Antigens of Streptococcus mutans: Characterization of a Polysaccharide Antigen from Walls of Strain GS-5

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A cell wall-associated polysaccharide antigen was isolated from Streptococcus mutans GS-5 and appeared to determine serotype c specificity. Ouchterlony double-diffusion analysis of crude formamide extracts derived from purified cell walls of two serotype c strains (GS-5 and JC-2) showed complete identity when reacted with anti-GS-5 sera. Immunoelectrophoresis of this extract demonstrated the typical mobility for this serotype as described by others. Column chromatography on BioGel P-100 of the crude formamide extracts derived from GS-5 walls resulted in a single antigenic peak being resolved. This material, when loaded onto a diethylaminoethylcellulose column and eluted with a linear gradient of ammonium carbonate (0.0 to 0.2 M), was resolved further into two serologically reactive peaks (I and II). Only two constituents, rhamnose and glucose, were detected in the purified column fractions. Peak I had a rhamnose-to-glucose molar ratio of 0.9:1.0, and peak II, the major resolvable fraction, had a molar ratio of 1.7:1.0. The peak II ratio was very similar to that found in the formamide extract residue pellet (1.6:1.0). Ouchterlony analysis of the crude formamide extract and the purified fractions revealed only partial identity between peaks I and II but complete identity between peak II and the crude extract. Likewise, immunoelectrophoresis showed no differences in mobility of peak II and the crude extract, whereas peak I moved towards the cathode. Possible structural relationships between the two antigenic fractions are discussed below. Hapten inhibition studies suggested that an α-glucosyl group is at the immunodeterminant site of the antigen.

In recent years much research has been focused on Streptococcus mutans, an etiological agent thought to be responsible for dental caries (12, 14, 16, 28). On the basis of serological or genetic criteria, Bratthall (3, 4), Coykendall et al. (9–11), deStoppelaar (12), and Jablon and Zinner (18, 27) divided S. mutans into several major groups. Bratthall (6) defined five serotypes (a–e) by using comparative immuno electrophoresis of Lancefield extracts. Recently, Perch et al. (22) proposed the establishment of serotypes f and g based on the biochemical characteristics of certain strains of S. mutans.

The immunochemical nature of the determinant antigens for serotypes a (21, 25), b (20, 26) and d (17, 19; T. Brown and A. S. Bleiweis, personal communication) have been established by several investigators. To this point one major serotype, c, remains undefined on an immunochemical basis. Of all the serotypes of S. mutans, serotypes c and d appear to be the most common on a world-wide basis (7). Indeed, in a study conducted in Denmark, over 90% of patients with subacute endocarditis contained serotype c strains in blood samples (22). Of late, success has been attained in reducing experimental carious infections in monkeys when a vaccine containing S. mutans serotype c organisms was administered (T. Lehner, personal communication). The nature of the protective antibodies and of the corresponding immunogens is being studied.

This paper describes the isolation, purification, and partial characterization of a polysaccharide antigen with serotype c specificity from purified cell walls of S. mutans GS-5.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In this study two strains of S. mutans originally isolated from human carious lesions were used. Strain GS-5 was obtained from A. L. Coykendall (Veterans Administration Hospital, Newington, Conn.) and JC-2 was obtained from D. Bratthall (University of Goteborg, Goteborg, Sweden). Each of these strains was characterized as to colonial morphology, ability to ferment sorbitol and mannitol, and serotype, using rabbit antisera to GS-5.

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Large-batch growth and harvesting conditions were as previously described (25).

**Extraction of antigens from whole cells.** In our preliminary studies, four extraction techniques were studied for relative abilities to remove the serotype-specific antigen from whole cells. In each case, 20 mg of lyophilized whole cells of GS-5 was extracted and final extracts were dialyzed, lyophilized, and then brought up to 1 ml, final volume, with distilled water.

In the phosphate buffer technique, whole cells were suspended in 1 ml of 0.1 M potassium phosphate buffer at pH 7.0 and heated in a boiling water bath for 10 min. The suspension was centrifuged at 2,000 × g for 20 min and the supernatant was treated as above.

In the Lancefield technique, whole cells were suspended in 1 ml of saline and the pH was adjusted to 2.5 with 4 M HCl. The suspension was heated in a boiling water bath for 10 min and quickly cooled. Debris was removed by centrifugation at 33,000 × g for 10 min and the supernatant fluid was neutralized before proceeding as above.

In the trichloroacetic acid technique, the lyophilized whole cells were suspended in 1 ml of cold 10% acid and stirred for 24 h at 4°C. The partially extracted whole cells were centrifuged at 33,000 × g for 10 min. After two additional extractions of the whole cells, the pooled supernatant fluids were shaken with 5 vol of ether to remove the acid from the aqueous phase until a pH of 4.0 was reached in the aqueous fraction; the ether was discarded.

In the formamide technique, the lyophilized whole cells were suspended in 1 ml of concentrated formamide and heated at 180°C for 30 min in an oil bath. The suspension was mixed with 2 vol of a solution containing 2 N HCl and absolute ethanol (1:19, vol/vol). After centrifugation at 350 × g for 20 min, the pellet was washed three times with 5 ml (total, 15 ml) of a solution consisting of 2 vol of the above acid-ethanol solution and 1 vol of distilled water. The pooled supernatants were then held at 4°C for 8 h, at which time 5 vol of acetone was added. The solution was then held at 4°C for 4 h and centrifuged at 350 × g for 20 min. The supernatant was discarded and, after drying to remove excess acetone, the white pellet was dissolved in 3 ml of distilled water, and the dissolved product was centrifuged at 27,000 × g for 10 min. Finally the supernatant was dialyzed against 200 vol of distilled water for 24 h before lyophilization. This extraction technique, based on that of Fuller (15), was found to be most effective for extraction of this antigen (see Fig. 1).

**Extraction of cell wall antigens by formamide.** Purified cell walls of GS-5 and JC-2 were obtained by the technique of Bleiwes et al. (1), using glass bead disruption in the Braun tissue homogenizer, followed by enzyme treatments with ribonuclease, deoxyribonuclease, and trypsin. Purified cell walls were extracted with concentrated formamide (5 ml/100 mg of cell walls) as described above for lyophilized whole cells. Routine yields of antigenic polysaccharide ranged from 30 to 35% of the recoverable fractions.

**Purification by gel column chromatography.** Columns (2.7 by 80 cm) of BioGel P-100 (100 to 200 mesh) (Bio-Rad Laboratories, Richmond, Calif.) were prepared by using distilled water. Samples (100 mg) were loaded and eluted with 0.85% NaCl (aqueous). The flow rates were approximately 10 ml/h, and 2-ml fractions were collected using a Gilson Mini-Escargot fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.). As described below, fractions were analyzed for absorbancy at 220 nm to detect chromophores common to polysaccharides (2), antigen content by the Ouchterlony technique, and rhamnose.

**Purification by diethylaminoethyl (DEAE)-cellulose column chromatography.** After dialysis and lyophilization of pooled fractions, antigen material derived from gel filtration was loaded onto a Whatman DE32 (Reeve Angel, Clifton, N.J.) column (2.7 by 30 cm). The ion exchange exchange had been washed first with distilled water and then with 0.45 M ammonium carbonate buffer. Finally, it was equilibrated with distilled water before loading. The samples (50 to 75 mg) were eluted with a linear gradient of 0.0 to 0.2 M ammonium carbonate buffer (pH 8.9). Selected 2-ml fractions were dialyzed before being assayed for absorbancy at 220 nm, rhamnose, and antigenic reactivity. Antigen fractions were pooled, dialyzed, and lyophilized.

**Quantitative chemical assays.** Phosphorus was determined by the method of Chen et al. (8) with absorbances measured at 820 nm. Glucose and galactose were determined by using the Glucostat and the Galactostat reagents (Worthington Biochemical Corp., Freehold, N.J.), respectively. The glycerol Stat-Pack (Calbiochem, Atlanta, Ga.) was used to determine the amount of glycerol present. The method of Dische and Shettles (13) was used to assay for rhamnose. Amino acids and amino sugars were measured on a JEOl model JLC-6AH automated amino acid analyzer (JEOl, Inc., Cranford, N.J.). The sample hydrolysates for all of the quantitative assays were prepared as previously described (25).

Gas-liquid chromatography. Antigen samples (10 mg) suspended in 0.5 ml of 1.0 N H2SO4, were hydrolyzed at 100°C for 8 h. After hydrolysis the samples were neutralized with barium carbonate and centrifuged at 350 × g for 10 min and the supernatants were lyophilized. Hydrolysates were converted to their trimethylsilyl derivatives by using TRI-SIL (Pierce Chemical Co., Rockford, Ill.) (23). Derivatives were separated on SE-30 (Pierce Chemical Co., Rockford, Ill.) on Chromosorb WHP), using a Packard model 803 (Packard Instrument Co., Inc., Downers Grove, III.) gas-liquid chromatograph. Nitrogen was used as the carrier gas, and isothermal (185°C) column temperatures were maintained. Detection was by hydrogen flame ionization.

**Immunological procedures.** The first paper in this series (25) presented details of the methods used for the preparation of rabbit antisera, quantitative precipitin assay, quantitative precipitin inhibition assay, gel diffusion, and immunoelectrophoresis. Except where noted below, these techniques were unchanged.

**Chemicals.** Organic solvents, acids, and most
common salts were obtained from Mallinckrodt (Scientific Products, Chambree, Ga.). Amino acids, amino sugars, D-glucose, maltose, β-methyl-D-glucopyranoside, and β-methyl-D-glucopyranoside were obtained from Sigma Chemical Co.; B-glucose was from Calbiochem.

RESULTS

Comparison of extraction techniques. Initial investigations were made with lyophilized whole cells of S. mutans GS-5 to determine which of several extraction techniques allows the greatest relative yield of antigenic material. In each case the same amount of lyophilized whole cells was used (see Materials and Methods), and after dialysis and lyophilization the resultant extracts were brought up to the same volume (1 ml) with distilled water. The formamide extraction technique allowed the highest relative yield when extracts were compared for antigenic reactivities by double-diffusion gel analysis (Fig. 1). This extraction technique was applied in all subsequent studies.

Extraction of antigen from purified cell walls. It was of obvious interest to identify the cellular location of the formamide-extractable antigen, as well as to provide preliminary evidence for its presence in other serotype c strains. Formamide extracts of purified cell walls of two serotype c strains (GS-5, JC-2) and one serotype d strain (B-13) were prepared and studied for immunological reactivity with anti-GS-5 serum. Figure 2 clearly illustrates the presence of identical cell wall-localized antigens in two c strains. The formamide extract of B-13 walls was unreactive with the heterologous antiserum. Figure 3 shows the immunoelectrophoresis precipitin patterns obtained with the GS-5 and JC-2 formamide extracts and a Lancefield extract of GS-5 walls after reacting with antiserum to GS-5. The lack of extensive mobility displayed is typical of this serotype as defined by Bratthall (6).

Purification of serotype-specific antigen. Crude formamide extract obtained from purified GS-5 cell walls was loaded onto a BioGel P-100 column and eluted with 0.85% sodium chloride. A single peak (Fig. 4) was obtained that contained all of the absorbancy at 220 nm, rhamnose, and the antigenic reactivity. Fractions 45 to 125 were pooled, dialyzed against distilled water, and lyophilized. The yields

![Fig. 1. Immunodiffusion in gel demonstrating the relative reactivities of four antigen extracts. In each case 20 mg of lyophilized whole cells of GS-5 was extracted (see text) and final extracts were dialyzed, lyophilized, and then brought up to 1 ml, final volume, with distilled water. The outer wells contain phosphate buffer (pH 7.0) extract (PB), Lancefield extract (LE), cold 10% trichloroacetic acid extract (TCA), and hot formamide extract (180 C, 30 min) (FE). The center well contains homologous (GS-5) antiserum. In this and subsequent double-diffusion analyses, each well contains a 5-μl volume of antiserum or extract.](http://iai.asm.org/)

![Fig. 2. Immunodiffusion in gel demonstrating the precipitin patterns formed by formamide extracts of two serotype c strains (GS-5 and JC-2) and one serotype d strain (B-13). In each case purified walls were extracted by hot formamide. These band formations indicate the presence of identical cell wall-localized antigens in the two serotype c strains. Again, antiserum to GS-5 was used to develop precipitin bands.](http://iai.asm.org/)
Fig. 3. Immunoelectrophoresis comparing the mobilities of formamide extracts (FE) of purified cell walls of GS-5 and JC-2 and a Lancefield extract (LE) of GS-5 cell walls. These identical patterns are consistent with previous reports (6).

Fig. 4. Elution profile of crude formamide extract of purified GS-5 cell walls on BioGel P-100 (100 to 200 mesh). Samples (100 mg) were loaded and fractions (2 ml) were eluted with 0.85% sodium chloride. Selected fractions were examined for absorbance at 220 nm (○), rhamnose (■), and antigenic reactivity (brackets). The void volume was determined at fraction number 15.

ranged from 70 to 85% of the material loaded onto the BioGel column. The symmetry of this single, antigenic peak indicates a lack of extensive molecular fragmentation as a result of the extraction techniques used.

The material recovered from the BioGel P-100 column was reloaded onto a DEAE-cellulose column and eluted with a linear gradient of 0.0 to 0.2 M ammonium carbonate (Fig. 5). Selected fractions were dialyzed to remove buffer salts and examined for absorbance at 220 nm, rhamnose, and antigenic reactivity. Here two peaks were resolved: a major peak (peak II) eluting between tubes 130 to 160 at approxi-
Elution profile on DEAE-cellulose of antigenic material recovered from BioGel P-100 indicating the resolution of two peaks. Samples (50 to 75 mg) were loaded and fractions (2 ml) were eluted with a linear gradient of ammonium carbonate (0.0 to 0.2 M), pH 8.9. Selected fractions were examined for absorbance at 220 nm (○), rhamnose (●), and antigenic reactivity (brackets). Two antigenic peaks are observed: I, tubes 30 to 55; and II, tubes 130 to 160. These were pooled separately and lyophilized.

Chemical and immunological characteristics of antigenic fractions. The two peaks recovered from the DEAE-cellulose column and the crude formamide extract were compared by using the Ouchterlony double-diffusion technique (Fig. 6). Although there were common antigenic determinants in all three fractions, only partial identity was observed between peak I material and the other fractions studied. Immunoelectrophoresis of the three antigenic constituents is shown in Fig. 7. Peak II and the crude formamide extract displayed identical precipitin patterns, whereas peak I showed mobility toward the cathode. The presence of only one band in each case may indicate a lack of gross contamination due to other antigenic moieties.

The chemical composition of the purified cell walls of GS-5 is shown in Table 1. Purified antigenic peaks I and II and the crude formamide extract of cell walls are chemically defined in Table 2. It is clear from these tables that formamide treatment of purified walls allowed the extraction of a rhamnose- and glucose-containing polymer, whereas the galactose moiety present in the native wall was absent from the isolated, soluble antigen fractions. Also, pepti-
FIG. 7. Immunoelectrophoretic precipitin band formation of peak I (I), peak II (II), and crude formamide extract of purified cell walls (FE). Peak II and the crude formamide extract have the same characteristic mobility, whereas peak I shows mobility toward the cathode. Precipitin bands were developed by using anti-GS-5 serum.

TABLE 1. Chemical compositions of purified cell walls of S. mutans GS-5

<table>
<thead>
<tr>
<th>Major component</th>
<th>Purified walls</th>
<th>μg/mg</th>
<th>μmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>292.1</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>137.6</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>19.3</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>75.0</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Muramic acid</td>
<td>103.6</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>113.1</td>
<td>1.27</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>50.7</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>50.9</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.5</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>9.7</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>9.1</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Total recovery</td>
<td>868.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Amino sugars are reported as acetylated derivatives.
* Trace amount not measured.

doglycan components, such as amino acids and amino sugars, remained in the residue fraction only. Peak II and the crude formamide extract were somewhat similar in their compositions, with rhamnose and glucose approaching 2:1 molar ratios. A similar ratio was found in the whole cell wall (Table 1). The chemical nature of peak I clearly differed from these, having a molar ratio of almost unity for these sugars. Amino acid analysis and gas chromatography were preformed on the crude formamide extract and failed to reveal any chemical moieties other than those listed in Table 2.

Quantitative precipitin reaction and hapten inhibition analysis. A quantitative precipitin experiment was done to determine the equivalence point of the serum to be used for hapten inhibition studies. The antiserum to whole cells was incubated with varying amounts of crude formamide extract for 4 days, and then the precipitates were collected and assayed for antibody protein content. Controls for nonspecific precipitating antibody were run and were negative. All samples were done in triplicate, and equivalence was obtained at approximately 75 μg of crude formamide extract.

After diluting the antiserum 1:3, a quantitative inhibition assay was run in triplicate with 75 μmol of potential inhibitor and 25 μg of crude formamide extract as antigen (Table 3). The α-linked diglucoside, maltose, gave the greatest amount of inhibition, with rhamnose showing a negligible value of precipitin inhibition. Values obtained using the methylated derivatives of glucose further indicated the hapten to be an α-glucosyl moiety.

**DISCUSSION**

A cell wall-associated polysaccharide antigen appears to be responsible for the serotype speci-
The resolution of the two antigenic fractions by DEAE-cellulose column chromatography presents still another problem. The major fraction (II) had a rhamnose-to-glucose molar ratio of 1.7:1.0, whereas that of the minor fraction (I) was close to unity. As mentioned previously, the residue remaining after extraction contained small amounts of polysaccharide with rhamnose-to-glucose molar ratios of 1.6:1.0. It is conceivable that the wall polysaccharide is composed of two polymeric types with respect to rhamnose-glucose molar compositions. Each fraction is antigenic, but II is present in far greater amounts than I and also binds more tightly to the peptidoglycan backbone. The partial identity between these two antigens shown in Fig. 6 indicates both common and uncommon immunological determinants. The possibility remains, however, that a common antigenic precursor polymer exists in the native cell wall and was fragmented into at least two resolvable moieties by the extraction procedure used here. It should be noted that gel column chromatography of crude extracts (Fig. 4) revealed only one peak, thereby precluding the possibility of extensive fragmentation into various sized sub-polymers. Splitting of this hypothetical precursor, therefore, may be restricted to certain vulnerable areas of the antigen. Studies are presently being carried out using various chemical extraction procedures to determine which hypothesis (i.e., multiple polymers versus single precursor polymer) is appropriate.

Bratthall (5) demonstrated the cross-reactivity of S. mutans serotypes c and e. Furthermore, serotype e cross-reacts with sera to Lancefield group E organisms. This latter cross-reaction is not exhibited when serotype c extracts are used. Analysis of the Lancefield group E antigen (24), a cell wall-localized polysaccharide, revealed a rhamnose-to-glucose ratio of 2:1, and a β-glucose moiety was determined to be the immunodeterminant sugar. The apparent presence of α-glucosyl haptenic groups in strain GS-5 would explain the inability of S.

### Table 2. Chemical compositions of various fractions of S. mutans GS-5 derived by formamide extraction of purified walls

<table>
<thead>
<tr>
<th>Compound</th>
<th>Crude extract</th>
<th>Residue</th>
<th>Peak I</th>
<th>Peak II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amt</td>
<td>Mole ratio</td>
<td>Amt</td>
<td>Mole ratio</td>
</tr>
<tr>
<td></td>
<td>µg/mg</td>
<td>µmol/mg</td>
<td>µg/mg</td>
<td>µmol/mg</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>474.0</td>
<td>2.85</td>
<td>2.2</td>
<td>117.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>231.0</td>
<td>1.28</td>
<td>1.0</td>
<td>78.0</td>
</tr>
</tbody>
</table>

* Chemical compositions were not corrected for waters of hydration or ash contents.
* See text for extraction techniques used.
* Peptidoglycan components present but not determined. Other fractions listed above contained only rhamnose and glucose.

### Table 3. Hapten inhibition of the quantitative precipitin reaction between crude formamide extract and antiserum against S. mutans GS-5

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>10.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>33.3</td>
</tr>
<tr>
<td>α-Methylglucopyranoside</td>
<td>29.8</td>
</tr>
<tr>
<td>β-Methylglucopyranoside</td>
<td>15.8</td>
</tr>
<tr>
<td>Maltose</td>
<td>45.6</td>
</tr>
</tbody>
</table>

* A 75-µmol amount was added in each case.
mutans serotype c to react strongly with antisera to Lancefield group E. The possible presence in S. mutans serotype e wall polysaccharides of both anomic linkages of glucose is an attractive basis for observed cross-reactions.

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LITERATURE CITED