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STRUCTURE OF THE SEROTYPE *f* POLYSACCHARIDE ANTIGEN OF *Streptococcus mutans**

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ABSTRACT

The structure of the serotype *f* polysaccharide antigen of *Streptococcus mutans* was determined by methylation analysis, periodate oxidation, and partial methanolysis, and the configuration of the anomeric linkages by ^{13}C-n.m.r. spectroscopy, indicating the trisaccharide repeating unit →3)-α-L-Rha*p*-(1→2)-[α-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→. The structure of the backbone of the polysaccharide was confirmed by demonstrating immunological identity between the product of Smith degradation of the *S. mutans* serotype *f* antigen and the group A-variant streptococcal polysaccharide.

INTRODUCTION

Rhamnose-containing polysaccharides are major components of the cell walls of all streptococci and their serological detection has been used extensively in classification. For example, *Streptococcus mutans*, an important cause of dental caries, can be divided into several serotypes based upon the immunological detection of a characteristic rhamnose-containing polysaccharide. This species, according to the proposed reclassification scheme of Coykendall[1], includes three serotypes, *c*, *e*, and *f*. The serotype-specific polysaccharides in each case are composed of rhamnose and glucose. The chemical structure of the serotype *e* polysaccharide was recently reported[2]. This paper describes the structural characterization of the serotype *f* polysaccharide of *S. mutans*.

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EXPERIMENTAL

Chemicals and reagents. — Sugars and alditols were obtained from Sigma Chemical Co. (St. Louis, MO 63178), and high-purity methanol, hexane, dimethyl sulfoxide, and *N,N*-dimethylformamide from Burdick & Jackson Laboratories Inc. (Muskegon, MI 49442). *N*-Methylbis(trifluoroacetamide) (MBTFA) was obtained from Pierce Chemical Co. (Rockford, IL 61105). (*R*)-(−)-2-Butanol, (*S*)-(+)-2-butanol, and acetyl chloride were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI 53233).

Methanolic HCl was prepared by passing dry HCl gas through methanol until the concentration was 1.4M (determined by titration with standard base). Glass ampules of the reagent were stored at −70°. Special, preheated methanolic HCl for partial methanolysis experiments was prepared by heating an ampule of the reagent at 80° for 24 h, and then cooling it to 4° before use. This treatment has been shown to convert most of the HCl present into chloromethane[3]. Butanolic HCl was prepared by adding 0.5 mL of acetyl chloride to 4.5 mL of the ice-cold, chiral alcohol and allowing the mixture to remain at room temperature for 2 h before use. A 10% (w/v) solution of MBTFA in *N,N*-dimethylformamide was prepared weekly. The trifluoroacetylation reagent was prepared immediately before use by adding pyridine (10 μL) to the 10% MBTFA solution (100 μL).

Analytical procedures. — The rhamnose content of column fractions was routinely monitored by use of the procedure of Dische and Shettles[4]. Phosphate was analyzed by the Ames and Dubin[5] modification of the procedure of Chen *et al.*[6]. Thin-layer chromatography was carried out on precoated glass plates of Silica gel 60, 0.25-mm layer thickness (EM Science, Cherry Hill, NJ 08034) in 8:3:1 ethyl acetate–pyridine–water. Sample spots were made visible by spraying the thoroughly dried plates with 1:3 H_2SO_4–methanol and charring at 120° for 10 min.

Monosaccharides were analyzed with a modification of the highly sensitive, gas-chromatographic procedure described previously[7]. Polysaccharide samples of 0.2 μg were routinely analyzed. They were dried in capillary tubes (90 × 0.8 mm). 1.4M Methanolic HCl (~10 μL) was added to each capillary tube by means of a glass micropipet. The capillaries were sealed and placed in a heating block for 24 h at 80°, after which they were opened and the samples rapidly dried *in vacuo*. Derivatization was accomplished by adding the trifluoroacetylation reagent (5 μL) to each capillary and allowing it to react at room temperature for at least 2 h.

The absolute configurations of the sugar residues was determined essentially as described by Leontein *et al.*[8] by g.l.c. of the (+)-2-butyl glycosides. However, HCl instead of trifluoroacetic acid was used as the catalyst and rather than using acetate derivatives, as in the original procedure, trifluoroacetate derivatives were prepared as described above. The retention times of the uncommon optical isomers of the sugars were determined by chromatography of the (−)-2-butyl glycosides of the common form of the sugar.

Gas–liquid chromatography analyses. — A Hewlett–Packard Model 5830A

gas chromatograph, equipped with a splitless, capillary-inlet system and a ^{63}Ni linear, electron-capture detector was used. A 30-m, fused-silica WCOT column coated with OV-105 was obtained from Universal Scientific Inc. (Atlanta, GA 30341). Hydrogen at an inlet pressure of 26.66 kPa was used as the carrier gas (38 cm/s linear flow), and for the septum (3 mL/min) and inlet (60 mL/min) purges. 19:1 Argon–methane was used for the detector make-up gas at a flow rate of 60 mL/min. The injector temperature was 195°, and the detector was operated at 280°. The chromatograph was held for 1 min at 100° and the temperature then programmed to 155° at 2°/min and to 225° at 5°/min. A 0.2-μL sample, followed by 1 μL of hexane was injected, and the inlet purge-gas flow was delayed for 18 s.

Methylation analysis. — The improved methylation analysis procedure of Harris *et al.*[9] employing potassium methylsulfinylmethanide in dimethyl sulfoxide was used. The resulting, partially methylated alditol acetates were separated on a 12.5-m, fused-silica WCOT column (0.2 mm i.d.) coated with a 0.33-μm film of cross-linked dimethyl silicone (Hewlett–Packard) in a Hewlett–Packard 5985A combined gas chromatography–mass spectrometry system. Electron-impact mass spectra were obtained at an ionizing voltage of 70 eV.

Nuclear magnetic resonance studies. — ^{13}C-n.m.r. spectra were recorded with a Bruker WH-400 spectrometer (9.4 Tesla) operating in the F.t. mode. *S. mutans* serotype *f* polysaccharide (50 mg) was dissolved in 2 mL of D_2O in a 10-mm tube. ^{13}C-n.m.r. spectra were obtained by use of broadband decoupling and a 3.3-s recycle delay. A minimum of 1500 free-induction decays were collected. ^{13}C-chemical shifts were measured with an internal standard of dimethyl sulfoxide (5 μL), whose chemical shift was set to δ 39.55 relative to the signal of tetramethylsilane.

Periodate oxidation. — Smith degradation of the serotype *f* polysaccharide was carried out with 40mM $NaIO_4$ in 50mM sodium acetate buffer, pH 4.0, for 48 h at 4° in the dark. Progress of the oxidation was monitored by measuring the absorbance at 223 nm of a 1:500 dilution of the reaction mixture. Excess periodate was destroyed by the addition of 1,2-ethanediol and the mixture dialyzed against distilled water. The oxidized polysaccharide was reduced with $NaBH_4$ (20 mg/mL in 0.2M NH_4OH) for 15 h at room temperature. The sample was acidified with glacial acetic acid, dialyzed against distilled water, and lyophilized. Mild acid hydrolysis of the resulting material was carried out in 0.5M trifluoroacetic acid for 24 h at room temperature. The product was loaded directly onto a column (1.5 × 90 cm) of Bio-Gel P-2 eluted with 1% (w/v) acetic acid and the rhamnose content of each fraction was determined.

Partial methanolysis. — The serotype *f* polysaccharide (20 mg) was suspended in previously heated methanolic HCl (5 mL, as described earlier) and sealed in a glass ampule. This was placed for 9 h in an oven at 80°. The ampule was cooled to −20° and the contents were removed and rapidly evaporated to dryness in a vacuum centrifuge. The partial-methanolysis products were fractionated on a Bio-Gel P-2 column (1.5 × 90 cm) eluted with 1% (w/v) acetic acid. Column fractions were examined by t.l.c.

Streptococcal strains. — Strain OMZ175 serotype *f S. mutans* was supplied by R. L. Gregory, Emory University, Atlanta, Georgia. Group A-variant streptococcal strain A486var was obtained from V. A. Fischetti of Rockefeller University, New York. Bacteria were grown in Todd–Hewitt broth (BBL Microbiology Systems, Cockeysville, MD 21030) for 18 h at 37° and harvested by centrifugation. The cell pellet was washed twice with phosphate-buffered saline, once with distilled water, and lyophilized.

Immunological methods. — Rabbit antisera to the group A-variant streptococcal polysaccharide was obtained from J. E. Coligan, National Institutes of Health, Bethesda, MD. Group A-variant polysaccharide was prepared as previously described[10]. Double-diffusion studies were carried out in 1% (w/v) agarose gels containing 0.10M Tris·HCl, pH 7.5.

Polysaccharide extraction and purification. — The *S. mutans* serotype *f* polysaccharide was extracted from lyophilized cells with a modified version of the nitrous acid-extraction procedure described by Swanson *et al.*[11]. A 2% (w/v) suspension of lyophilized cells was stirred for 1 h at room temperature in a mixture composed of 1 part of 4M $NaNO_2$, 1 part of glacial acetic acid, and 3 parts of water. A few drops of antifoam B (Sigma Chemical Co., St. Louis, MO) were also added. The cells were removed by centrifugation and the polysaccharide in the supernate was precipitated by the addition of ice-cold ethanol (3 vol.) and recovered by centrifugation. The precipitate was dissolved in 0.1M LiCl and partially purified on a column (2.5 × 90 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) eluted with 0.1M LiCl. The single peak of rhamnose-containing material was pooled, lyophilized, desalted on a column (2.5 × 25 cm) of Bio-Gel P-2 eluted with 1% acetic acid, and once again lyophilized. The product was dissolved in ice-cold 49% HF (1 mL) and placed in an ice bath for 17 h to cleave phosphoric diester bonds[12]. The HF was neutralized by the addition of a slurry of $CaCO_3$ in water and the mixture was centrifuged. The supernate, together with two water-washes of the precipitate were pooled, lyophilized, and rechromatographed on the Sephacryl S-200 column. The large rhamnose-containing peak was pooled, desalted on Bio-Gel P-2, and lyophilized. This material was dissolved in 10mM NH_4HCO_3 (pH 8) buffer, loaded onto a column (1.6 × 75 cm) of DEAE-Sephacel, and eluted with the same buffer (300 mL). Bound material was then eluted with 0.3M NaCl in 10mM NH_4CO_3, pH 8. All tubes were analyzed for rhamnose and phosphate. The two peaks eluted with the starting buffer were separately pooled and lyophilized. The material eluted with the high-salt buffer was desalted by gel filtration and lyophilized.

RESULTS AND DISCUSSION

A single rhamnose-containing peak was obtained upon Sephacryl S-200 chromatography of the yellow, crude extract of the *S. mutans* serotype *f* polysaccharide. Peak tubes were pooled, desalted, and lyophilized to yield a fluffy,

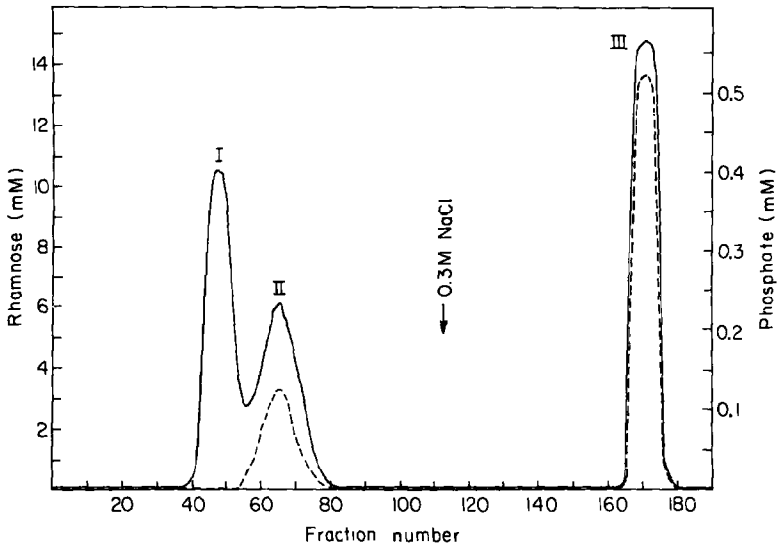


Fig. 1. Fractionation on DEAE-Sephacel of the product of hydrofluoric acid treatment of the nitrous acid-extracted *S. mutans* serotype *f* polysaccharide. All fractions were analyzed for rhamnose (——) and phosphate (------). The arrow indicates the position at which elution with 0.3M NaCl was begun.

white powder. Analysis of this material indicated the presence of rhamnose and glucose in approximately a 2:1 molar ratio, plus very much smaller proportions of *N*-acetylglucosamine, *N*-acetylmuramic acid, and phosphate.

The chemical basis for the nitrous acid extraction procedure is not clear, but it is thought to involve cleavage at or near the linkage region of the rhamnose-containing polysaccharide to the peptidoglycan lattice of the cell wall. In gram-positive

TABLE I

METHYLATION ANALYSIS OF THE *S. mutans* SEROTYPE *f* POLYSACCHARIDE, THE GLUCOSE-RHAMNOSE DISACCHARIDE PRODUCT OF PARTIAL METHANOLYSIS, AND THE SMITH-DEGRADED SEROTYPE *f* POLYSACCHARIDE

Substance	*Methylated sugars*[a]	*Retention time*[b]	*Relative peak area*
Serotype *f*	2,4-Me_2-Rha	10.45	1.00
	2,3,4,6-Me_4-Glc	10.59	0.87
	4-Me-Rha	10.89	0.89
Disaccharide	2,4-Me_2-Rha	10.51	1.00
	2,3,4,6-Me_4-Glc	10.94	0.91
Smith-degraded	3,4-Me_2-Rha	10.37	0.88
serotype *f*	2,4-Me_2-Rha	10.48	1.00
polysaccharide	4-Me-Rha	10.92	0.07

[a]As peracetylated alditols. [b]Retention time in min on a 12.5-m, cross-linked dimethyl silicone WCOT, fused-silica column; temperature programmed from 50 to 270° at 15° per min. [c]Relative peak-areas in the total ion chromatogram.

bacteria, the cell-wall polysaccharides are linked directly or indirectly *via* a phosphoric diester bond to O-6 of the *N*-acetylmuramic acid residue[13]. In order to obtain a polysaccharide free of the small amount of phosphate and peptidoglycan linked to the reducing terminal residue of the chains, the material was treated with 49% hydrofluoric acid for 17 h in an ice bath. These conditions were found to give an acceptable yield of dephosphorylated serotype *f* polysaccharide without extensive cleavage of the polysaccharide chains. Gel filtration of the hydrofluoric acid-treated antigen on Sephacryl S-200 gave a large rhamnose-containing peak with a retention volume essentially the same as that of the untreated material. Figure 1 illustrates the results of fractionation of the partially purified, hydrofluoric acid-treated antigen on DEAE-Sephacel. The first peak to be eluted was the desired, phosphate-free serotype *f* polysaccharide and the material in the second peak contained 0.27% phosphorous (w/w) but no peptidoglycan components. The tightly bound third peak appeared to be undegraded starting material.

The D-configuration was assigned to the glucose residues in the serotype *f* polysaccharide and the L-configuration to the rhamnose residues on the basis of the retention times of their (+)-2-butyl glycosides, as described by Leontein *et al.*[8].

Methylation analysis of the *S. mutans* serotype *f* polysaccharide gave the partially methylated alditol acetates listed in Table I. These results indicated the presence of a nonreducing, terminal D-glucopyranosyl group, an L-rhamnopyranosyl residue linked at O-2, and a branched L-rhamnopyranosyl residue linked at O-2,3.

The ^{13}C-n.m.r. data for the serotype *f* polysaccharide are given in Table II. The chemical shift at δ 96.7 and coupling constant $J_{C,H}$ 171 Hz indicated that the terminal D-glucopyranosyl group has the α configuration[14]. The chemical shifts and the observed splittings of 176 Hz for the two L-rhamnose residues in the ^{1}H-coupled, ^{13}C-n.m.r. spectrum indicated that they are both α linked[14].

Partial methanolysis of the polysaccharide was carried out in order to deter-

TABLE II

^{13}C-N.M.R. DATA FOR THE SEROTYPE *f* POLYSACCHARIDE OF *S. mutans*

Chemical shift (δ)[a]	*Coupling constant* $J_{C,H}$ *(Hz)*	*Assignment*
102.4	176	C-1 of →3)-α-L-Rha*p*
101.7	176	C-1 of →2,3)-α-L-Rha*p*
96.7	171	C-1 of α-D-Glc*p*
61.2		C-6 of α-D-Glc*p*
17.8		CH_3 of α-L-Rha*p*
17.4		

[a]Relative to the signal of an internal standard of dimethyl sulfoxide set at δ 39.55 relative to the signal of tetramethylsilane.

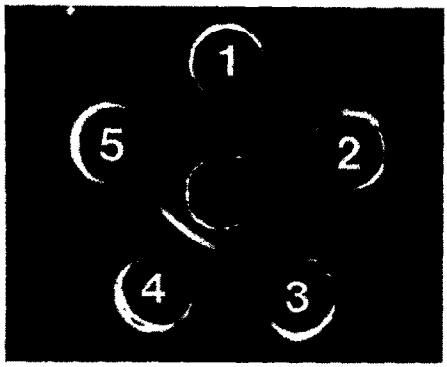
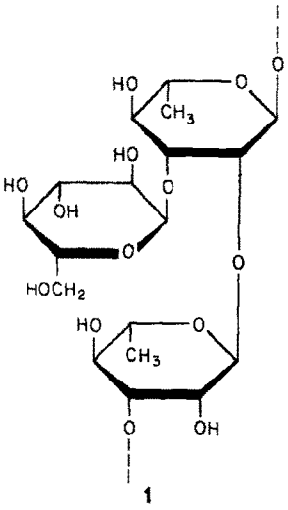


Fig. 2. Immunodiffusion reactions in a 1% agarose gel between rabbit antisera to the group A-variant streptococcal polysaccharide (center well) and various polysaccharides. Purified *S. mutans* serotype *f* polysaccharide (well 1), the product of Smith degradation of the serotype *f* polysaccharide (well 3), and the group A-variant polysaccharide (well 4) were all used at a concentration of 1 mg/mL.

mine whether the terminal α-D-glucopyranosyl groups were linked to O-2 or O-3 of the branched, 2,3-linked L-rhamnose units. The products of partial methanolysis were separated on a Bio-Gel P-2 column and the fractions examined by thin-layer chromatography. A D-glucose–L-rhamnose disaccharide was identified and subjected to methylation analysis with the results shown in Table I. Approximately equimolar amounts of terminal D-glucosyl residues and O-3-linked L-rhamnosyl units were observed.

Sequential periodate oxidation, borohydride reduction, and mild acid hydrolysis (Smith degradation) of the serotype *f* polysaccharide completely destroyed the glucose residues. Gel filtration of the product of Smith degradation on Bio-Gel P-2



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resulted in 89% of the rhamnose present being eluted in the void volume of the column. The results of methylation analysis of this material are given in Table I and indicate the presence of approximately equimolar amounts of O-2-linked and O-3-linked L-rhamnosyl residues. The very small proportion of O-2,3-linked rhamnosyl units observed is probably the result of incomplete hydrolysis of the periodate oxidation fragments of the terminal glucosyl groups. These results corroborated the results of the partial methanolysis experiment and indicated that the glucosyl residues are attached to O-3 of the 2,3-linked rhamnosyl residues.

The product of Smith degradation of the *S. mutans* serotype *f* polysaccharide reacted strongly with rabbit antisera to the group A-variant streptococcal polysaccharide in an immunodiffusion experiment. Figure 2 shows that a line of complete identity was obtained between the group A-variant polysaccharide and the Smith-degraded serotype *f* polysaccharide.

The group A-variant streptococcal polysaccharide has been shown to be a linear homopolymer of alternating O-2- and O-3-linked L-rhamnosyl units[15] and the structure confirmed by n.m.r. studies[16].

The aforementioned results support the conclusion that the serotype *f* polysaccharide antigen of *S. mutans* possesses the trisaccharide repeating-unit **1**. Assuming that the material present in the second peak illustrated in Fig. 1 contains only a single phosphate group per polysaccharide chain, then this would indicate a molecular weight of 11 400 and the presence of approximately 25 repeating units per chain. This structure represents one more example of a streptococcal polysaccharide possessing a polyrhamnose backbone corresponding to the group A-variant streptococcal polysaccharide. Other streptococcal polysaccharides shown to possess this backbone include the group-specific polysaccharides of groups A (ref. 15), C (ref. 17), and E (ref. 18) streptococci, and the serotype-*e* polysaccharide[2] of *S. mutans*.

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