INFECTION AND IMMUNITY, May 1987, p. 1264–1273
0019-9567/87/051264-10$02.00/0

Vol. 55, No. 5

Characterization of a Rat Salivary Sialoglycoprotein Complex Which Agglutinates *Streptococcus mutans*

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Received 14 October 1986/Accepted 29 January 1987

Rat saliva agglutinated *Streptococcus mutans* Ingbritt and NCTC 10449 and *Streptococcus sanguis* NCTC 7864 but not *S. mutans* NCTC 10921, GS 5, or LM 7, *Streptococcus sobrinus* 6715-13 or OMZ 65, or *Streptococcus cricetus* HS 6, as measured turbidometrically. The specificity of agglutination by rat saliva was the same as that by human saliva. Agglutination was associated with a mucin complex (rat salivary agglutinin complex [rS-A]) of sulfated sialoglycoproteins, with a trace of associated lipid and an apparent M_r of 1.6×10^6, isolated by gel-filtration Fast Protein Liquid Chromatography. The complex was dissociated in a high-ionic-strength buffer containing 6 M urea and then fractionated by gel filtration and anion-exchange Fast Protein Liquid Chromatography into four sulfated sialoglycoprotein components, designated rS-A-1Q1, rS-A-1Q2, rS-A-1Q3, and rS-A-2, with rS-A-1Q2 being polydisperse through differential glycosylation of the polypeptide backbone. The dissociation destroyed agglutination activity. The polypeptide backbones contained up to 42% serine plus threonine and up to 40% glycine plus alanine plus proline plus valine. The carbohydrate moiety of the rS-A sialoglycoproteins consisted of *N*-acetylgalactosamine, sialate, galactose, fucose, *N*-acetylglucosamine, and small amounts of mannose, with the predominant sugar being *N*-acetylgalactosamine. Agglutination was inhibited by 1 mM EDTA but was restored by 1.5 mM $CaCl_2$. Agglutination was also inhibited by 5 mM $CaCl_2$; nonimmune sera; cationic polymers; and wheat germ, lentil, soybean, and peanut lectins. However, agglutination was not affected by lipoteichoic acid, various anionic proteins, or various sugars. Neuraminidase treatment of rS-A did not affect activity, but tryptic digestion of *S. mutans* did prevent agglutination. The results are consistent with calcium bridging the negative groups within the rS-A complex and allowing the approach of rS-A to the bacterial cell surface to effect a specific conformational attachment.

Human saliva agglutinates oral bacteria in species- and strain-specific interactions (11). Agglutinins from human saliva interactive with *Streptococcus mutans* (9) and *Streptococcus sanguis* (14) have been isolated and chemically characterized. These salivary agglutinins are sialoglycoproteins with greater than 45% carbohydrate and a characteristically high content of serine, threonine, proline, alanine, and glycine (35). Sialoglycoproteins from monkey saliva with compositions similar to the human salivary agglutinins also agglutinate *S. mutans* and *S. sanguis* (20). These agglutinins are all sialoglycoprotein complexes (mucins) with molecular weights greater than 10^6. However, recently Babu et al. (1) have isolated a sialoglycoprotein from human saliva with a molecular weight of only 6×10^4 that agglutinates *S. mutans*. Although this agglutinin is much smaller, it does have a composition similar to that of the high-molecular weight agglutinin and could be a functional fragment of one of the subunits of the larger complex.

The interactions between human salivary agglutinins and oral streptococci have been studied. The agglutination of *S. mutans* is calcium dependent (11, 31) and requires a pH above 5.0 (31). The calcium requirement of *S. mutans* agglutination has resulted in a proposed mechanism of agglutination based on the bridging by calcium of negatively charged groups on the surface of bacteria (such as the phosphate of lipoteichoic acid [LTA]) with similar groups on the agglutinin (30). The agglutination of *S. sanguis* is not calcium dependent (although it is enhanced by calcium) and is stable between pH 3.9 and 8.7 (11, 14, 16, 31). Sialyl residues of the agglutinin are involved in the agglutination of *S. sanguis* but not of *S. mutans*, and a mechanism of agglutination of *S. sanguis* based on protein-carbohydrate binding has been proposed by Levine et al. (20).

In this paper we report the presence of a high-molecular weight, mucous sialoglycoprotein complex in rat saliva (rat salivary agglutinin complex [rS-A complex]) which agglutinates *S. mutans* with strain specificity. The rS-A complex was isolated, dissociated, fractionated, chemically characterized, and compared with previously characterized salivary agglutinins. Inhibition studies and enzyme digestion were used to characterize the bacterium-agglutinin interaction.

MATERIALS AND METHODS

Bacteria. *S. mutans* Ingbritt, NCTC 10449, NCTC 10921, GS 5, and LM7 (27); *Streptococcus sobrinus* 6715-13 and OMZ 65 (4); *Streptococcus cricetus* HS 6; and *S. sanguis* NCTC 7863 and NCTC 7864 (2) were stored at −20°C in Todd-Hewitt broth (Oxoid Australia) containing 0.5% yeast extract (THYE) and 30% glycerol and were grown in either THYE or THYE–2% (wt/vol) sucrose at 37°C for 18 h. Cell growth was monitored turbidometrically at 650 nm.

Saliva. Rat whole saliva (rWS) was collected from adult male Sprague-Dawley rats by the method of Reynolds and del Rio (28). The pooled saliva was centrifuged at 5,500 × *g* for 20 min at 4°C, and the supernatant was stored in aliquots at −20°C. The clarified saliva was frozen and thawed once before use. Human whole saliva (hWS) was collected on ice from six donors without stimulation. The hWS was maintained at 4°C during collection, pooled, and then treated as described for rWS.

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Agglutination and cell sedimentation assays. The turbidometric assay of Ericson et al. (8) was modified and used to measure cell agglutination. Cells were harvested from an 18-h culture by centrifugation (1,000 × *g*, 15 min, 4°C), washed twice with buffer (50 mM imidazole hydrochloride [pH 7.0], 25 mM NaCl), and resuspended to an optical density at 700 nm (OD_{700}) of 1.5. All manipulations were carried out at 4°C. The suspension was brought to 37°C, and 0.4 ml was added to 0.2 ml of the rS-A (0.2 mg/ml) or to rWS or hWS diluted 1/2 with buffer in disposable cuvettes. The contents of the cuvettes were mixed, and then incubated at 37°C, and the OD_{700} was measured at 5-min intervals for 2 h. A control, cells in the absence of saliva, was included, and 0.4 ml of the bacterial suspension was added to 0.2 ml of buffer. Cell sedimentation was studied by using the same turbidometric assay in the absence of saliva, and the OD_{700} was monitored for 3 h. The effects of various proteins, lectins, carbohydrates, and antisera on agglutination (by saliva and rS-A) and cell sedimentation of bacteria were determined. Proteins were either purchased from Sigma Chemical Co., St. Louis, Mo., or obtained as described previously (29). The chemical characteristics of the various proteins have been described by Reynolds and Wong (29). Wheat germ, soybean, lentil, and peanut lectins were from P-L Biochemicals, Inc., Milwaukee, Wis., as were the sugars *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-mannosamine, *N*-acetylneuraminic acid (NANA), fucose, galactose, mannose, lactose, maltose, cellobiose, and *N*-acetylneuramin–lactose. Dextran T-10 and T-70 were from Pharmacia Australia. LTA from *Lactobacillus casei* and anti-LTA antiserum (rabbit) were gifts from K. W. Knox, Institute of Dental Research, United Dental Hospital, New South Wales, Australia, and have been described by Hardy et al. (12). The protein concentrations of antisera and normal rabbit (nonimmune) serum (NRS) were determined after precipitation with 10% trichloroacetic acid by the method of Lowry et al. (21) by using bovine serum albumin as a standard.

Isolation of the rS-A complex and its components. (i) Gel-filtration FPLC. Clarified rWS (1.5 ml), concentrated threefold by ultrafiltration using a YM-5 membrane (Amicon, Australia), was applied to a Fast Protein Liquid Chromatography (FPLC) Superose-6 column HR 10/30 (Pharmacia Australia) equilibrated with 50 mM Tris hydrochloride (pH 8)–150 mM NaCl at 25°C at a flow rate of 0.5 ml/min. Absorbance of the column effluent was monitored at 214 nm. Fractions corresponding to peaks were collected, desalted by ultrafiltration, lyophilized, and then stored with desiccant at −20°C.

(ii) Gel-filtration FPLC with urea. Samples (0.5 ml each, 10 mg/ml) in 50 mM Tris hydrochloride (pH 8.0)–640 mM NaCl–6 M urea–1 mM dithiothreitol–1 mM EDTA were applied to a Superose-6 column at a flow rate of 0.3 ml/min. Light scattering and absorbance of the column effluent were monitored at 280 nm. Peaks were treated as described above.

(iii) Anion-exchange FPLC. An FPLC Mono-Q column HR 5/5 (Pharmacia Australia Pty. Ltd.) was equilibrated with 20 mM Tris hydrochloride (pH 8.0)–200 mM NaCl–6 M urea at a flow rate of 1.0 ml/min. Samples (0.5 ml each, 5 mg/ml) in equilibration buffer were applied and eluted with a linear gradient of 200 to 290 mM NaCl over 30 min, followed by a steeper linear gradient of 290 to 400 mM NaCl over 3 min. Light scattering and absorbance of the eluate were monitored at 280 nm. Fractions were dialyzed against Milli-Q water by using dialysis tubing with a molecular weight cutoff of 12,000 to 14,000 (Union Carbide), concentrated by ultrafiltration using a YM-5 membrane, and then lyophilized.

Chemical analyses. Amino acid compositions were determined after vapor hydrolysis of samples (10 μg each) by using 6 N (constant-boiling) HCl at 110°C for 24 h, as recommended in the Picotag procedure (Waters Associates Pty. Ltd.). This procedure minimized hydrolysis losses. The amino acids were derivatized with phenyllisothiocyanate, and the derivatives were separated by reverse-phase high-performance liquid chromatography (Picotag procedure). Total protein was calculated from residue weights.

Analyses of neutral sugars were performed by means of borate-complex anion-exchange chromatography by using a modification of the method of Kesler (17). Samples were hydrolyzed with trifluoroacetic acid (TFA) by the method of Lee et al. (19). The hydrolized samples were lyophilized, dissolved in 75 mM sodium borate, pH 7.5 (buffer A), and injected onto a Mono-Q HR 5/5 column that had been equilibrated with buffer A at 0.5 ml/min. Sample components were eluted with linear gradients prepared by mixing buffer A with 400 mM sodium borate, pH 10.0 (buffer B): 0 to 10% B over 4 min, 10% B for 4 min, 10 to 100% B over 20 min, and then 100% B for 80 min. Fractions collected (0.4 ml each) were analyzed for hexoses by the phenol-sulfuric acid method (6) by using glucose, galactose, mannose, and fucose as standards.

Sialic acids were determined by the thiobarbituric acid assay (40) after hydrolysis of the sample in 200 mM sulfuric acid at 80°C for 1 h with NANA as a standard.

Sulfate was analyzed by the method of Dodgson and Price (5) by using the modification of Clarke and Denborough (3).

Glucosamine and galactosamine were determined by reverse-phase high-performance liquid chromatography after derivatization with phenylisothiocyanate of hydrolyzed samples (1 M HCl, 100°C, 2 h) by the Picotag procedure.

Lipid was separated from samples by extraction (twice) with chloroform-methanol (2:1, vol/vol). The extracts were pooled, dried under a stream of nitrogen, and assayed for lipid by using the potassium dichromate-sulfuric acid assay of Tourtelotte et al. (38), with cholesterol as a standard. Hydrophobic proteins were used as negative controls.

Amino-terminal residue analysis. Amino-terminal residues were analyzed by a modification of the manual Edman degradation procedure. Each sample (0.4 mg) was dissolved in 100 μl of coupling buffer in a borosilicate glass tube. Coupling buffer was prepared by titrating a 0.4 M solution of dimethylallylamine in 1-propanol–H_2O (3:2, vol/vol) to pH 9.5 with TFA. Phenylisothiocyanate (5 μl) was added under a stream of nitrogen, and the solution was mixed thoroughly and incubated at 50°C for 20 min. This mixture was then extracted once with 0.5 ml of benzene. The upper phase was removed, and the lower phase was dried under a stream of nitrogen. TFA (100 μl) was added under nitrogen, and the mixture was incubated at 50°C for 7 min. The TFA was then evaporated under a stream of nitrogen, and 300 μl of 0.01 N HCl containing 2% (vol/vol) ethanethiol was added and mixed. The thiazolinone derivative was extracted with 0.5 ml of diethyl ether containing 10^{-4} M dithiothreitol. The upper ether phases were collected, pooled, and evaporated to dryness under nitrogen. Conversion to the phenylthiohydantoin (PTH) was achieved by the addition of 200 μl of 1 M HCl containing 2% (vol/vol) ethanethiol and by incubation at 80°C for 10 min. The PTH derivative was extracted twice with 0.5 ml of ethyl acetate. The upper phases were collected, pooled, and dried under a stream of nitrogen. PTH

amino-terminal derivatives were identified by a modification of the high-performance liquid chromatography procedure of Zimmerman et al. (42) with a Waters Associates M-680 automated gradient controller and a Zorbax ODS column (25 by 0.46 cm).

Immunological procedures. Enzyme-linked immunosorbent assays were performed by the method of Voller et al. (39). Wells of microtiter trays were coated with antigen (50 μl, 3 μg/ml) for 3 h at 37°C and blocked with 10 mg of bovine serum albumin per ml in phosphate-buffered saline–0.05% Tween 20 for 3 h at 37°C. The first antibody was either rabbit anti-rat immunoglobulin (immunoglobulin G [IgG], IgM, IgA [Fc + Fab]), rabbit anti-rat IgM (Fc-specific), rabbit anti-rat IgA (Fc-specific), or rabbit anti-rat IgG (heavy and light chains) diluted 1/100, 1/1,000, 1/10,000, 1/50,000, and 1/100,000. Antisera were from Nordic Immunological Laboratories, Tilburg, The Netherlands. The second antibody (diluted 1/3,000) was horseradish peroxidase-conjugated, affinity-purified, goat anti-rabbit IgG (heavy and light chains) from Bio-Rad Laboratories Australia. Color development was achieved by adding 200 μl of a solution of *o*-phenylenediamine (0.4 mg/ml)–0.1 M citrate-phosphate buffer (pH 5.0)–0.01% (vol/vol) H_2O_2 to each well. The enzyme reaction was stopped by the addition of 4 N H_2SO_4.

Proteolytic and dextranase treatment of *S. mutans* cells. A suspension of buffer-washed *S. mutans* Ingbritt cells (OD_{700}, 1.5) was incubated with 2 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Sigma) per ml in 50 mM imidazole hydrochloride (pH 7.0)–25 mM NaCl at 25°C for 2 h. Cells were then washed with buffer and assayed for their ability to sediment and to undergo agglutination. Control cells not pretreated with trypsin were also assayed for agglutination in the presence of heat-treated trypsin in order to exclude nonproteolytic inhibition by the protease.

Bacterial cells (OD_{700}, 1.5) were incubated with 5 μg of dextranase per ml (grade I; Sigma) in 50 mM acetate (pH 5.5) at 37°C for 30 min. Cells were washed with 50 mM imidazole hydrochloride (pH 7.0)–25 mM NaCl and assayed for agglutination. Control cells were included as described above.

Treatment of rS-A. (i) Neuraminidase and dextranase. Clarified rWS and rS-A (0.2 mg/ml of 50 mM acetate [pH 5.5]) were incubated with 2 μg of neuraminidase type X or dextranase grade I (Sigma) per ml. The enzyme (0.1 mg of enzyme per ml of 50 mM acetate [pH 5.5]) was added, and the solutions were incubated at 37°C for 18 h. Each solution was ultrafiltered with agglutination assay buffer (CS-15 ultrafiltration membrane; Amicon Scientific, Sydney, Australia), and the retentate was then assayed for its ability to agglutinate *S. mutans* Ingbritt. A control solution in the absence of enzyme was similarly treated. The effect of neuraminidase on the bacteria was also assessed. Cells (OD_{700}, 1.5) were incubated with neuraminidase (0.33 μg of enzyme per ml of 50 mM acetate [pH 5.5]) at 37°C for 2 h, washed with assay buffer, and then tested for their ability to undergo agglutination and to sediment.

(ii) Anti-immunoglobulin antisera. Rabbit antisera against rat IgG (heavy and light chains), IgM (Fc-specific), and IgA (Fc-specific) were from Nordic Immunological Laboratories. Antiserum (0.5 ml) and either clarified rWS (0.6 ml) or rS-A (0.2 mg/ml; 0.6 ml) were mixed and incubated at 37°C for 1 h and centrifuged at 5,000 × *g* for 20 min at 4°C, and the supernatant was assayed for agglutinating activity.

(iii) Hydroxyapatite. Hydroxyapatite (Bio-Gel HTP; Bio-Rad Laboratories, Sydney, Australia) was washed three times with agglutination assay buffer. Clarified rWS (0.5 ml) and rS-A (0.2 mg/ml of assay buffer; 0.5 ml) were incubated with the washed hydroxyapatite (50 mg [dry weight]) at 37°C for 1 h with end-over-end shaking in a rotary shaker at 60 cycles per min. After centrifugation (1,000 × *g*, 1 min), the supernatant (undiluted and diluted 1/2) was assayed for agglutination activity. Clarified rWS and rS-A in the absence of hydroxyapatite were treated similarly.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (SDS-PAGE) was performed by the method of Laemmli (18). Gradient slab gels (T = 4 to 12%, C = 2.6%) were used with a stacking gel (T = 3%, C = 2.6%). Proteins in gels were silver stained by the method of Wray et al. (41).

RESULTS

Stimulated rWS and hWS agglutinated *S. mutans* Ingbritt and NCTC 10449 and *S. sanguis* 7864 but did not agglutinate *S. mutans* (NCTC 10921, GS 5, or LM 7), *S. sobrinus* 6715-13 or OMZ 65, *S. cricetus* HS 6, or *S. sanguis* 7863. Glucose-grown *S. mutans* (NCTC 10921, LM 7, and GS 5), *S. sobrinus* 6715-13 and OMZ 65, *S. cricetus* HS 6, and *S. sanguis* 7863 displayed normal cell sedimentation characteristics in the presence of saliva. Although saliva did not agglutinate *S. mutans* NCTC 10921 or LM 7, *S. sobrinus* OMZ 65, or *S. cricetus* HS 6, these organisms removed agglutination activity from saliva. The bound agglutinin could be eluted with EDTA (3 mM) from strains Ingbritt, NCTC 10449, NCTC 10921, and HS 6, with activity being restored by the addition of calcium (1.5 mM). Saliva-mediated agglutination of *S. mutans* Ingbritt was slightly inhibited at an ionic strength of 0.5 and did not occur at all at an ionic strength of 0.64. Self-agglutination in the absence of saliva occurred at low ionic strengths ($I < 0.01$). The assays were performed at the ionic strength of 0.05, which approximates that of saliva and at which the specific agglutination by saliva is accentuated. Agglutination mediated by rat saliva in this assay system was maximal at pH 8.0 and did not occur at pH values below 6.0. The pH of stimulated rWS was 8.0; however, at this pH cell sedimentation was enhanced, so assays were performed at pH 7.0, at which effects on agglutination and sedimentation could be clearly differentiated. Agglutinated cells could be washed repeatedly (at least five times) with buffer without losing the ability to reagglutinate after resuspension.

Isolation and characterization of the rS-A. Agglutination activity of clarified rWS was found in the first peak after gel-filtration FPLC (elution volume [V_e], 7.68 ± 0.08 ml), and this fraction was designated rS-A (Fig. 1). Fractions (0.5 ml) from the gel-filtration FPLC corresponding to the respective peaks were pooled and concentrated to the original saliva concentration by ultrafiltration and assayed for agglutination activity (undiluted and serially diluted to 1/16) by using the turbidometric assay. Only the fraction rS-A (Fig. 1) agglutinated *S. mutans* Ingbritt. Gel-filtration FPLC of molecules of known molecular weight (blue dextran [2.0×10^6] V_e, 6.4 ml; thyroglobulin [669,000] V_e, 11.3 ml; ferritin [440,000] V_e, 13.0 ml; catalase [232,000] V_e, 14.9 ml; and lactate dehydrogenase [140,000] V_e, 16.1 ml) enabled the determination of the relative molecular weight [M_r] of rS-A. A plot of log molecular weight versus V_e gave an M_r of (1.57 ± 0.03) × 10^6. This large apparent M_r suggested that the agglutinin was a mucin complex. To dissociate the complex, rS-A was dissolved in a high-ionic-strength buffer ($I = 0.67$) containing 6 M urea, 1 mM EDTA, and 1 mM dithiothreitol and rechromatographed by gel-filtration FPLC under the same dissociating conditions. This procedure separated rS-A into three components, designated rS-A-1, rS-A-2, and rS-

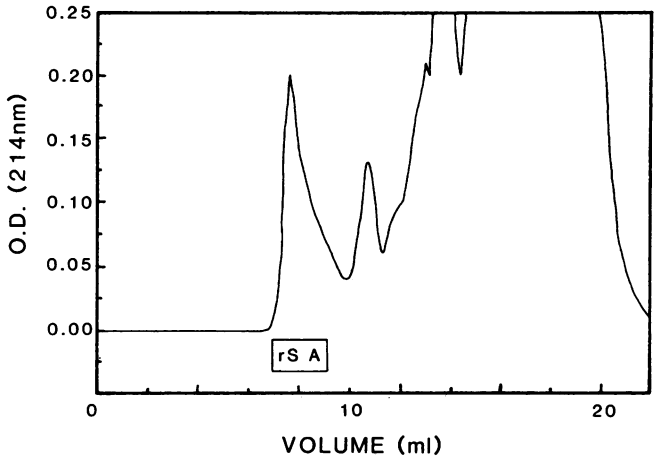

FIG. 1. Gel-filtration FPLC of clarified rWS. Clarified rWS (1.5 ml, concentrated threefold) was applied to the Superose-6 column. The fraction rS-A contained the agglutination activity.

A-3 (Fig. 2). The fraction rS-A-3 did not contain protein and was later identified as lipid. The M_r for rS-A-1 and rS-A-2 from the gel-filtration FPLC were $(1.46 \pm 0.06) \times 10^6$ and $(4.4 \pm 0.2) \times 10^4$, respectively. SDS-PAGE of rS-A-1 produced one diffuse band which just entered the separating gel corresponding to an M_r of approximately 1.3×10^6, and rS-A-2 produced one sharp band upon SDS-PAGE corresponding to an M_r of 4.4×10^4 (Fig. 3). The M_rs were obtained from a plot of log molecular weight versus relative mobility of protein standards; the values compared well with those from gel filtration, although the M_r of rS-A-1 could not be determined accurately with this method. The fraction rS-A-2 produced only one peak upon anion-exchange (Mono-Q) FPLC, which confirmed the SDS-PAGE result and suggested homogeneity. The fraction rS-A-1, however, separated upon anion-exchange FPLC into three peaks, designated rS-A-1Q1, rS-A-1Q2, and rS-A-1Q3 (Fig. 4).

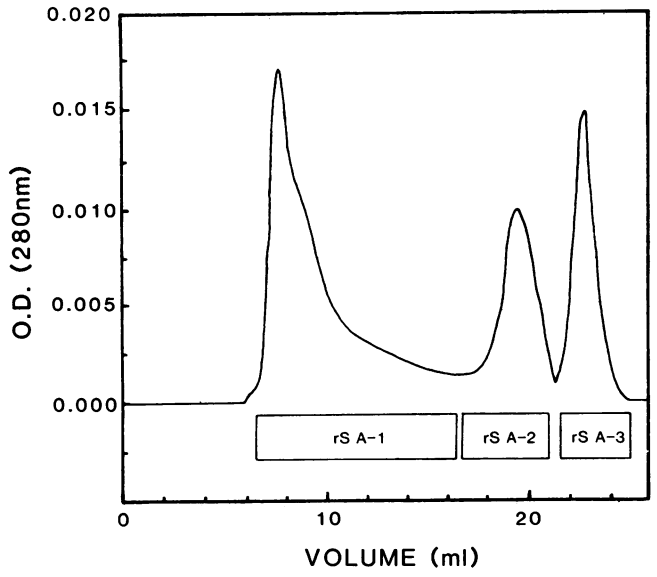

FIG. 2. Urea gel-filtration FPLC of rS-A. The rS-A (10 mg/ml of urea buffer) was applied to the Superose-6 column. The fractions rS-A-1, rS-A-2, and rS-A-3 were collected.

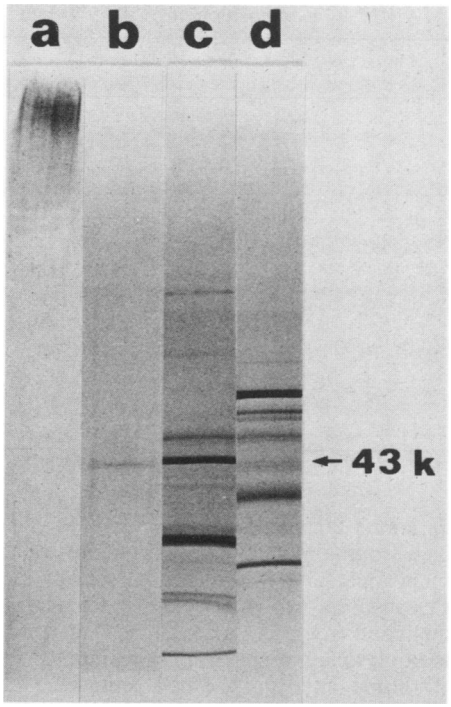

FIG. 3. Discontinuous SDS-PAGE gradient gel of the agglutinin components. Lanes: a, rS-A-1; b, rS-A-2; c and d, molecular weight markers (ovalbumin [43,000] marked by arrow). A 20-μl sample of each fraction (0.5 mg/ml in 0.0625 M Tris hydrochloride [pH 8.8]–2.3% SDS–5% β-mercaptoethanol–20% glycerol–0.05% bromphenol blue) was loaded into wells in the stacking gel (T = 3%, C = 2.6%). The electrophoretic conditions were 150 V for 20 min and then 200 V for 3 h. Proteins were silver stained.

However, rS-A-1Q2 was still heterogeneous, so fractions of the peak (rS-A-1Q2a to rS-A-1Q2f) were collected which corresponded approximately to six overlapping peaks. The homogeneity of each of the fractions was checked by amino-terminal residue analysis, as isoelectric focusing proved unsatisfactory. The fraction rS-A-1Q1 contained one main amino-terminal residue (glycine) and trace amounts of con-

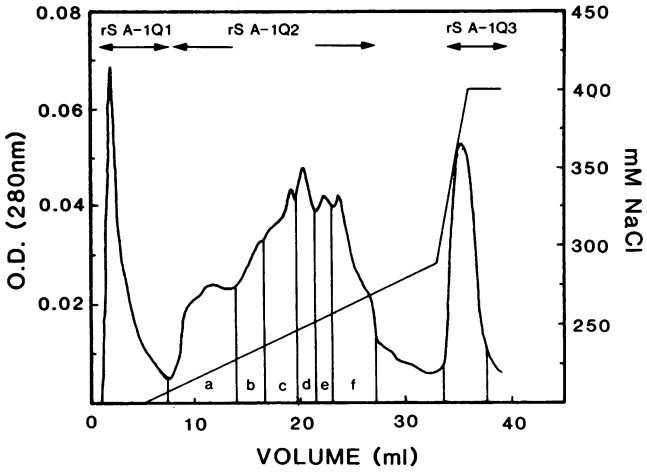

FIG. 4. Anion-exchange FPLC of rS-A-1 in the presence of 6 M urea. The rS-A-1 (5 mg/ml of urea buffer) was applied to the Mono-Q column, and fractions rS-A-1Q1 to rS-A-1Q3 were collected.

TABLE 1. Composition of rS-A by weight

Component	% of rS-A complex
rS-A-1-Q1	5.1
rS-A-1-Q2a	20.7
rS-A-1-Q2b	4.7
rS-A-1-Q2c	3.7
rS-A-1-Q2d	3.1
rS-A-1-Q2e	10.2
rS-A-1-Q2f	12.5
rS-A-1-Q3	4.3
rS-A-2	35.6
rS-A-3	0.1

taminating amino-terminal residues (alanine, proline, and serine). The contaminating proteins were unlikely to be breakdown products due to the lack of proteolytic activity in rS-A (with casein as substrate). The other agglutinin components analyzed (rS-A-1Q2a to rS-A-1Q2d, rS-A1Q3, and rS-A-2) had only one amino-terminal residue (glycine), consistent with homogeneity of the polypeptide chain.

The rS-A complex made up 2.5% of rWS dialyzed solids by weight and was present at 190 μg/ml of stimulated rWS. The proportions of each component of the rS-A complex, on the basis of weight, are shown in Table 1. None of the rS-A components separately or in combination, at their concentrations in saliva, in the absence or presence of calcium, agglutinated *S. mutans* Ingbritt in the turbidometric assay. *S. mutans* Ingbritt was agglutinated by rS-A at 0.2 mg/ml; however, fraction rS-A-1 at 0.16 mg/ml failed to agglutinate *S. mutans* Ingbritt, as did rS-A-2 at 0.10 mg/ml. Fractions rS-A-1 and rS-A-2 at higher and lower concentrations and mixtures of rS-A-1, rS-A-2, and rS-A-3 at their concentrations in saliva in the presence of 1 mM $CaCl_2$ also failed to agglutinate *S. mutans* Ingbritt, suggesting an irreversible denaturation of the rS-A complex.

The chemical compositions of the components of rS-A are shown in Table 2. The proportion of carbohydrate to protein in the glycoprotein components is characteristic of high-molecular weight mucous glycoproteins, as are the amino acid and carbohydrate compositions (Table 3). The polypeptide backbones consisted of up to 42% serine plus threonine and up to 40% glycine plus alanine plus proline plus valine. They also had low or nondetectable amounts of histidine, tyrosine, phenylalanine, methionine, and cysteine. The very similar amino acid compositions of rS-A-1Q2 fractions, together with the common amino-terminal residue, glycine, suggest that these fractions have a common polypeptide backbone. The polydispersity that rS-A-1 displayed upon anion-exchange (Mono-Q) FLPC reflected the differential sialylation and sulfation of the oligosaccharide chains (Table 3). In fact, the order of elution of rS-A-1 components from the Mono-Q column corresponded to the negative charge at pH 8.0 on the components, arising from their content of sulfate and sialyl residues (Table 3). The carbohydrate moiety of the rS-A sialoglycoproteins consisted of GalNAc, sialate, galactose, fucose, GlcNAc, and small amounts of mannose (Tables 3 and 4). The predominant sugar was GalNac, and the amount of this sugar in rS-A-1Q1, rS-A-1Q2, rS-A-1Q3, and rS-A-2 was correlated ($r = 0.973$) with the serine-plus-threonine content. The high proportions of GalNAc and of serine plus threonine suggest that the carbohydrate-protein linkages are predominately O-glycosidic.

Characterization of the agglutination interactions. The presence of EDTA (1 mM) inhibited the agglutination of *S. mutans* Ingbritt and NCTC 10449 by rS-A, but activity was restored by the addition of 1.5 mM $CaCl_2$. Inhibition of agglutination of *S. sanguis* 7864 occurred at 5 mM EDTA. The EDTA acted on the agglutinin rather than on the bacteria, as cells pretreated with EDTA (5 mM, 37°C, 1 h) and then washed with buffer agglutinated upon addition of fresh rS-A or saliva. Addition of $CaCl_2$ in the absence of EDTA inhibited the agglutination of the *S. mutans* strains; 2.5 mM $CaCl_2$ slightly reduced agglutination, but 100 mM $CaCl_2$ was required to completely inhibit the agglutination in this assay. Saliva or rS-A treated with hydroxyapatite no longer agglutinated *S. mutans* cells. The presence of a 100 mM concentration of phosphate, sulfate, or acetate had no effect on the agglutination or cell sedimentation of *S. mutans*.

LTA (1 mg/ml with or without added 1 mM $CaCl_2$) did not affect agglutination or cell sedimentation of *S. mutans* Ingbritt. Anti-LTA antiserum at low dilutions agglutinated cells and inhibited rS-A-mediated agglutination. However, nonimmune serum at the same dilutions also inhibited rS-A-mediated agglutination and cell sedimentation (Table 5).

The effects of various proteins and polypeptides of known chemical characteristics on agglutination and cell sedimentation of *S. mutans* Ingbritt are shown in Table 6. Anionic proteins and polypeptides had no effect on agglutination, and only the very hydrophobic anionic protein, β-casein, and the glycoprotein κ-casein inhibited cell sedimentation. Cationic proteins and polypeptides inhibited both processes. Cells pretreated with cationic proteins (37°C for 1 h, then washed with buffer) were unable to undergo agglutination or to sediment, indicating that the cationic proteins and polypeptides acted on the bacteria rather than on the agglutinin.

S. mutans cells preincubated with lectins (lentil, peanut, wheat germ, and soybean [20 μg/ml, 37°C, 30 min]) did not agglutinate upon addition of saliva or rS-A. Soybean and

TABLE 2. Protein, carbohydrate, and lipid compositions of rS-A components

Material	% Wt of component:									
	rS-A-1 peak:									
	Q1	Q2 fraction:						Q3	rS-A-2	rS-A-3
		a	b	c	d	e	f			
Protein	25.6	19.8	19.2	22.1	16.9	23.1	21.4	23.9	37.8	ND[a]
Carbohydrate	73.0	80.1	80.3	75.6	81.7	76.1	77.4	69.7	58.3	ND
Lipid	0	0	0	0	0	0	0	0	2.0	100

[a] ND, Not detectable.

TABLE 3. Chemical compositions of rS-A components

Constituent(s)	Composition[a] (mol/100 mol of amino acid residues) of component:								
	rS-A-1 peak:								rS-A-2[b]
	Q1	Q2 fraction:						Q3	
		a	b	c	d	e	f		
Amino acids									
Asx	6.3	5.5	5.8	5.8	5.9	6.2	6.5	7.3	10.0 (12)
Glx	7.0	5.5	7.0	6.1	6.4	6.9	6.7	7.7	9.8 (12)
Ser	17.0	23.3	23.1	24.1	22.1	22.1	20.7	17.7	12.5 (15)
Gly	8.3	7.9	9.2	9.1	7.9	8.6	8.4	8.4	9.1 (11)
His	1.4	ND	ND	ND	ND	ND	ND	ND	ND
Arg	2.7	1.4	1.1	1.5	1.9	2.0	2.2	2.8	2.3 (3)
Thr	13.7	19.0	18.0	18.2	17.5	16.8	15.9	14.7	10.3 (13)
Ala	8.7	11.2	10.6	11.2	10.4	10.5	9.9	8.3	6.7 (8)
Pro	7.3	7.2	7.2	6.9	6.9	7.5	7.2	7.4	9.6 (12)
Tyr	1.2	ND	ND	ND	ND	ND	ND	ND	1.7 (2)
Val	9.0	12.8	11.7	10.4	15.2	11.4	14.7	17.6	12.0 (15)
Met	0.9	ND	ND	ND	ND	ND	ND	ND	ND
Cys	2.9	ND	ND	ND	ND	ND	ND	ND	ND
Ile	2.3	1.9	1.7	1.9	2.0	2.0	2.1	1.6	2.5 (3)
Leu	4.4	2.6	2.6	2.8	2.9	3.4	3.3	3.8	8.5 (10)
Phe	0.9	ND	ND	ND	ND	ND	ND	ND	1.8 (2)
Lys	2.3	1.7	2.1	1.9	1.8	2.3	2.5	2.7	3.3 (4)
Hexoses	37.9	57.8	50.8	54.3	47.8	59.0	45.9	50.3	29.3 (36)
Sialate[c]	17.9	34.5	44.1	36.6	35.2	37.9	38.8	21.6	10.9 (13)
GlcNAc	11.6	14.4	13.4	13.6	12.6	12.4	11.3	12.3	5.6 (7)
GalNAc	57.3	74.7	73.6	50.2	116.4	72.9	82.3	56.1	28.9 (35)
Sulfate	5.3	0.6	2.8	7.2	8.1	7.5	7.6	29.0	3.9 (5)
Negative charge[d]	28.5	35.0	49.7	51.0	51.4	52.9	54.0	79.6	

[a] Means of at least three analyses; maximum coefficient of variation, 21%. Trp was not analyzed. ND, Not detected.
[b] Numbers in parentheses are residues per molecule, assuming a molecular weight of 44,000.
[c] Determined as NANA.
[d] Negative charge per 100 amino acid residues from sulfate and sialate at pH 8.0.

wheat germ lectins also inhibited cell sedimentation (Table 7). The effect was further investigated by assaying agglutination in the presence of the sugars which are bound specifically by the lectins. Other carbohydrates were also tested. GalNAc, GlcNAc, *N*-acetylmannosamine, fucose, galactose, mannose, lactose, and maltose at 10 and 100 mM did not affect agglutination or cell sedimentation. Cellobiose and *N*-acetylneuramin–lactose at 10 mM also failed to inhibit agglutination and cell sedimentation, as did 0.83 mM dextran T-10 and dextran T-70. NANA from some but not all batches of the sugar agglutinated *S. mutans* Ingbritt and *S. sanguis* 7864 at 10 and 100 mM NANA, respectively. This NANA-dependent agglutination was inhibited by 5 mM EDTA, and the agglutination of *S. sanguis* was also inhibited by 1 mM $CaCl_2$. The agglutination was not due to contamination by colominic acid [poly-(2-8)-*N*-acetylneuraminic acid], from which NANA was prepared, and the results remain inconclusive. However, neuraminidase-treated and ultrafiltered rS-A retained agglutination activity, suggesting no involvement of NANA.

TABLE 4. Neutral sugar composition of selected components of rS-A

Sugar	mol/100 mol of amino acid residues[a] of peak rS-A-1Q2 fraction:			
	a	c	e	f
Fucose	22.6	18.5	23.8	22.1
Mannose	1.9	2.5	3.5	4.8
Galactose	33.3	33.3	31.7	19.0

[a] Corrected for loss due to hydrolysis.

Dextranase treatment of cells did not affect their ability to sediment or to undergo subsequent agglutination. However, tryptic digestion of cells destroyed their ability to be agglutinated by rat saliva and rS-A.

Enzyme-linked immunosorbent assay with anti-rat immunoglobulin antisera indicated the presence of IgG, IgM, and IgA (presumably secretory IgA) in rat saliva. However, immunoglobulins could not be detected in the agglutinin complex by enzyme-linked immunosorbent assay. This was consistent with the fact that the anti-immunoglobulin antisera only inhibited agglutination at the same dilutions as nonimmune sera (Table 8).

DISCUSSION

Rat saliva agglutinated *S. mutans* with the same strain specificity as human saliva. Agglutination was associated with a mucin complex (rS-A) of sulfated sialoglycoproteins with a trace of associated lipid and an apparent M_r of 1.6×10^6. The lipid component is consistent with previous reports of the association of lipids with salivary mucins (33, 34). The mucin complex was dissociated with a high-ionic-strength buffer containing 6 M urea and was fractionated by FPLC into four sulfated sialoglycoprotein components, designated rS-A-1Q1, rS-A-1Q2, rS-A-1Q3, and rS-A-2, with rS-A-1Q2 being polydisperse through differential glycosylation of the polypeptide backbone. The dissociation caused a loss of agglutination activity which could not be regained on removal of dissociating agents, suggesting that rS-A was

TABLE 5. Agglutination of *S. mutans* Ingbritt in the presence of anti-LTA antiserum and NRS[a]

Dilution[b]	Cell sedimentation		Agglutination			
			rS-A		Human whole saliva	
	NRS	Anti-LTA	NRS	Anti-LTA	NRS	Anti-LTA
1/4	I	E	I	I	I	I
1/8	I	E	I	I	I	I
1/16	I	E	I	I	NA	NA
1/32	I	I	I	I	NA	NA
1/64	I	I	NA	NA	NA	NA
1/128	I	I	NA	NA	NA	NA
1/256	NA	NA	NA	NA	NA	NA
1/512	NA	NA	NA	NA	NA	NA

[a] Anti-LTA antiserum and NRS were adjusted to the same protein concentration with the assay buffer. E, Enhanced; I, inhibited; NA, not affected.
[b] A 0.1-ml amount of each dilution was added to the agglutination assay to yield a total volume of 0.6 ml.

irreversibly denatured. The irreversible denaturation of mucous sialoglycoproteins has been reported previously (26). It would appear, therefore, that the rS-A components associate to produce a specific conformation which interacts with the cell surface of *S. mutans*.

The rS-A, being a high-molecular weight sialoglycoprotein complex, is similar to the agglutinins isolated from human and monkey saliva that interact with *S. mutans* (9, 20) and *S. sanguis* (15, 20). However, rS-A differed slightly from its human and monkey salivary counterparts in protein and carbohydrate compositions; in particular, it contained a much higher proportion of GalNAc than of GlcNAc. Mucins from rat, porcine, ovine, and bovine salivary glands contain high proportions of GalNAc (26, 37), and the mucin 2B-2C, purified from the rat submandibular gland (RSMG) by Tabak et al. (37), is similar to rS-A in its protein and carbohydrate compositions. The RSMG mucin 2B-2C contained 39.8% serine plus threonine and 25.2% proline plus glycine plus alanine and no or trace amounts of tyrosine, phenylalanine, methionine, cysteine, or histidine, being similar to the amino acid composition of rS-A-1Q2. The carbohydrate moiety of RSMG mucin 2B-2C consisted of GalNAc, galactose, fucose, and sialate with trace amounts of GlcNAc and mannose, thus being similar in composition to the carbohydrate moieties of the agglutinin sialoglycoproteins. Furthermore, the molar ratio of GalNAc to serine plus threonine was 1.3 for RSMG mucin 2B-2C; this is the same as the ratio for rS-A-1Q2a. Tabak et al. (37) did not report on whether their RSMG mucin 2B-2C contained sulfate or agglutinated *S. mutans*.

TABLE 6. Effect of various proteins with known chemical characteristics on agglutination and cell sedimentation of *S. mutans* Ingbritt[a]

Protein[b]	Agglutination	Sedimentation
α_{s1}-Casein	NA	NA
β-Casein	NA	I
κ-Casein	NA	I
Phosvitin	NA	NA
Poly-L-lysine	I	I
α-Lactalbumin	NA	NA
β-Lactoglobulin	NA	NA
Histone VIII S (H3)	I	I
BSA	NA	NA
Histone III S (H1)	I	I
Poly-L-glutamate	NA	NA

[a] I, Inhibited; NA, not affected.
[b] Protein and polypeptide concentrations were 0.67 mg/ml. BSA, Bovine serum albumin.

TABLE 7. Effect of lectins on agglutination and cell sedimentation of *S. mutans* Ingbritt[a]

Lectin	Sugar specificity	Cell sedimentation
Lentil	D-Mannose, D-glucose	NA
Soybean	GalNAc	I
Wheat germ	Tri-GlcNAc	I
Peanut	Gal(β-1,3)GalNAc	NA

[a] Lectins were added to the agglutination assays to yield a final concentration of 20 μg/ml; agglutination was inhibited in all cases. I, Inhibited; NA, not affected.

The presence of the relatively low-molecular weight rS-A-2 in the agglutinin complex is consistent with reports of the association of relatively low-molecular weight proteins with mucins by Hill et al. (13) and Tabak et al. (37). These investigators removed contaminating protein by dansylation and reductive methylation, respectively, and noted that upon purification the mucins lost aromatic and sulfur-containing amino acids and histidine, as they were associated with the contamination. This is confirmed by the present work; the fraction rS-A-1Q1, shown by amino-terminal analysis to be contaminated with small amounts of protein, contained tyrosine, phenylalanine, cysteine, methionine, and histidine, whereas the other rS-A-1Q fractions did not.

The association of the agglutinin with lipid can be attributed to the extremely high hydrophobicity of the glycoprotein components. The proportion of nonpolar residues of the sialoglycoprotein fractions ranged from 42 to 50%. These results suggest the presence of distinct hydrophobic and glycosylated domains. The negative charge associated with the sulfated carbohydrates and sialyl residues, together with the hydrophobicity, would make the glycoprotein components distinctly amphipathic and would certainly explain the propensity to produce homotypic and heterotypic complexes. The amphipathic nature would allow stabilization of complexes via intra- and intermolecular hydrophobic interactions, as well as ionic interactions (possibly Ca^{2+} bridging between sulfates and carboxyls), Van der Waals interactions, and hydrogen bonding between sugars.

The agglutination of *S. mutans* by the rS-A occurred in the same stages as those described by Ericson et al. (8) for agglutination of the organism by human saliva, i.e., initial formation of core aggregates followed by extensive clump-

TABLE 8. Effect of anti-immunoglobulin antisera on *S. mutans* Ingbritt agglutination[a]

Antiserum	Dilution range	Agglutination
Anti-IgG	Undiluted–1/50	I
	1/75–1/200	NA
Anti-IgM	Undiluted	I
	1/10–1/200	NA
Anti-IgA	Undiluted–1/10	I
	1/20–1/50	NA

[a] Antisera protein concentrations: anti-IgG, 61 mg/ml; anti-IgM, 25 mg/ml; anti-IgA, 100 mg/ml. I, Inhibited; NA, not affected.

ing. The component on the cells which interacts with the salivary agglutinin could be protein in nature as agglutination was abolished by tryptic digestion of cells. The susceptibility to proteolysis of the bacterial binding site for the human salivary agglutinin has been demonstrated by Simonson and Reiher (32). Agglutination was not affected by ionic strength between 0.01 and 0.05, but at an ionic strength of 0.01 sedimentation of *S. mutans* was enhanced to the extent of agglutination. This suggests that, when the ionic strength is low and the electrical double layers surrounding the bacteria are small, cells can approach each other to allow short-range interactions resulting in agglutination. The ionic strength of saliva limits the approach required for these interactions.

The failure of nonagglutinating dextrans or dextranase treatment to abolish agglutination suggests that salivary agglutination is distinct from dextran-mediated agglutination (10).

Rat and human salivary agglutination of *S. mutans* were similarly calcium dependent. Agglutination by rS-A did not occur in the absence of calcium and was inhibited by relatively high levels of calcium. This is consistent with the involvement of Ca^{2+} in bridging between the agglutinin components and the agglutinin and bacteria (30). In the absence of Ca^{2+} there would be electrostatic repulsion between negative groups on bacteria and agglutinin components, and when Ca^{2+} was in excess all negative groups would have associated Ca^{2+} and no bridging would occur. Carboxyl, phosphate, and sulfate groups on the cells and agglutinin components most likely bind calcium. Agglutination was not disrupted by excess acetate, phosphate, or sulfate, but EDTA did inhibit the process. This suggests that the agglutinin components bound the calcium with higher affinity and specificity than did the individual negative groups and possibly that polydentate binding between agglutinin components and bacteria, including a specific combination of interactions, was involved. The agglutination of only some *S. mutans* strains also indicated that the interaction between the agglutinin and the bacterial cell surface was specific. LTA on the surface of bacteria binds calcium and could also participate in hydrophobic interactions with the agglutinin, possibly via hydrophobic domains or the lipid component. LTA is therefore a candidate for one of the molecules on the surface of the bacterial cell which could bind the salivary agglutinin, and it has been suggested that LTA is involved in the interaction of a human salivary agglutinin with *S. sanguis* (15). In our work, agglutination was not affected by the addition of LTA, suggesting that LTA alone was not responsible for the interactions with the agglutinin. However, an anti-LTA antiserum, which agglutinated cells, inhibited rS-A-mediated agglutination. One possible interpretation of these results is that the two agglutinins, LTA-specific antibodies and rS-A, were antagonistic by both binding to or near LTA, thus preventing intercell bridging through steric hindrance. However, as nonimmune sera also inhibited saliva-mediated agglutination at the same dilutions as the anti-LTA antiserum, these results give little information on the interaction of the bacterial cell surface with the rS-A. The presence of agglutination-inhibitory activity in serum has been previously reported (7) and is possibly associated with nonspecific binding of immunoglobulins or other serum proteins (7, 23).

The effect of lectins and proteins of known primary sequence and chemical characteristics on agglutination and sedimentation can give some indication of the chemical nature of the interactions between the agglutinin and bacteria. The various anionic proteins had no effect on agglutination. There would be electrostatic repulsion between the electrical double layers of the negatively charged surface of the bacteria and anionic proteins which would hinder their approach, leaving the bacteria free to interact with salivary agglutinin. The anionic protein κ-casein contains the trisaccharide NANA(α-2,3)Gal(β-1,3)GalNAc(β-1)Thr (35), and its ability to inhibit sedimentation was most likely the result of interaction between the carbohydrate moiety and components on the bacterial cell surface, increasing the net negative charge on the bacterial surface and therefore increasing the electrostatic repulsion between bacteria. The very hydrophobic anionic protein β-casein also inhibited sedimentation of bacteria, and this inhibition was attributed to an increase in the bacterial surface negative charge resulting from the binding of the protein to the cell surface via hydrophobic interactions. The fact that neither κ-casein nor β-casein inhibited agglutination shows that an increase in net negative surface charge of the bacterial cell did not hinder the approach of the agglutinin in the presence of calcium. The results also suggest that the nonspecific binding of the caseins could not compete with the specific binding of the agglutinin. Various cationic proteins and polymers inhibited both agglutination and sedimentation. The inhibition of both processes has been attributed to the ability of these proteins to bind to the cell surface and thereby to sterically hinder the specific short-range interactions involved in both agglutination and cell sedimentation. The same mechanism may be involved in the nonspecific inhibition of both processes by serum.

The lectins from wheat germ, lentil, soybean, and peanut inhibited agglutination, but the sugars to which the lectins specifically bind had no effect on agglutination. Soybean and wheat germ lectins inhibited cell sedimentation, as well as agglutination, suggesting that these lectins bound to bacterial surface components. The inhibition was considered to be the result of steric hindrance rather than direct competition for binding sites on bacteria or agglutinin. The work of Mirth et al. (24) and Nonaka et al. (25) with a fucose-binding protein, concanavalin A, and the human salivary agglutinin also suggests that the inhibition of agglutination by these lectins was not a result of direct competition for binding sites on the agglutinin or bacteria.

Sialyl residues did not appear to be directly involved in agglutination as neuraminidase treatment of the rS-A did not affect its ability to agglutinate *S. mutans*. There is evidence of direct participation of sialyl residues in the interaction of a human salivary agglutinin with *S. sanguis* (36), but no evidence for their participation in that with *S. mutans* (20).

Results of this work are consistent with the proposal (30) that calcium binds to negative groups on bacteria and the agglutinin components (presumably sulfate). This situation would stabilize the agglutinin complex and allow the approach of the agglutinin to the bacterial cell surface, bringing together complementary sites involving ionic (calcium-bridging), hydrophobic, Van der Waals, and hydrogen-bond interactions with conformational specificity. Neither the individual carbohydrates of the agglutinin nor sulfate inhibited agglutination, suggesting that several of these groups and/or amino acid residues in a specific conformation are involved in binding of the agglutinin to bacteria. The large size, similarity in composition of components, amphipathic nature, and propensity to associate suggest a multivalency of the agglutinin with respect to bacterial binding. The multivalency would allow cell-cell bridging, resulting in agglutination. The interactions between the agglutinin components were specific such that upon disruption of the complex,

agglutination activity was lost and could not be regained upon recombination of components.

Adsorption of the agglutinin out of rat saliva onto hydroxyapatite is consistent with its calcium dependence. Human salivary agglutinins also adsorb onto hydroxyapatite (22), and this adsorption has led to the speculation that they bind to enamel surfaces in vivo, thereby forming part of the pellicle. If bound agglutinins were able to interact with bacterial surfaces, they could enhance adherence of the bacteria to the tooth surface. Alternatively, the agglutination of bacteria by agglutinin free in saliva could enhance the clearance of the bacteria from the oral cavity. The similarity between the rS-A and its human salivary counterpart suggests that a rat model could be appropriate for the study of the potential role of salivary agglutination in colonization of enamel surfaces by *S. mutans*.

ACKNOWLEDGMENTS

This work was supported by the Victorian Dairy Industry Authority. C.M.B. was a recipient of a Commonwealth Postgraduate Research Award.

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