum ether. I.r. spectroscopy was used to test for completeness of methylation.

Methanolysis. — Methylated carbohydrates (4 mg) were subjected to methanolic HCl (0.55M, 0.8 mL) for 72 h at 100°. *tert*-Butyl alcohol (0.5 mL) was added to the methyl glycosides and then they were dried under N_2. The material was divided into two fractions. One was used for reductive cleavage and the other

was acetylated with 1:1 acetic anhydride–pyridine (1 h, 40°) and analyzed by gas chromatography.

Reductive cleavage. — The methanolyzed, methylated carbohydrate was subjected to reductive cleavage as described by Rolf and Gray[8]. The reducing agent was prepared from boron trifluoride etherate (310 μL), triethylsilane (400 μL), trifluoroacetic acid (64 μL) and dichloromethane (226 μL). The reducing agent (500 μL) mixture was added to the methanolysis product (1 mg) at 0° and allowed to react for 24 h. Acetic anhydride (50 μL) was added and the temperature was raised to 40° for 1 h. The acetylated–methylated products were extracted against water with CH_2Cl_2. The combined extracts were washed (3×) with H_2O and dried down under nitrogen. Dichloromethane was added and the products were analyzed by g.l.c.–m.s.

Gas chromatography–mass spectroscopy. — A Hewlett–Packard 5790A series capillary gas chromatograph was used to analyze all products before g.l.c.–m.s. analysis. A SE-30 coated, fused-silica capillary column (30 m × 0.25 mm i.d.) and a temperature program [150° (2 min), 4°/min, 250° (2 min)] were used. Subsequent analysis by g.l.c.–m.s. (e.i., 70-eV source) was performed on an OV-1 coated, fused-silica capillary column (12 m × 0.2 mm i.d.) in a Hewlett–Packard 5970A mass-selective detector, using the temperature program already described. Spectra were acquired by scanning from *m/z* 40–400.

Dextranase digestion. — Carbohydrate samples (100 mg) were dissolved in acetate buffer (6 mL, 50mM, pH 5.1) which contained dextranase (100 μL, 1700 units/mg, Miles Scientific, Naperville, Il) from *Chaetomium gracile*. The samples were incubated overnight at 40°. The digestion was monitored by the glucose oxidase–peroxidase assay for glucose production and a total reducing-sugar assay[16]. The mixture was centrifuged free of insoluble material, concentrated, and analyzed by column chromatography on Sephadex G-15 and by cellulose t.l.c.

Concanavalin A–water-insoluble glucan binding. — Binding studies were conducted essentially according to Shibata and Goldstein[17]. Water-insoluble glucan (27 mg) was added to phosphate buffer (3 mL, 0.1M, pH 7.2) that contained $CaCl_2$ (0.1 mM), NaN_3 (0.01%), NaCl (0.15M), and concanavalin A (300 μg, Sigma Chemical Co. St. Louis, Mo.) isolated from *Canavalia ensiformis*. The mixtures were allowed to react for 2 days at 40°. Following incubation, the mixture was centrifuged (10,000*g*, 15 min). The supernatant solution was decanted and tested for protein by the method of Lowry[11]. This was repeated until the supernatants contained <10 μg of protein. The complex was then disassociated with methyl α-D-mannopyranoside (50mM) and the protein concentration was determined.

RESULTS

Total acid hydrolysis. — Total acid hydrolysis of the polysaccharide revealed only one component, indistinguishable from glucose upon paper chromatography. Comparison of the glucose concentration in the total hydrolyzate by total carbo-

hydrate analysis and by the glucose oxidase–peroxidase assay verified that all of the carbohydrate was D-glucose. The polysaccharide was therefore a homopolymer of glucose.

I.r. spectroscopy. — The water-insoluble glucan was shown to be α-linked from its strong absorption at 850 cm^{-1} and the lack of absorption at 891 cm^{-1} (which would have been indicative of a β-linked polymer). In addition, both ^{13}C-n.m.r. spectroscopy and concanavalin A-binding to the glucan confirmed the α orientation.

Periodate oxidation and Smith degradation. — The water-insoluble glucan consumed 0.70 mol of periodate and produced 0.33 mol of formate per mol of glucose residues, which corresponds to 67% α-(1→3) and 33% α-(1→6) linkages in the glucan. The percentage of α-(1→3) residues in the oxidized polysaccharide was determined directly by complete acid hydrolysis. The glucose remaining closely correlated with the percentage of α-(1→3) linkages calculated from the periodate analysis. Smith degradation of the water-insoluble glucan yielded fractions of high and low molecular weight upon gel filtration on Sephadex G-15 (Fig. 1). The high-molecular-weight fraction (solubilized in pH 11 buffer) had mol. wt. ~3500, corresponding to 20 glucose residues. The low-molecular-weight fraction was shown by t.l.c. to be composed primarily of glycerol. The high-molecular-weight fraction was reoxidized by periodate. Only 0.026 mol of periodate was consumed per mol of glucose residues, suggesting that ~95% of the glucose in the high-molecular-weight fraction was α-(1→3)-linked. These data are indicative of a contiguously linked α-(1→3) backbone with α-(1→6) residues as linear side-chains.

Dextranase digestion. — Endodextranase digestion of the water-insoluble

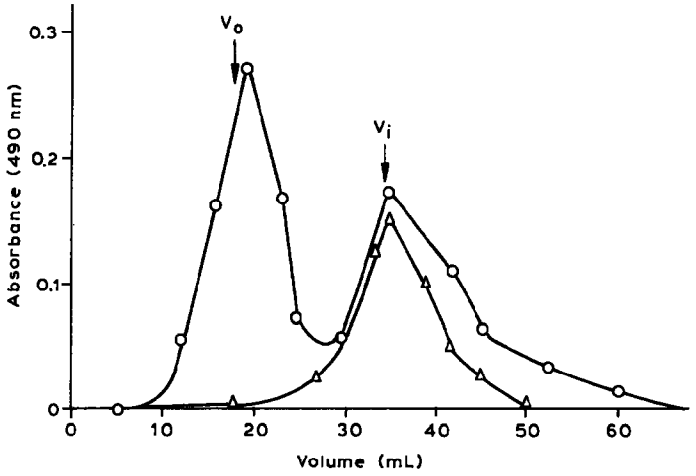


Fig. 1. Elution profile on Sephadex G-15 of the products from the partial acid hydrolysis of the Smith degradation. The curve with circles represents the products from the water-insoluble glucan from the broth of *Streptococcus mutans* 6715 and that with triangles represents products from the standard *Leuconostoc* dextran.

glucan gave soluble and insoluble fractions. The soluble fraction eluted with the inclusion volume of a Sephadex G-15 column and was found by t.l.c. to contain glucose and isomaltose. The insoluble material remaining after dextranase digestion was 74% of the original mass of the glucan; it had mol. wt. ~13,000 by gel filtration in a pH 11 buffer on a column of Bio-Gel P60. Studies by ^{13}C-n.m.r. spectroscopy showed the insoluble material to be devoid of α-(1→6) linkages[18]. This evidence suggests that the insoluble glucan has a contiguously linked α-(1→3) backbone with α-(1→6) residues extending linearly from α-(1→6) branch points.

Molecular weight studies. — The water-insoluble glucan had mol. wt. 18,000–20,000, as determined by gel filtration in a pH 11 buffer on a column of Bio-Gel P60, using *Leuconostoc* dextrans for standardization. This value was substantiated by results of end-group analysis.

The discrepancy between the molecular weights by dextranase digestion (13,000) and by Smith degradation (3,500) most probably was attributable to partial acid hydrolysis of the oxidized–reduced polymer.

Hakomori methylation and methanolysis. — The water-insoluble glucan was methylated for 24 h and then remethylated. The extent of methylation was determined by the i.r. absorption at 3500 cm^{-1}. The gas chromatogram of the products from the methylated, water-insoluble polysaccharide, after methanolysis and acetylation, is shown in Fig. 2. The products gave the expected α,β-mixtures. Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside had a longer retention time than its anomer.

Methanolysis and reductive cleavage. — The water-insoluble glucan gave rise to four sets of peaks following methanolysis: these correspond to derivatives

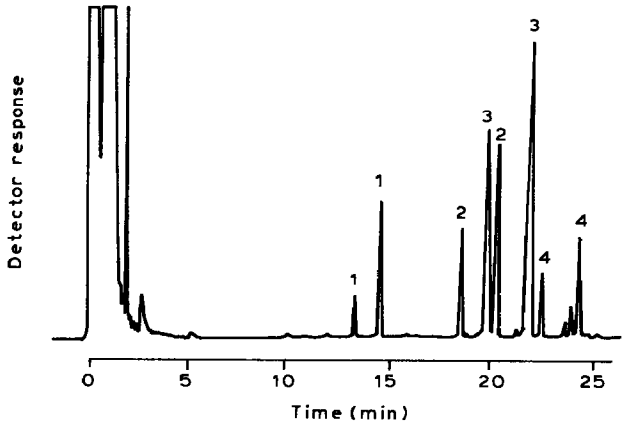


Fig. 2. Gas chromatogram of the products of the methylated water-insoluble glucan following methanolysis and acetylation: 1, methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-glucopyranoside; 2, methyl 6-*O*-acetyl-2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside; 3, methyl 3-*O*-acetyl-2,4,6-tri-*O*-methyl-α,β-D-glucopyranoside, and 4, methyl 3,6-di-*O*-acetyl-2,4-di-*O*-methyl-α,β-D-glucopyranoside. Unlabeled peaks were not identified, but were shown by their mass spectra to be devoid of carbohydrate. The identity of each compound was confirmed by e.i.–m.s. Refer to Experimental for conditions.

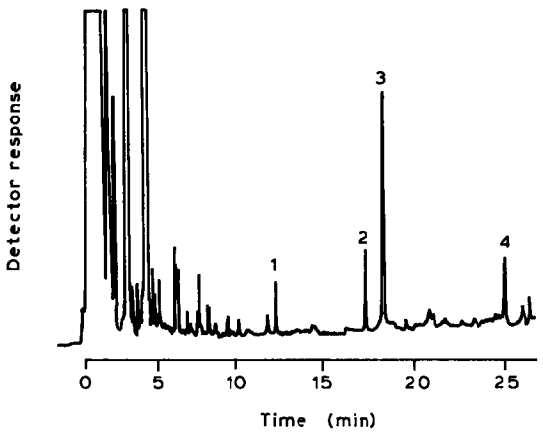


Fig. 3. Gas chromatogram of the carbohydrate derivatives resulting from the reductive cleavage method: 1, 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol; 2, 6-*O*-acetyl-1,5-anhydro-2,4,3-tri-*O*-methyl-D-glucitol; 3, 3-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol; and 4, 3,6-di-*O*-acetyl-1,5-anhydro-2,4-di-*O*-methyl-D-glucitol. Unlabeled peaks were not identified but were shown by their mass spectra to be devoid of carbohydrate.

produced from the non-reducing ends, from the linear α-(1→6) and α-(1→3) residues, and from the branch points. The methanolysis products were analyzed by g.l.c.–m.s. The e.i. fragments were identical to published data[19]. The methanolysis derivatives were then subjected to reductive cleavage to simplify the g.l.c. interpretation, since only the 1,5-anhydroglucitol derivatives would result. The gas

TABLE I

ELECTRON-IMPACT FRAGMENTATIONS OF THE DERIVATIVES PRODUCED BY REDUCTIVE CLEAVAGE AND ACETYLATION[a]

1,5-Anhydro-2,3,4,6-tetra-O-*methyl*-D-*glucitol*
41(11), 42(3), 43(40), 44(8), 45(47), 55(6), 56(4), 57(4), 58(7), 59(9), 65(3), 67(8), 69(6), 71(42), 72(4), 73(8), 75(21), 83(5), 85(9), 87(5), 88(19), 89(4), 96(11), 98(5), 99(12), 101(100), 102(11), 109(12), 110(4), 111(14), 115(6), 124(11), 125(5), 127(14), 143(10), 149(52), 175(31), 176(5), 177(12), 188(4), 191(47), 192(2), and 193(2).

3-O-*Acetyl-1,5-anhydro-2,4,6-tri*-O-*methyl*-D-*glucitol*
41(17), 43(78), 44(50), 45(44), 55(21), 59(26), 63(11), 66(12), 69(22), 71(41), 74(63), 75(100), 83(12), 85(14), 87(5), 97(6), 99(4), 101(57), 102(4), 111(7), 114(11), 115(12), 116(4), 125(14), 127(11), 129(8), 138(3), 139(4), 141(5), 143(34), 156(4), and 158(9).

6-O-*Acetyl-1,5-anhydro-2,3,4-tri*-O-*methyl*-D-*glucitol*
43(100), 45(41), 58(31), 59(13), 69(5), 71(41), 73(14), 75(53), 87(55), 88(42), 101(51), 102(52), 130(16), 156(9), and 188(3).

3,6-*Di*-O-*acetyl-1,5-anhydro-2,4-di*-O-*methyl*-D-*glucitol*
43(100), 45(11), 57(7), 58(2), 71(8), 74(14), 75(11), 87(10), 88(4), 101(24), 102(3), 117(13), 153(3), 154(2), and 156(4).

[a]The mass number is given first followed by the relative abundance in parentheses.

TABLE II

RELATIVE PERCENTAGES[a] OF CARBOHYDRATE DERIVATIVES PRODUCED BY METHANOLYSIS AND REDUCTIVE CLEAVAGE

1,5-Anhydro derivatives of D-*glucopyranoses*	*Insoluble glucan*	Leuconostoc *dextran*
2,3,4,6-Tetra-*O*-methyl-	12	4
3-*O*-Acetyl-2,4,6-tri-*O*-methyl-	53	0
6-*O*-Acetyl-2,3,4-tri-*O*-methyl-	21	89
3,6-Di-*O*-acetyl-2,4-di-*O*-methyl-	14	7

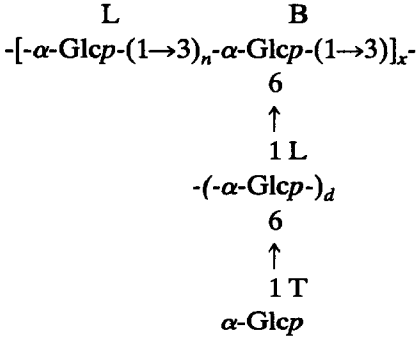
[a]By integration of peak areas.

chromatogram and the mass spectra of the reductively cleaved, permethylated water-insoluble glucan are shown in Fig. 3 and Table I, respectively. The *Leuconostoc* dextran contained 89% of linear, extending α-(1→6) residues and 7% of branching residues, according to methylation analysis (Table II). The water-insoluble glucan has, on the average, 5 linear α-(1→3) residues to 2 linear α-(1→6) residues, with ~14% of the α-(1→3) backbone involved as branch points.

Concanavalin A–insoluble glucan binding studies. — It was determined that 33 μg of concanavalin A binds to 1 mg of insoluble glucan. As concanavalin A has high affinity for terminal α-D-glucopyranosyl residues, it was concluded that the glucan contains α-D-glucopyranosyl terminal residues.

DISCUSSION

The results from F.t.–i.r. spectroscopy, dextranase digestion, periodate oxidation, Smith degradation, concanavalin A binding studies, and methylation analysis suggest the water-insoluble glucan to have the structure:



where α-Glc*p* indicates an α-D-glucopyranosyl residue; the superscripts L, B, and T indicate a linear extending residue, a branching residue, and terminal group, respectively; and the subscripts *n*. *d*. and *x* indicate lengths of the sequences in the

polymer. The ratio of n to d of 5:2 was obtained from methylation analysis. The side chains are α-(1→6)-linked and are bonded to a contiguously linked, α-(1→3) backbone. These results are typical of some water-insoluble glucans isolated from streptococcal species such as that from strain Ingritt A[20], from OMZ 176[21], and from a strain isolated from the caecum of a weanling male rat[22]. Another glucan[2] produced from *Strep. mutans* 6715–15 contains 93% of α-(1→3) linkages and 7% of α-(1→6) linkages, that occur primarily as branch points.

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