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Serological Characterization of *Streptococcus mutans* Serotype Polysaccharide *g* and Its Different Molecular Weight Forms

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The serotype polysaccharide *g* from *Streptococcus mutans* 6715 was found to cross-react with serotype polysaccharide *a* from *S. mutans* HS6 and serotype polysaccharide *d* from *S. mutans* B13. Double immunodiffusion experiments indicated that the serotype polysaccharide *g* consisted of the following: (i) the type-specific *g* site; (ii) a cross-reactive site *g-a* that was in common with polysaccharide *a*; (iii) a cross-reactive site *g-d* that was in common with polysaccharide *d*; and (iv) a cross-reactive site *g-(a-d)* that was in common with both polysaccharides *a* and *d*. Moreover, by a procedure involving several column chromatography steps, six polysaccharide-containing fractions showing reactivity with anti-*g* serum were found. By gel filtration, the molecular weight estimates of fractions LI, LII, LIII, SI, SII, and SIII were 2×10^6, 5×10^5, 6×10^4, 3×10^4, 1.4×10^4, and 1×10^4, respectively. Double immunodiffusion analysis indicated that LI, LII, and LIII contained the four antigenic sites of the putative polysaccharide *g*. LII also contained another additional immunodominant region, designated site x. The analysis also suggested that fraction SI lacked the type-specific site *g*, fraction SII lacked sites *g* and *g-a*, and fraction SIII lacked sites *g*, *g-a*, and *g-d*.

Detailed study with *Streptococcus mutans* serotype *a* (15, 16) and *S. mutans* serotype *e* (7) of the adherence of *S. mutans* cells to smooth surfaces led to the implication of serotype polysaccharides and cell-associated glucans in adherence (20). Serologically, *S. mutans* comprises seven serotypes (1, 18). Strains belonging to serotypes *a* and *d* were found to cross-react (2, 12–14, 16), and this common cross-reactive site has been designated *a-d*. Moreover, strains belonging to serotype *g* were also found to share cross-reactivity with strains of serotypes *a* and *d* (5, 10, 18), and serotype polysaccharide *g* has been proposed to consist of a type-specific *g* site and common cross-reactive sites *g-a*, *g-d*, or *g-(a-d)* (or a combination).

During our biochemical studies of a polysaccharide *g* defective-mutant of *S. mutans* 6715 (8, 17), it became necessary to clarify the proposed cross-reactive sites of polysaccharide *g* from strain 6715. It is the purpose of this communication to present data on two aspects of polysaccharide *g*. The first aspect is further documentation for the presence of sites of polysaccharide *g* that cross-react with polysaccharide *a* from *S. mutans* HS6 and with polysaccharide *d* from *S. mutans* B13 (5, 9, 18). The second aspect is evidence for the existence of not only the complete antigenic polysaccharide *g* forms, but also three incomplete forms of polysaccharide *g*.

MATERIALS AND METHODS

S. mutans strains. *S. mutans* strains HS6 (serotype *a*), B13 (serotype *d*), 6715 (serotype *g*) and C307 (a mutant defective in polysaccharide *g*, derived from strain 6715) (8, 17) were maintained in brain heart infusion agar (Difco Laboratories) supplemented with solid $CaCO_3$. For experimental purposes, cultures were grown in PD-glucose medium (11).

Preparation of sera. Anti-6715, anti-B13, anti-HS6, and anti-C307 sera were prepared in New Zealand white rabbits. The immunoglobulin G (IgG) fraction was obtained as previously described (8), except 0.0175 M phosphate buffer (pH 8.3) was employed in the DEAE-cellulose chromatography step. Anti-*g* serum was obtained by absorbing an IgG fraction of anti-6715 serum with lyophilized cells of mutant C307 (8). Additional absorbed sera employed in this study, such as anti-*g* serum absorbed by cells of B13 or HS6 (or both), an IgG fraction of anti-B13 serum absorbed by cells of HS6 or 6715, and an IgG fraction of anti-HS6 serum absorbed by cells of B13 or 6715, were obtained by methods similar to that described for anti-*g* serum (8).

Antigens. Rantz-Randall extracts were obtained by autoclaving dry cell suspensions in saline (19). Our usual practice to prepare serotype polysaccharides *a*, *d*, and *g* was to extract 10 g of lyophilized cells of strains HS6, B13, and 6715, respectively. The extracts were processed by a three-column chromatography procedure suggested by Hamada and Slade (6), but modified by employing carboxymethyl Sephadex for the second chromatography step and substituting Ultrogel AcA 34 (LKB Instruments Inc.) for Sephadex G-200 in gel filtration, with water used as the eluent.

Inhibition of precipitin reaction between gel filtration fractions and anti-*g* serum. Anti-*g* serum in saline with no inhibitors (controls) or 5 μmol of various carbohydrates dissolved in saline were incubated at room temperature for 15 min. Gel filtration fractions LI, LII, LIII, SI, SII, and SIII (6 μg of carbohydrate dissolved in saline) were then added, and the total final volumes were adjusted to 0.1 ml with saline. The mixtures were incubated at room temperature for 2 h with a rack rotator (10 rpm; Fisher Scientific Co.). The tubes were allowed to stand at 5°C for 18 h and then centrifuged. Pellets were washed twice with saline and dissolved in 0.1 N NaOH, and protein contents were determined.

Molecular weight estimates. Molecular weight estimates of polysaccharide *g* and its intermediary forms were obtained

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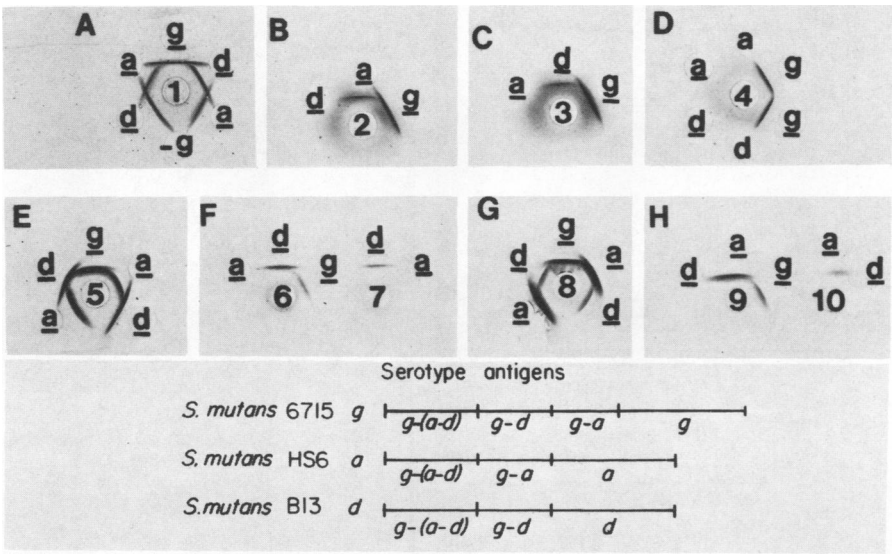

FIG. 1. Double immunodiffusion analysis of antigen preparations with various absorbed sera. A, Precipitin pattern obtained with DEAE-treated Rantz-Randall extract of C307 (12 μg of polysaccharide) designated −g, three-column purified polysaccharides *a* (4 μg), *d* (4 μg), and *g* (1 μg) with anti-*g* serum (40 μg of protein, designated 1). B, Precipitin pattern obtained with polysaccharides *a* (2 μg), *d* (2 μg), and *g* (1 μg) with anti-*g* serum absorbed with cells of B13 (26 μg of protein, designated 2). C, Same as in B, except anti-*g* serum absorbed with cells of HS6 (30 μg of protein, designated 3) was used. D, Precipitin pattern obtained with Rantz-Randall extracts (13 μg of polysaccharide) each of HS6, B13, and 6715 (designated a, d, and g) and polysaccharides *a*, *d*, and *g* with anti-*g* serum absorbed with cells of both B13 and HS6 (20 μg of protein, designated 4). E, Precipitin pattern obtained with polysaccharides *a*, *d*, and *g* (see B) with an IgG fraction of anti-B13 serum (24 μg of protein designated 5). F, Precipitin pattern obtained with polysaccharides *a*, *d*, and *g* (see B) with an IgG fraction of anti-B13 serum absorbed with cells of either HS6 (20 μg of protein, designated 6) or 6715 (20 μg of protein, designated 7). G, Precipitin pattern obtained with polysaccharides *a*, *d*, and *g* (see B) with an IgG fraction of anti-HS6 serum (40 μg of protein, designated 8). H, Precipitin pattern obtained with polysaccharides *a*, *d*, and *g* (see B) with IgG fraction of anti-HS6 serum absorbed with cells of either B13 (35 μg protein, designated 9) or HS6 (32 μg protein, designated 10). The proposed schematic representations for polysaccharides *a*, *d*, and *g* are also presented.

by gel filtration. For fractions LI, LII, and LIII, an Ultrogel A4 (fractionation range of 55,000 to 9,000,000; LKB) column was standardized with blue dextran and dextrans T500, T110, and T10 (Pharmacia Fine Chemicals). For fractions SI, SII, and SIII, Ultrogel AcA 44 (fractionation range of 10,000 to 130,000; LKB) was used, and the column was standardized with blue dextran and dextrans T70, T40, and T10. Purified fractions of Pharmacia dextrans used for molecular weight estimation were generous gifts from H. Hidaka of Meiji Laboratories, Kanagawa, Japan.

Serological analysis. Serological analyses of various antigen and anti-serum preparations were done by a double immunodiffusion agar method; plates were dried and stained with Coomassie blue to improve visualization. The polysaccharide content in antigen preparations was determined by a phenol-sulfuric acid method (3) with glucose as the standard, and protein in antiserum preparations was determined by a dye binding method (Bio-Rad Laboratories) with crystallized bovine serum albumin as the standard.

RESULTS

Double immunodiffusion analysis for antigenic sites of serotype polysaccharides *a*, *d*, and *g*. Figure 1A shows a double immunodiffusion precipitin reaction pattern obtained with anti-*g* serum (designated 1) tested against polysaccharides *a*, *d*, and *g* and against an extract of C307 designated −*g*. The pattern shows nonidentity between polysaccharides *a* and *d*; this nonidentity suggested that polysaccharides *a* and *d* contained the cross-reactive sites *g-a* and *g-d*, respectively. The partial identity seen between polysaccharide *g* and polysaccharide *a* or *d* indicated that the former may be due to the type specific *g* and the cross-reactive *g-d* and the latter may be due to the specific *g* and the cross-reactive *g-a* of polysaccharide *g*.

Anti-*g* serum absorbed with cells of B13 (designated 2, Fig. 1B), cells of HS6 (designated 3, Fig. 1C), and cells of both types (designated 4, Fig. 1D) were tested against serotype polysaccharide preparations of *a*, *d*, and *g*. The pattern of partial identity precipitin lines seen in Fig. 1B was likely due to sites *g* and *g-a* of polysaccharide *g*, and those seen in Fig. 1C were likely due to sites *g* and *g-d* of polysaccharide *g*. Figure 1D shows that absorption of anti-*g* serum with both types of cells resulted in a monospecific anti-*g* serum.

The nature of the cross-reactivity between polysaccharides *a*, *d*, and *g* was studied further by using anti-sera to HS6 and B13. The results presented in Fig. 1E showed that an IgG fraction of anti-B13 serum (designated 5) gave precipitin lines with the three polysaccharide antigens and that the polysaccharide *d* flanked by polysaccharides *a* and *g* showed partial identity due to the presence of type-specific *d*. Similarly, when polysaccharides *g* and *d* were in juxtaposition to polysaccharide *a* (Fig. 1G) and these polysaccharides were tested against an IgG fraction of anti-HS6 serum (designated 8), partial identity was seen, believed due to the type specific *a* site on polysaccharide *a*. In Fig. 1E, the precipitin pattern showing partial identity between polysaccharides *g* and *a* was suggestive of site *g-d* on the former antigen and site *g-(a-d)* on both of these antigens. Likewise (Fig. 1G), the pattern of precipitin bands given by polysaccharide *g* adjacent to polysaccharide *d* indicated the presence of site *g-a* on polysaccharide *g* and the common site *g-(a-d)* on both polysaccharides. In Fig. 1F, an IgG

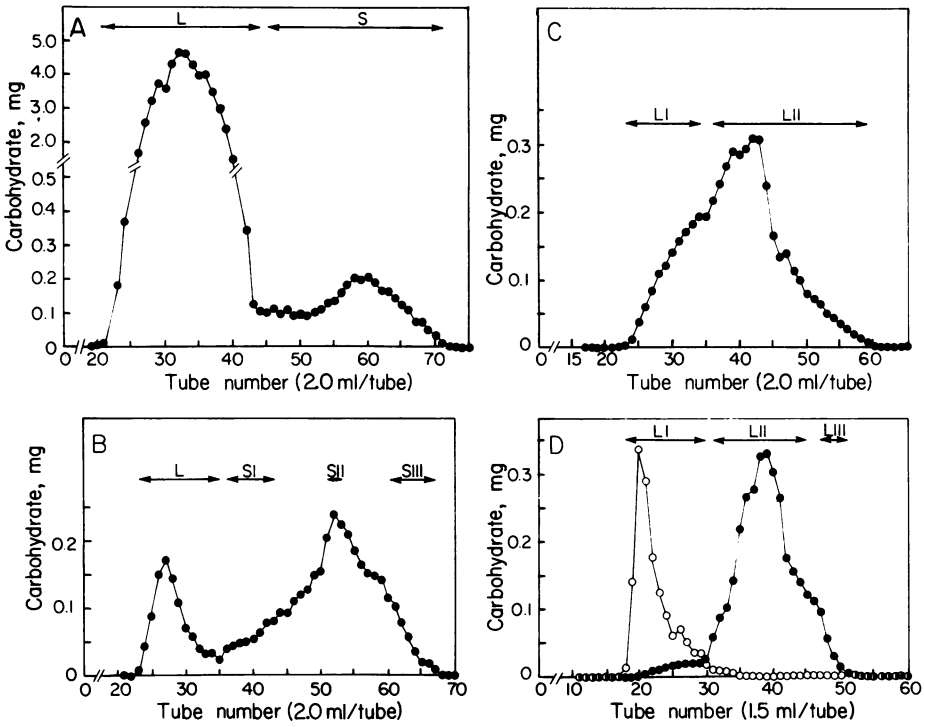

FIG. 2. Gel filtration of a three column purified Rantz-Randall extract of *S. mutans* 6715. A, A three-column purified Rantz-Randall extract (60 mg) that was treated with an IgG fraction of anti-C307 serum was applied to an Ultrogel AcA 44 column (1.7 by 66 cm). The column was washed with water, and the contents of the tubes were assayed for carbohydrate; tubes representing the profile obtained were also tested against anti-*g* serum by a double immunodiffusion method. Pooled fractions L and S were then prepared. B, The pooled fraction S obtained as described for Fig. 2A was concentrated and applied to an Ultrogel AcA 44 column (1.7 by 66 cm). Pooled fractions L, SI, SII, and SIII were prepared after a double immunodiffusion test. C, Pooled fractions L obtained as described for Fig. 2A and B were combined, concentrated, and applied to an Ultrogel AcA 22 column (1.7 by 66 cm), and pooled fractions LI and LII were prepared. D, Concentrated pooled fractions LI and LII (Fig. 1C) were applied separately to an Ultrogel A4 column (1.7 by 66 cm), and a composite of the two chromatography steps is presented. Pooled fraction LI yielded a single pooled fraction LI, but pooled fraction LII yielded pooled fractions LII and LIII.

fraction of anti-B13 absorbed by cells of HS6 (designated 6) showed no bands with polysaccharide *a* and partial identity between polysaccharides *d* and *g*. The spur, then, is regarded to be due to the specific *d* site of polysaccharide *d* and to the common *g-d* site. When an IgG fraction of anti-B13 serum was absorbed by strain 6715 (Fig. 1F), the absorbed serum (designated 7) did not react with polysaccharides *g* and *a*, but did react with polysaccharide *d*. This suggested the removal of antibodies to sites *g-a* and *g-(a-d)*, resulting in a monospecific anti-*d* serum. Anti-HS6 serum absorbed by B13 (designated 9) gave no bands (Fig. 1H) with polysaccharide *d* and a band of partial identity against polysaccharides *a* and *g*. The partial identity is thought to be due to the specific *a* site on polysaccharide *a* and to the common *g-a* site on both polysaccharides. The absorption of an IgG fraction of anti-HS6 serum by cells of 6715 (designated 10), produced monospecific anti-*a* serum (Fig. 1H).

Gel filtration chromatography to obtain intermediary forms of polysaccharide *g*. To obtain intermediary forms of polysaccharide *g*, a polysaccharide *g* preparation (70 mg), isolated by a three-column chromatography purification procedure from Rantz-Randall extract of strain 6715, was treated with 10 ml of an IgG fraction of anti-C307 serum for 2 h at room temperature and then for 18 h at 5°C. The mixture was centrifuged (10,000 × *g* for 15 min), and the pellet was discarded. An equal volume of 12% trichloroacetic acid was added to the supernatant fluid, and the mixture was held at 5°C for 2 h and centrifuged as before. After the pH of the supernatant fluid was adjusted to 7.0 with NaOH, the fluid was dialyzed overnight and lyophilized. Minimum water was added to dissolve the dried polysaccharide *g* material (60 mg), and it was then subjected to successive Ultrogel filtration column chromatography steps (1.7 by 66 cm). In these steps, all columns were washed with water, and the profiles of the gel filtration based on the carbohydrate content of the tubes collected were determined. Selected samples from the tubes representing the profile were tested against anti-*g* serum by a double immunodiffusion agar method to determine the tubes to be pooled. In Fig. 2A, the contents of tubes containing the large-molecular-weight polysaccharide fraction (L) from an Ultrogel AcA 44 column were pooled, as were the contents of tubes containing the small-molecular-weight polysaccharide fraction (S). The pooled L and S fractions were concentrated by lyophilization. The S fraction was again subjected to an Ultrogel AcA 44 gel filtration (Fig. 2B), and tubes were pooled to give fractions L, SI, SII, and SIII. The L fraction was combined with the previously obtained L fraction (Fig. 2A), and the combined L fractions were added to the top of an Ultrogel AcA 22 column. This gel filtration step produced pooled fractions LI and LII (Fig. 2C). Each was then applied separately to an Ultrogel A4 column; fraction LI gave a single fraction, again designated LI, whereas fraction LII was divided into pooled fractions LII and LIII (Fig. 2D).

Analysis of gel filtration fractions by a double immunodiffusion method. The gel filtration fractions of polysaccharide

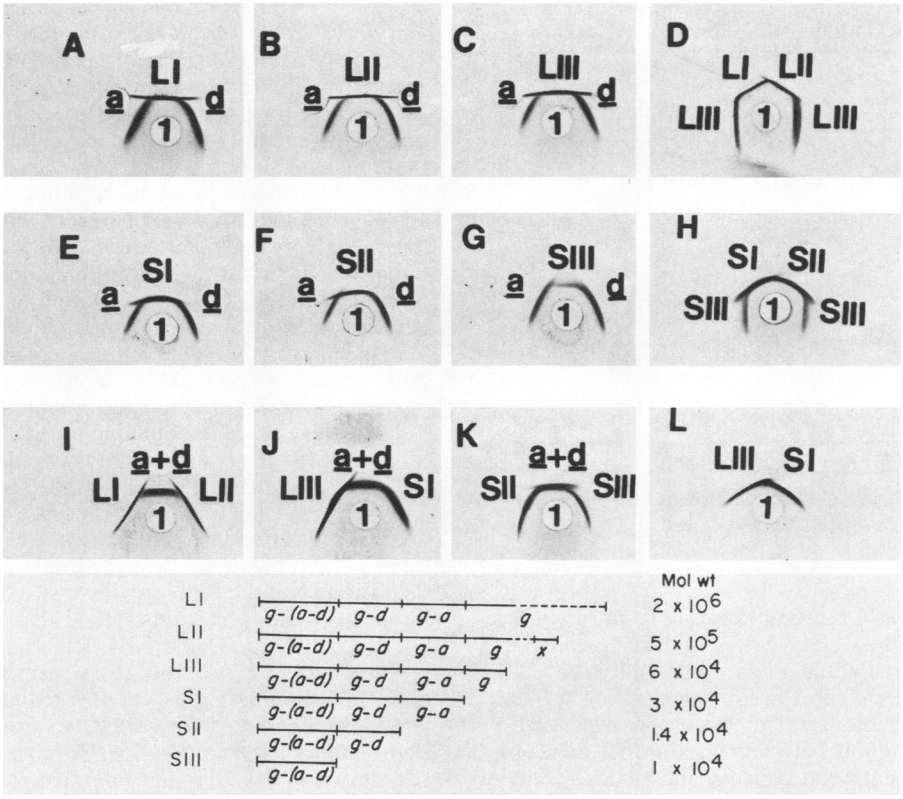

FIG. 3. Analysis of gel filtration by a double immunodiffusion method. Fractions LI, LII, LIII, SI, SII, and SIII and polysaccharides *a* and *d* were tested against anti-*g* serum (designated 1, see Fig. 1A), as indicated. The proposed schematic representations for polysaccharides *g* and its intermediary forms and their approximate molecular weights are also presented.

g obtained above (Fig. 2B and D) were tested against anti-*g* serum by a double immunodiffusion method. The pattern of the precipitin bands obtained with fractions LI (Fig. 3A), LII (Fig. 3B), LIII (Fig. 3C), and SI (Fig. 3E) flanked on either site by polysaccharides *a* and *d* suggested that these four fractions were antigenically similar to polysaccharide *g*. However, when fractions LI, LII, and LIII were tested against anti-*g* serum (Fig. 3D), fractions LI and LIII appeared to be identical, whereas fraction LII appeared to contain an additional distinct immunogenic region, designated *x*. Fractions LI, LII, and LIII differed in molecular weights, estimated at 2×10^6, 5×10^5, and 6×10^4, respectively. Also, the subtle changes in the shape of the arc of precipitin bands, concave up for fraction LI (Fig. 3A), horizontal for fraction LII (Fig. 3B), and concave down for fraction LIII (Fig. 3C), reflected the decreasing order of molecular weights of these fractions. In Fig. 3I, the partial identity seen with LI and LII when adjacent to a well containing a mixture of polysaccharides *a* and *d* suggested that LI and LII may be similar to the putative polysaccharide *g*.

Fraction SI, on the other hand, showed identity when adjacent to a well containing polysaccharides *a* and *d* (Fig. 3J) and partial identity when adjacent to fraction LIII (Fig. 3L). These results suggested that SI lacked the type-specific *g* site of LIII (putative polysaccharide *g*), but retained the cross-reactive sites *g-(a-d)*, *g-d*, and *g-a*. Thus, the partial identity between fraction SI and either polysaccharide *a* or *d* (Fig. 3E) was likely due to the cross-reactive sites *g-d* or *g-a*, respectively, of fraction SI.

Fraction SII (Fig. 3F) showed identity with polysaccharide *d* and nonidentity with polysaccharide *a*. These results suggested that fraction SII lacked both the type-specific *g* and the cross-reactive *g-a* sites. The partial identity seen with fraction SII when adjacent to a well containing both polysaccharides *a* and *d* supported the absence of both the type-specific *g* and the cross-reactive *g-a* sites from fraction SII (Fig. 3K). Figure 3G shows that fraction SIII, when flanked by polysaccharide *a* or *d*, showed partial identities. This result indicated that fraction SIII probably lacked three antigenic sites, the type-specific *g* site and the cross-reactive *g-a* and *g-d* sites. The pattern of precipitin bands obtained with fractions SI, SII, and SIII arranged as in Fig. 3H suggested that fraction SIII lacked antigenic sites present on fractions SII and SI and that fraction SII lacked a site present as fraction SI. Thus, we propose that fraction SIII contains only the *g-(a-d)* site, fraction SII contains the *g-(a-d)* and *g-d* sites, and fraction SI contains the *g-(a-d)*, *g-d*, and *g-a* sites. In Fig. 3K, the partial identity between fraction SII and the well containing both polysaccharides *a* and *d* indicated the absence of the *g-a* site on fraction SII. The larger spur produced by the mixture of antigens *a* and *d* when adjacent to fraction SIII suggested the lack of both *g-a* and *g-d* sites for fraction SIII. The molecular weight estimates of fractions SI, SII, and SIII were found to be 3×10^4, 1.4×10^4, and 1×10^4, respectively; this decreasing ranking of the molecular weights probably corresponded to the increasing absence of antigenic sites of the putative polysaccharide *g*. Analyses for sugar composition and protein, amino sugar, and phosphorus content of these polysaccharide prepara-

TABLE 1. Inhibition of precipitin reactions between gel filtration fractions and anti-*g* serum

Inhibitor[a]	% Inhibition of gel filtration fractions					
	LI	LII	LIII	SI	SII	SIII
Melibiose (Gal$\xrightarrow{\alpha 1,6}$Glc)	41	39	40	46	38	44
Stachyose (Gal$\xrightarrow{\alpha 1,6}$Glc$\xrightarrow{\alpha 1,6}$Glc$\xrightarrow{\beta 1,2}$Fru)	35	36	25	42	25	38
Lactose (Gal$\xrightarrow{\beta 1,4}$Glc)	43	20	39	29	25	32
D-Galactose	49	33	26	52	13	21
β-Gentiobiose (Glc$\xrightarrow{\beta 1,6}$Glc)	38	26	0	0	5	12
β-Methylglucoside	29	31	5	4	0	0
α-Methylglucoside	9	5	8	5	0	2
α-Rhamnose	15	4	3	0	1	12
Cellobiose (Glc$\xrightarrow{\beta 1,4}$Glc)	54	12	10	23	0	14
Isomaltose (Glc$\xrightarrow{\alpha 1,6}$Glc)	17	8	7	0	9	16
Maltose (Glc$\xrightarrow{\alpha 1,4}$Glc)	18	9	6	0	14	2
D-Glucose	10	11	2	16	0	7

[a] The abbreviations used for galactose, glucose, and fructose are Gal, Glc, and Fru, respectively.

tions were not possible due to insufficient amounts of preparations obtained.

Inhibition of precipitin reaction between gel filtration fractions and anti-*g* serum. Galactose and galactose-containing oligosaccharides were in the main good inhibitors of the precipitin reactions between the six gel filtration fractions and anti-*g* serum (Table 1). The inhibition produced by galactose for the reaction between fraction SII and anti-*g* serum was ranked the lowest. Glucose and glucose-containing compounds were less effective than galactose-containing compounds as inhibitors of the precipitin reactions involving LIII, SI, SII, and SIII. Compounds containing the β-glucosidic linkage inhibited the precipitin reaction with fractions LI and LII, with cellobiose being most active with LI.

DISCUSSION

A single molecule of serotype polysaccharide *a* from *S. mutans* strain HS6 or AHT and also a single molecule of serotype polysaccharide *d* from *S. mutans* B13 have been shown conclusively to contain the specific antigenic sites *a* and *d*, respectively, and also to contain the common *a-d* sites (2, 12–14, 16). Subsequently, serotype polysaccharide *g* from *S. mutans* strains OMZ65 and 6715 has been reported to cross-react with strains HS6 and B13 (5, 10, 18). These results suggest that polysaccharide *g* probably contains, in addition to the serotype-specific *g* site, the cross-reactive sites *g-a*, *g-d*, and *g-(a-d)*, the latter being the same as the previously mentioned site *a-d*. The results we have obtained confirm the proposal for the presence of such cross-reactive sites on polysaccharide *g*. In Fig. 1, the proposed schematic representations for polysaccharides *g*, *a*, and *d* are shown to contain four, three, and three immunogenic regions, respectively. Thus, the cross-reactive site *g-(a-d)* of polysaccharide *g* finds its counterpart on both polysaccharides *a* and *d*, whereas sites *g-a* and *g-d* find their counterparts only in polysaccharide *a* and in polysaccharide *d*, respectively.

The natural occurrence of different molecular weight forms of polysaccharide *g* in *S. mutans* 6715 may represent products of degradation or intermediates of biosynthesis. Experiments have not been performed to address this problem. In growth of gram-positive bacteria, autolytic enzymes have been suggested to play a role in hydrolyzing cell wall linkages to allow insertion of newly synthesized wall components (4). A similar process may take place for polysaccharide *g*, which occurs as a microcapsule of cells of *S. mutans* 6715 (9). Whatever the mechanism, the process yields polysaccharide fractions containing sequences of sugars that maintain the expressions of the immunogenic regions found for the completed polysaccharide *g*. The relationship between the molecular weight of the fractions and the antigenic sites found on these fractions suggest that if these fractions are products of autolysis, then the regions sensitive to hydrolysis are specific. On the other hand, if these products represent intermediates of biosynthesis, then the order of synthesis of immunodominant sites of the fractions and the subsequent ligation of these fractions must occur in a highly specific manner. Based on these possibilities, one can envision the order of scission of the completed polysaccharide *g* to yield the fractions found or the order of ligation of fractions to yield the completed polysaccharide *g*.

We believe that the obtaining of the L fraction from the S fraction (Fig. 2B) is due to cross-contamination rather than to aggregation of smaller S fraction. The L fractions in Fig. 2A and were combined and processed further by two additional gel filtration steps resulting in LI, LII, and LIII. These three L fractions when tested against anti-*g* serum showed the presence of the type-specific *g* site (Fig. 3). On the other hand, all of the S fractions lacked the type-specific *g* site.

Fractions LI, LII, and LIII, which appear to contain the type-specific site and the cross-reactive sites, differed in their molecular weights, and fraction LII contained an additional immunogenic region, *x*. Presently, we have no information on the nature of *x*. Moreover, the length of the *g* site drawn in Fig. 3 is not intended to be reflective of the different molecular weights of the L forms.

The inhibition experiments performed suggested that polysaccharide *g* and its derivatives contain immunogenic regions specified by the presence of galactose. Polysaccharide *g* has been previously shown to contain the sugars galactose and glucose in a ratio of 6:1 (9, 10). The *a-d* site of serotype *a* (and serotype *d*), which is proposed to be identical to the *g-(a-d)* site, was also suggested to be galactose rich by inhibition studies (2, 14). Fractions LI and LII, which were inhibited by β-gentibiose and β-methylglucoside, and fraction LI, which was inhibited by cellobiose, suggested that these larger forms of polysaccharide *g* expressed glucose in β-linkages as a component of the immunodominant region. Iacono et al. (9) also showed that glucose containing glucosides, including cellobiose and β-methylglucoside, were effective inhibitors of purified, but not fractionated, polysaccharide *g* from *S. mutans* 6715.

ACKNOWLEDGMENTS

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