INFECTION AND IMMUNITY, Nov. 1985, p. 583–585
0019-9567/85/110583-03$02.00/0
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Vol. 50, No. 2

# NOTES

## Structural Studies of the Rhamnose-Glucose Polysaccharide Antigen from *Streptococcus sobrinus* B13 and 6715-$T_2$

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**The rhamnose-glucose polysaccharide antigens from the cell walls of *Streptococcus sobrinus* B13 and 6715-$T_2$ (formerly *Streptococcus mutans* serotypes *d* and *g*, respectively) were structurally examined by using gas chromatography-mass spectroscopy. These data confirmed earlier chemical and serological studies suggesting that these polysaccharides had identical structures. The polysaccharides appeared to have a backbone of alternating 1,2- and 1,3-linked rhamnose units. Branching occurred at 1,2,3-linked rhamnose units. Side chains appeared to be composed of 1,2- and 1,6-linked glucose units with glucose as the only terminal carbohydrate.**

Recent animal studies have demonstrated the efficacy of vaccination with purified proteins or ribosomal preparations from *Streptococcus mutans* as a means of reducing dental caries (6, 11, 21). However, proteins associated with cell walls or membranes of *S. mutans* have been implicated in cross-reactions with heart tissue (1, 4). Therefore, polysaccharide antigens are of interest as potential alternative vaccines. The cell walls of *Streptococcus sobrinus* (formerly *S. mutans* serotypes *d* and *g*) (2) possess two distinct polysaccharide antigens, the galactose-glucose polymers, which have been used in the serological classification of these organisms (9, 15), and the rhamnose-glucose polymers (RGPs), which compose 40 to 50% of the dry weight of the cell walls (13, 18). The RGPs of strains B13 and 6715-$T_2$ (representing serotypes *d* and *g*, respectively) have previously been shown to be antigenic and appear to be chemically and serologically identical (17, 18). The present study used analysis by gas chromatography-mass spectroscopy to verify the structural identity of the RGPs in organisms of the *d* and *g* serotypes.

Batch cultures of *S. sobrinus* B13 and 6715-$T_2$ were grown in dialysate of Todd-Hewitt broth supplemented with glucose and salts (17). Cultures were grown at 37°C for 18 h, harvested by centrifugation, washed twice with distilled water, and lyophilized. RGP polysaccharides were prepared from cells or cell walls by acid extraction and purified by sequential chromatography on columns of DEAE-Sephadex A-25 and Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.), as previously reported (17, 18). In addition, RGP was prepared from a wall-enriched preparation of strain 6715-$T_2$ by mutanolysin digestion. A 5-g amount of cells (25 mg/ml) was homogenized in 2% sodium dodecyl sulfate, centrifuged, and washed four times with distilled water. The sodium dodecyl sulfate-treated cells were digested with RNase and protease, as previously described (18). A sample of the wall-enriched fraction (1 g) was suspended in 100 ml of 0.01 M Tris-hydrochloride, pH 7.0, and was incubated in the presence of 10 mg of purified M-1 *N*-acetylmuramidase from *Streptomyces globisporus* 1829 (24) for 18 h at 37°C. The enzyme was inactivated by heat treatment at 70°C for 10 min, and the reaction mixture was centrifuged at 27,000 × *g* for 20 min. The supernatant was dialyzed against distilled water and lyophilized. Purified RGP polysaccharide was prepared from the M-1 digest by chromatography on columns of Sepharose 4B (Pharmacia Fine Chemicals) and DEAE-Trisacryl M (LKB Instruments, Inc., Rockville, Md).

The compositions of the acid-extracted and M-1 digest polysaccharides are shown in Table 1. Total sugars were assayed by the phenol-sulfuric acid method of Dubois et al. (3). Rhamnose was measured by the methylpentose assay of Gibbons (5), substituting thioglycolic acid for cysteine hydrochloride. Protein was measured by the method of Lowry et al. (16). Quantitative determination of specific sugars was done by using a Varian model 2740 gas chromatograph, as previously described (18). Amino acids were characterized by using a Beckman model 120C amino acid analyzer. Samples for amino acid analysis were hydrolyzed in constantly boiling HCl under $N_2$ at 105°C for 28 h. The acid-extracted RGP preparations from strains B13 and 6715-$T_2$ contained >90% rhamnose and glucose by weight. In contrast, the M-1 digest polysaccharide contained 65% rhamnose and glucose, with the remaining dry weight being

TABLE 1. Chemical composition of purified RGP preparations

| Component | mg/100 mg (dry wt) | | |
|---|---|---|---|
| | B13 | 6715-$T_2$ | |
| | | Acid extract | M-1 digest |
| Rhamnose | 54 | 54 | 38 |
| Glucose | 37 | 42 | 27 |
| Protein | 1.4[a] | 2.2[a] | 19[b] |

[a] Determined by the method of Lowry et al. (16).
[b] Determined by amino acid analyzer. Glutamic acid, alanine, lysine, and threonine constituted >97% of the amino acids detected and were present in a ratio of 1:2:0.7:0.7.

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TABLE 2. Comparison of methylation derivatives for RGP polysaccharides

| Derivative | Substitution site | Presence in strain: | | |
|---|---|---|---|---|
| | | B13 | 6715-$T_2$ | |
| | | | Acid extract | M-1 digest |
| 1,2,3,5-Tetra-*O*-acetyl-4-*O*-methyl rhamnitol | 1,2,3 | + | + | + |
| 1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methyl rhamnitol | 1,2 | + | + | + |
| 1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl rhamnitol | 1,3 | Tr | Tr | Tr |
| 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol | 1 | + | + | + |
| 1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol | 1,6 | + | + | + |
| 1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl glucitol | 1,2 | + | + | + |

peptidoglycan components. The RGP polysaccharides appeared to be covalently bound to the peptidoglycan since they cochromatographed on columns of Sepharose 4B and DEAE-Trisacryl M.

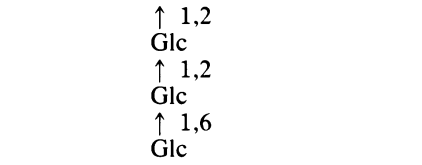
The molecular weights of the chromatographically purified RGP samples from strain 6715-$T_2$ were examined by filtration on a standardized column of Sephadex G-150 (Pharmacia Fine Chemicals). The acid-extracted polysaccharide eluted at a position similar to that of bovine serum albumin (molecular weight, 67,000). This is in agreement with a molecular weight of 50,000, which was suggested by analysis of the reducing potential of the polysaccharide, using a dinitrosalicylic acid reagent (10). The polysaccharide from the M-1 digest appeared to have a molecular weight of ≥150,000. This larger molecule represents the polysaccharide bound to peptidoglycan.

Methylation of the polysaccharide samples (200 μg) was carried out by the method of Hakomori (8). The permethylated oligosaccharides were converted to partially methylated hexitol acetates and 6-deoxy-hexitol acetates by the acetolysis-hydrolysis procedure described by Stellner et al. (19). Derivatives were identified by using a Hewlett-Packard model 5992 gas chromatograph-mass spectrometer with a glass column (0.2 by 200 cm) packed with 3% OV-225 (for details of these procedures, see reference 20). The methylation derivatives detected in the RGP polysaccharides are summarized in Table 2. All three samples demonstrated similar methylation derivatives. Glucose was the only terminal carbohydrate noted in the samples; 1,2,3-linked rhamnose was the only branch point detected. Also present were 1,2-linked rhamnose and 1,6- and 1,2-linked glucose units. Trace amounts of 1,3-linked rhamnose were also noted.

A sample of the RGP of strain 6715-$T_2$ (10 mg) was subjected to partial acid hydrolysis in 0.1 N HCl (2 ml) at 100°C for 3 h. After lyophilization, the hydrolysis products were fractionated by using descending paper chromatography on Whatman 3MM paper with butanol-pyridine-water (6:4:3, vol/vol) as the solvent. Standard oligosaccharides run parallel with the RGP sample were detected by silver nitrate staining (22) after a periodate dip (20). Fractions eluted from the paper with water and subjected to methylation analysis contained disaccharides of glucose-1,2-rhamnose and glucose-1,6-glucose. Insufficient quantities of disaccharides were recovered for serological inhibition studies.

Previous quantitative precipitin assays with antiserum prepared against cells of strain 6715-$T_2$ suggested that the determinants on the RGP antigen of strains 6715-$T_2$ and B13 included a terminal α-linked glucose unit. For example, isomaltose (α-1,6-diglucose) inhibits the precipitin reaction 93%, whereas an equimolar concentration of gentiobiose (β-1,6-diglucose) inhibits the precipitin reaction 45% (17). The precipitin assay could not distinguish between 1,4- and 1,6-linked diglucose units, which inhibited in a similar manner. The precipitin reaction was not significantly inhibited by rhamnose, suggesting that terminal rhamnose was not a component of the RGP determinant. The present structural studies suggest that a terminal 1,6-linked glucose unit is a key component of the antigenic determinants on the RGP molecules. However, additional specificity appears to be contributed by adjacent saccharide units since dextran reacts very weakly with anti-RGP sera (7).

A possible structure for the RGP polysaccharide is as follows (Rha, rhamnose; Glc, glucose):




The terminal 1,6-diglucose would be α-linked as suggested by inhibition assays (17).

The *S. mutans* serotype *c* antigen has also been identified as a wall-associated rhamnose-glucose polysaccharide (12, 23). Although antiserum to *c* antigen has been shown to partially cross-react with the RGP polysaccharides from serotypes *d* and *g*, anti-RGP serum does not react with *c* antigen (7). Structural studies in progress should help elucidate the relationships among these bacterial surface antigens.

This work was supported by Public Health Service grant DE 05017 from the National Institute of Dental Research.

We thank Lynette M. Guindon for technical assistance.

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