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Isolation and Characterization of a 60-Kilodalton Salivary Glycoprotein with Agglutinating Activity against Strains of *Streptococcus mutans*

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A bacterial agglutinin specific for strains of *Streptococcus mutans* was isolated from human saliva. Physiochemical analyses showed the agglutinin to be a glycoprotein with a molecular weight of 60,000. The agglutinin aggregated four of the eight strains of *Streptococcus mutans* tested but did not aggregate the strains of *Streptococcus salivarius*, *Streptococcus sanguis*, and *Streptococcus mitis* tested. Chemical modification of carbohydrate moieties of the agglutinin with sodium metaperiodate had no effect on aggregation, whereas modification of the polypeptide portion with trypsin abolished aggregating activity. A set of five murine hybridoma antibodies was employed to further analyze the agglutinin. Two carbohydrate-specific antibodies, directed against D-mannose and *N*-acetylgalactosamine moieties, respectively, failed to block agglutinin- or whole saliva-mediated aggregation of *S. mutans* cells. In contrast, two antibodies directed against pronase-sensitive antigenic sites blocked both agglutinin- and saliva-mediated aggregation of *S. mutans* cells. Western blot analysis with the agglutinin-specific hybridoma antibodies demonstrated the agglutinin in whole saliva and in artificial tooth pellicles formed on hydroxyapatite beads incubated with saliva. These results suggest that a 60-kilodalton glycoprotein of human saliva is a bacterial agglutinin with specificity for certain strains of *S. mutans*. They further suggest that aggregation is mediated by polypeptide rather than carbohydrate determinants of the glycoprotein.

The relationship between the colonization of tooth surfaces by a variety of bacteria and the pathogenesis of dental caries has been studied extensively (10–12, 31). The ecology of the oral cavity and dental tissues is modulated by a number of local factors. One of these is the presence of bacterial agglutinins in salivary secretions (8, 12, 13, 16, 17, 19). Human saliva has been shown to contain a number of glycoproteins (1, 23, 29), some of which are rich in dicarboxylic amino acids, proline, and glycine (1, 3, 24, 29) and shown to contain bacterial agglutinating properties (7, 8, 16, 17, 19). Some salivary agglutinins are high-molecular-weight mucinous glycoproteins (16, 28, 34), some of which are blood group reactive glycoproteins (32), lysozyme (30), or immunoglobulins. These agglutinins may prevent bacterial colonization by promoting clearance of the clumped organisms (11, 15, 19, 34), or, alternatively, the same agglutinins attached to mucosal cell or tooth surfaces may promote colonization (8, 12).

Streptococcus mutans has been implicated as one of the major cariogenic organisms present in dental plaque (11, 21, 25). The present study concerns the isolation and characterization of a low-molecular-weight (60,000) salivary glycoprotein that specifically agglutinates certain strains of *S. mutans*. We present data to show that the agglutinin is adsorbed from whole saliva onto hydroxyapatite to form a component of artificial tooth pellicles. The agglutinin was detected in whole saliva as well as in saliva collected from parotid and submandibular glands. Two of five murine monoclonal antibodies raised against the purified agglutinin blocked agglutinin-mediated and whole saliva-mediated aggregation of *S. mutans* 6715. In studies utilizing monoclonal antibody and sodium metaperiodate ($NaIO_4$)-treated agglutinin or trypsin-treated agglutinin, we obtained evidence to suggest that the aggregation of *S. mutans* is mediated by polypeptide rather than oligosaccharide determinants of the agglutinin molecule. Our studies suggest that this salivary glycoprotein may play a role in the streptococcal ecology of the oral cavity and dental tissues.

MATERIALS AND METHODS

Microorganisms. The *S. mutans* strains used were the same as those previously described (2). In addition, strains GS-5, B14, OMZ26, BHT, and B421 were kindly furnished by J. Olson (University of Tennessee Center for the Health Sciences, Memphis). The other species of oral streptococci, *Streptococcus sanguis* Challis, *Streptococcus salivarius* 13419, and *Streptococcus mitis* 9811, were kindly furnished by R. Doyle (University of Kentucky, Louisville) and B. Rosan (University of Pennsylvania, Philadelphia). Stock cultures prepared by growing the microorganism in Todd-Hewitt broth (Difco Laboratories, Inc., Detroit, Mich.) for 18 h (stationary phase) at 37°C were aliquoted and frozen at −70°C. For each experiment, a sample was thawed and grown in fresh broth for 18 h at 37°C. The cells were harvested by centrifugation and washed three times with 0.02 M phosphate–0.15 M NaCl (pH 7.4) (PBS). A standard bacterial suspension was prepared by diluting the cell pellet in PBS to an absorbance of 0.35 at 560 nm (10^9 cells per ml) in a Coleman Junior spectrophotometer. For some experiments, microorganisms were grown in Todd-Hewitt broth at 37°C containing 0.5 μCi of [^{3}H]thymidine (Research Products International Corp., Mt. Prospect, Ill.) per ml for 18 h, at which time the

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bacteria incorporated approximately 35% of the radiolabel. The radiolabeled cells were stored at −70°C and thawed before use in aggregation experiments.

Agglutination assay. The assays for bacterial aggregation were performed as described by Golub et al. (13). Briefly, 0.1 ml of the standard suspension of [^{3}H]thymidine-labeled bacteria was incubated with 0.1 ml of test material at 37°C for 10 min. The reaction was terminated by the addition of 0.2 ml of 2.5% glutaraldehyde in PBS, and aggregates were separated from free bacteria by centrifugation (1,100 × *g*) for 5 min over a 1.0-ml cushion of 25% (wt/vol) sucrose. Pellets were suspended in Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) and assayed for radioactivity in a scintillation counter (Tri-Carb liquid scintillation spectrometer; Packard Instrument Co., Inc., Rockville, Md.). The results are expressed as percent aggregation, calculated as: Percent bacterial agglutination = [(counts per minute experimental − counts per minute control)/total counts per minute of bacteria added] × 100. The experimental values in all cases were 10- to 15-fold higher than the blank control values (500 to 1,200 cpm).

Isolation and purification of the agglutinin. Paraffin-stimulated whole saliva from several donors were collected in ice-chilled cups and pooled. Donors of both sexes were used; the donors ranged in age from 26 to 36 and were without unusual caries activity. The saliva was clarified by centrifugation at 12,000 × *g* for 20 min, filtered through an Amicon XM-100 filter disk (Amicon Corp., Lexington, Mass.), and lyophilized. The lyophilized material was dissolved in PBS (10 mg/ml) and further purified by molecular sieve chromatography over a Sephadex G100 column (40 by 1.75 cm). The column eluates were monitored at 230 nm with a Beckman spectrophotometer. The materials in the fractions of each peak were pooled, dialyzed overnight against distilled water, lyophilized, and tested for bacterial agglutinating activity. The lyophilized material obtained from the second peak was dissolved in deionized water (5 mg/ml) and further purified by ion-exchange chromatography over a DEAE-Biogel column (1.5 by 22 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with deionized water. The first peak material eluted from this column was reconstituted (2 mg in 0.3 ml of water) and further purified by reverse-phase high-pressure liquid chromatography (HPLC) on an ultrasphere ODS2 column (Whatman, 9.4 mm by 25 cm) with a 10 to 90% acetonitrile gradient in water supplemented with 0.01 M trifluoroacetic acid for elution. Two distinct peaks were eluted and tested for bacterial aggregating activity. The active second-peak fractions were pooled and lyophilized and hereafter are designated as the agglutinin. Purity and relative molecular mass (M_r) were assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel (7.5%) electrophoresis (22). For certain studies, parotid saliva was collected from two laboratory individuals by placing capillary pipettes over the papilla of the Stenson duct on the side of the subject's mouth. Pep-O-Mint Life Savers (Life Savers Inc., New York, N.Y.) were administered as a salivary stimulus. The contents of the pipette were emptied immediately into tubes placed on ice.

Agglutinin binding assays. The purified agglutinin was radiolabeled with ^{125}I (specific activity, 17 Ci/mg; New England Nuclear Corp., Boston, Mass.) by the lactoperoxidase method (27). The specific activity of radiolabeled agglutinin was 20,000 cpm/μg. A 100-μl volume of standard bacterial suspension (10^9 cells per ml) prepared from an 18-h culture of organisms was incubated with 20 μg of ^{125}I-labeled agglutinin for 30 min at 37°C. The cell pellet was washed three times with PBS and assayed for radioactivity in a gamma scintillation counter (Packard Instruments). The specificity of the binding of ^{125}I-agglutinin to the bacterial cells was determined by pretreating the microorganisms with 10 to 40 M excess of unlabeled agglutinin for 30 min at 37°C before incubating them with ^{125}I-agglutinin. In control experiments, bovine serum albumin (BSA) was substituted for the unlabeled agglutinin.

$NaIO_4$ oxidation. The purified agglutinin (2 mg) was dissolved in 1.0 ml of 0.05 M sodium acetate buffer (pH 4.5). An equal volume (0.5 ml) of 0.2 M $NaIO_4$ (Sigma Chemical Co., St. Louis, Mo.) dissolved in the same buffer was mixed with the agglutinin solution and incubated for 24 h at ambient temperature in the dark. Excess $NaIO_4$ was neutralized with 4 M sodium borohydride in acetate buffer. In control experiments, the agglutinin was treated as described above except that $NaIO_4$ was deleted from the reaction mixture.

Enzymatic treatments. A 200-μl sample of a 2-mg/ml solution of agglutinin in PBS was mixed with an equal volume of PBS containing 2 mg of pronase (Calbiochem) per ml and incubated at 37°C for 4 h. The reaction was terminated by incubating the mixture at 60°C for 15 min. In control experiments, the agglutinin sample was incubated with heat-inactivated (60°C for 15 min) pronase.

The effect of trypsin on the activity of the agglutinin against streptococci was tested by treating 2 mg of the agglutinin per ml with 1 mg of trypsin (bovine pancreas type 1; Sigma) per ml for 60 min at 37°C in 0.5 M Tris (pH 8.2). Samples (100 μl) were removed from the reaction mixture at 10-min intervals, heat inactivated (60°C for 60 min), and then tested for the agglutination of *S. mutans* 6715 as described earlier. A sample of agglutinin similarly treated with heat-inactivated (60°C for 60 min) trypsin served as a control.

Chemical analyses. The agglutinin was hydrolyzed under N_2 in 6.0 M hydrochloric acid for 24 h at 110°C and analyzed with a Beckman 121 MB automatic amino acid analyzer using a two-column technique (18). The protein content of the agglutinin also was determined by the method of Lowry et al. (26). Fucose was determined by the method of Gibbons (9), and sialic acid was determined according to Warren (36) after hydrolysis of the sample in 0.1 N sulfuric acid at 80°C for 60 min using *N*-acetylneuraminic acid as a standard. Hexoses were determined quantitatively by gas-liquid chromatography after trimethylsilylation of the deionized hydrolysate with addition of α-*methyl*-D-mannoside as an internal standard as described previously (33). Glucosamine and galactosamine were determined after hydrolysis in 1 N HCl at 100°C. After removal of HCl, the amino sugars were absorbed on and eluted from Dowex 50-X4 H^+ (200- to 400-mesh) columns with 2 N HCl and then quantitated on a Beckman automatic analyzer.

Production of monoclonal antibodies. Murine hybridoma antibodies against the purified agglutinin were prepared by methods previously described in detail (14). Five different hybridoma clones were obtained, and ascites fluids containing high concentrations of antibodies were produced in mice primed with Pristane (Aldrich Chemical Co., Milwaukee, Wis.). The immunoglobulin G (IgG) isotype of each monoclonal antibody was determined by double immunodiffusion in agarose gel against mouse IgG subclass-specific antisera (Miles Laboratories, Elkart, Ind.).

ELISA for antibody reactivity. Antibody activities in culture medium supernatants or ascites fluids were measured by an enzyme-linked immunosorbent assay (ELISA) as previously described (14). Purified agglutinin (5 μg/ml) in carbonate buffer (0.1 M, pH 9.6) absorbed to polystyrene assay

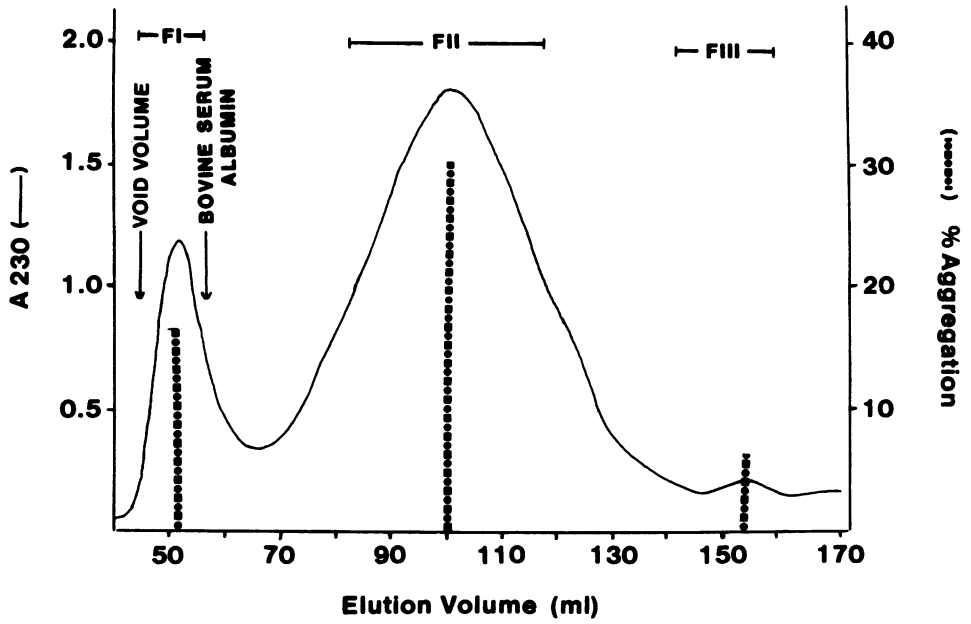

FIG. 1. Molecular sieve chromatography of saliva ultrafiltrate (XM-100) on Sephadex G-100. The samples eluted with PBS were pooled into three fractions (FI, FII, and FIII) and tested for bacterial aggregation. The vertical bars represents the percent aggregation of *S. mutans* 6715 induced by 100 μg of each of the three fractions.

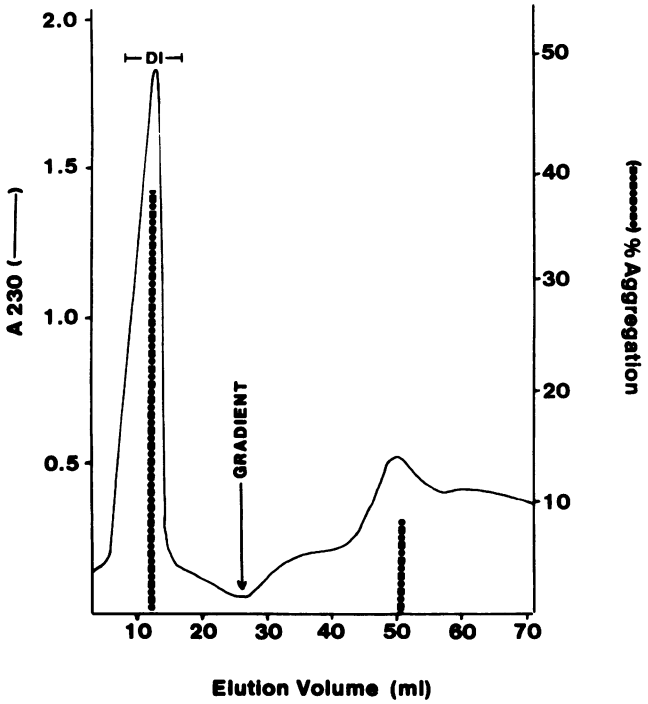

FIG. 2. DEAE-Biogel column chromatography of FII obtained from Sephadex G-100 gel filtration (see Fig. 1). The sample was applied to a column (1.5 by 22 cm) of DEAE-Biogel equilibrated with deionized water. The first peak was eluted with deionized water, and the remaining material was eluted with a linear gradient of 0 to 0.6 M NaCl. The major agglutinin activity was found in the first peak and was pooled as indicated by the bar into fraction DI. The vertical bars represent the percent aggregation of *S. mutans* 6715 by individual fractions.

plates (EIA plates; Costar, Cambridge, Mass.) served as the solid-phase antigen.

ELISA inhibition experiments were performed by preincubating serial twofold dilutions of the inhibitor in 0.1 ml of PBS with an equal volume of diluted ascites fluid for 60 min at 37°C. The incubation mixtures were then added to antigen-coated trays, and the standard ELISA procedures were followed.

Immunoblot analyses. Purified agglutinin, clarified whole saliva, parotid saliva, and an SDS extract of saliva-coated hydroxyapatite beads (artificial tooth pellicle) prepared by the method of Clark and Gibbons (5) were electrophoresed on a 7.5% SDS gel (22). After electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose paper by the method of Towbin et al. (35). After transfer, the nitrocellulose paper was soaked in buffer (0.05 M Tris, 0.15 M NaCl, pH 7.3) containing 3% BSA (Tris-BSA) for 15 min. The strips of nitrocellulose were then incubated for 3 h at 37°C with diluted (1:800 in Tris-BSA) ascites fluids containing agglutinin-specific hybridoma antibodies. The strips were then washed extensively with 0.9% NaCl–0.05% Tween 20 and then incubated for 2 h at 37°C with peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories, Downington, Pa.) diluted 1:1,500 in Tris-BSA buffer. The strips were washed again, and the reactive bands were visualized by treating the nitrocellulose strips with HRP color development reagent (Bio-Rad Laboratories).

Assays of the agglutination inhibitory activities of the monoclonal antiagglutinin antibodies. Samples (100 μl) of clarified, pooled whole saliva or purified agglutinin in PBS (2 mg/ml) were incubated at 37°C for 60 min with an equal volume of a 1:500 dilution of ascites fluids. The mixtures were then tested for the aggregation of *S. mutans* 6715 as described above.

Estimation of agglutinin content of saliva samples. An ELISA inhibition experiment was performed to estimate the concentration of the agglutinin in samples of whole, clarified saliva collected from 10 different individuals as described

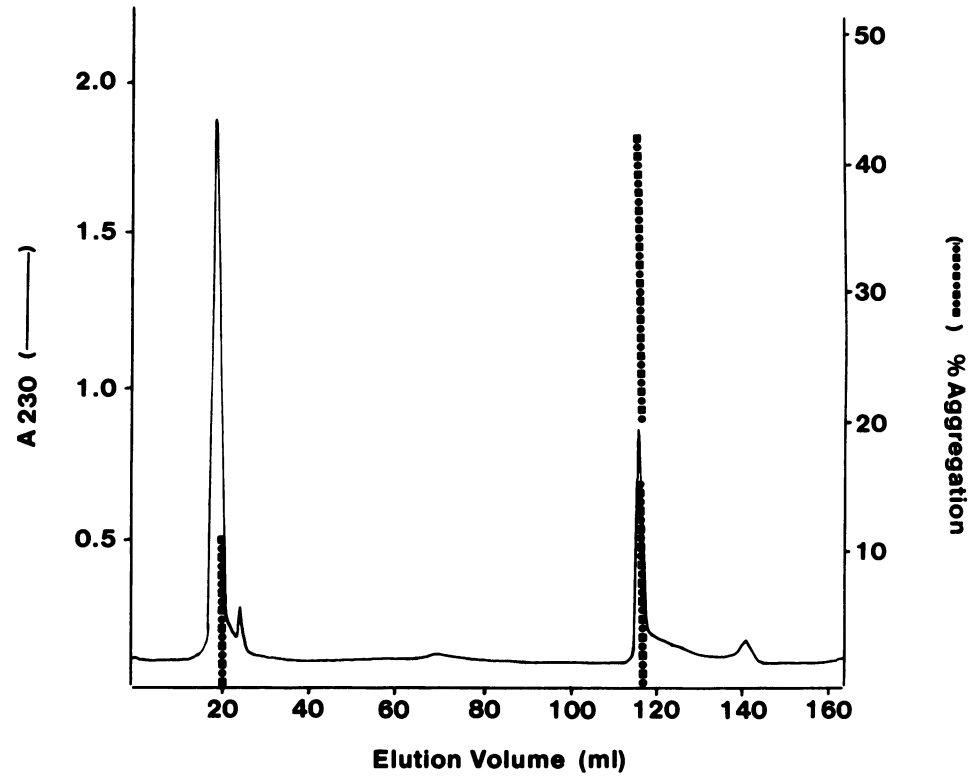

FIG. 3. Reverse-phase HPLC purification of fraction DI eluted from DEAE-Biogel (see Fig. 2) on a column of ultrasphere ODS2 beads, using a gradient of 10 to 90% acetonitrile. The second major peak, containing most of the agglutinating activity as represented by the vertical bar, was lyophilized and is designated the purified agglutinin.

earlier. Assay plates were coated with purified agglutinin as described above, and monoclonal antibody IB9 diluted 1:1,000 was preincubated for 60 min at 37°C with serial twofold dilutions of saliva before adding to the assay plates. The concentration of agglutinin present was then estimated from a standard curve obtained with the purified agglutinin in an identical ELISA inhibition test.

RESULTS

Isolation and purification of the agglutinin. Pooled whole saliva was fractionated by filtration through an XM-100 Amicon filter. The filtrate and the retentate were collected, lyophilized, redissolved to a concentration of 1 mg/ml in PBS, and tested for agglutinating activity. The *S. mutans* 6715 agglutinating activity of the rententate (molecular weight, ≥100,000) was 12%, and that of the filtrate (molecular weight, ≤100,000) was 28%.

The filtrate was further fractionated by gel filtration into three UV light-absorbing peaks (Fig. 1). Individual peak materials were pooled, dialyzed, lyophilized, and tested for their ability to agglutinate *S. mutans* 6715. The most active peak (FII) was further fractionated by ion-exchange chromatography (Fig. 2). The first-peak (DI) material contained most of the bacterial agglutinating activity. The active material was further separated by reverse-phase HPLC (Fig. 3). The second peak eluted from the HPLC column contained most of the bacterial agglutinating activity. SDS-polyacrylamide gel electrophoresis of the active fractions from each of the purification steps is shown in Fig. 4. The agglutinin obtained after the final step of purification by HPLC migrated as a single Coomassie blue-staining band with an apparent M_r of 60,000. Treatment of an identical gel with periodic acid-Schiff reagent (8) resulted in staining only of material coinciding with the Coomassie blue-staining band, suggesting that the purified agglutinin was a glycoprotein. Purification of 10 mg of the ≤100,000-molecular-weight saliva filtrate yielded 1.2 mg of the final product (agglutinin).

Chemical characterization of the agglutinin. Quantitative amino acid analysis showed that the agglutinin was rich in glycine (25%) and the content of proline was low (6%) (Table 1). The carbohydrate-conjugable amino acids (Ser, Thr, Asp) accounted for 24% of the total amino acid residues in the molecule. Further chemical analysis indicated that the agglutinin is composed of neutral hexoses (28.7%), hexosamines (30.1%), protein (32%), fucose (6.3%), and sialic acid (2.4%) (Table 2). Taken together, these results indicate that the agglutinin is a glycoprotein.

Interaction of the agglutinin with oral streptococci. The interaction of the agglutinin with various species and strains of streptococci was examined by agglutination and direct binding assays. The agglutinating activity was variable against different strains of *S. mutans*, with strains 6715, GS-5, AHT, and OMZ26 showing the greatest agglutination (Table 3). These four strains also bound the greatest amounts of radiolabeled agglutinin (Table 3). Pretreatment of the streptococcal cells with a 40 M excess of unlabeled agglutinin resulted in 87% inhibition of the binding of ^{125}I-agglutinin, whereas a 40 M excess of BSA had no effect. Agglutination of *S. mutans* 6715 was dose dependent (Fig. 5). Maximum agglutination was reached at 150 μg of agglutinin per ml. These results suggest that the agglutination of streptococci is brought about by the binding of the agglutinin to the surfaces of the bacterial cells.

Effect of $NaIO_4$ or trypsin treatment. Because the aggluti-

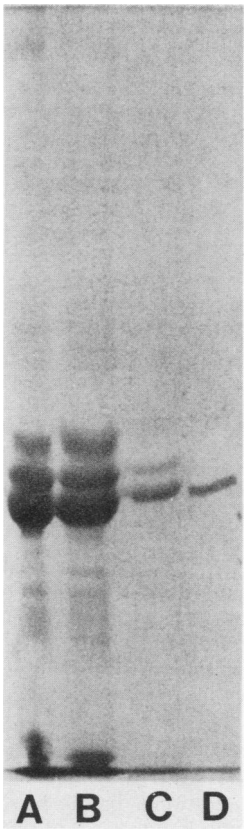

FIG. 4. SDS-gel electrophoresis at various stages of purification of the streptococcal agglutinin from saliva. Lane A, Ultrafiltrate (XM-100) of expectorated saliva; lane B, FII eluted from Sephadex G-100; lane C, DI eluted from DEAE-Biogel; lane D, second-peak material eluted from the HPLC column, constituting the purified agglutinin.

nin was shown to be a glycoprotein, it was of interest to determine whether the carbohydrate or the polypeptide portions mediated the interaction of the molecule with cells of streptococci. To test these alternatives, we treated samples of the agglutinin with $NaIO_4$ to destroy the carbohydrate moieties and treated other samples with trypsin to hydrolyze the polypeptide portion of the molecule. $NaIO_4$ treatment had no effect on the aggregating activity of the agglutinin, whereas trypsin digestion abolished aggregating activity (Table 4). That the $NaIO_4$ modified carbohydrate moieties of the agglutinin was confirmed by its loss of reactivity with carbohydrate-specific monoclonal antibodies (see below). Although these studies are not conclusive, the results suggest that the active site for streptococcal agglutination resides in the polypeptide rather than the oligosaccharide portion of the salivary glycoprotein.

TABLE 1. Amino acid composition of purified streptococcal agglutinin

Amino acid	mol/100 mol[a]
Aspartic acid	8
Threonine	5
Serine	11
Glutamic acid	9
Proline	6
Glycine	24
Alanine	10
Valine	5
Methionine	2
Isoleucine	4
Leucine	4
Tyrosine	1
Phenylalanine	2
Lysine	6
Histidine[b]	—
Arginine	3

[a] Expressed as assumed integral values.
[b] Amino acid not detectable or not significant.

TABLE 2. Chemical composition of purified streptococcal agglutinin

Component	% Composition[a]
Protein	32.0
Galactose	8.4
Glucose	3.7
Mannose	16.6
N-Acetylglucosamine	20.7
N-Acetylgalactosamine	9.4
Fucose	6.3
Sialic acid	2.4

[a] The protein and carbohydrate composition of the agglutinin is reported as a percentage (wt/wt) of the total weight of the components in the streptococcal agglutinin.

Production and characterization of antiagglutinin monoclonal antibodies. To gain further insight into the active regions of the agglutinin molecule, we prepared monoclonal antibodies against the molecule in mice. Spleen cells from BALB/c mice hyperimmunized with purified agglutinin were fused with SP2 myeloma cells. Five different hybridoma clones were selected for the following studies. Two of the hybridoma antibodies (AO7 and FA12) were of the IgG3 isotype, two (IB9 and D6) were of the IgG2 isotype, and the remaining one (C7) was of the IgG2a isotype. None of these antibodies precipitated the purified agglutinin, as determined

TABLE 3. Interaction between purified agglutinin and oral streptococci

Organism and strain	Serotype	% Aggregation[a]	^{125}I-agglutinin bound[b] (ng)
S. mutans			
6715[c]	g	41	108
GS-5	c	37	89
AHT[c]	a	34	97
OMZ26		33	62
BHT[d]	b	8	10
B14		5	11
B421		11	13
OMZ176	d	10	8
S. salivarius		2	16
S. sanguis		8	15
S. mitis 9011		3	17

[a] A 0.1-ml sample of [^{3}H]thymidine-labeled bacteria (10^9 cells per ml) was incubated with 0.1 ml of a 2-mg/ml solution of purified agglutinin, and the degree of aggregation was determined as a percentage of the radioactivity associated with the aggregates, as described in the text.
[b] A 0.1-ml sample of bacterial suspension (10^9 cells per ml) was incubated with 20 μg of ^{125}I-agglutinin for 30 min at 37°C as described in the text. All values are corrected for nonspecific binding by subtracting the values obtained in control samples incubated with 10 M excess of unlabeled agglutinin.
[c] Synonymous with *S. sobrinus*.
[d] Synonymous with *S. rattus*.

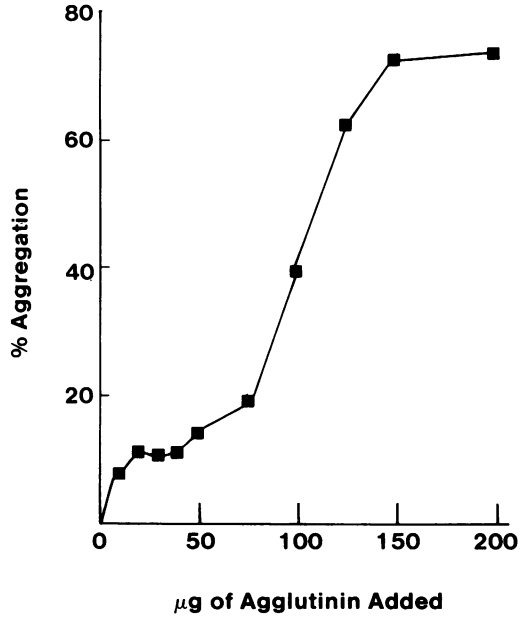

FIG. 5. Dose-dependent aggregation of *S. mutans* 6715 by the purified agglutinin. Various amounts of the agglutinin (10 to 200 μg) were mixed with 0.1 ml of [^{3}H]thymidine-labeled bacteria (10^8 cells) and tested for aggregation as described in the text.

by double immunodiffusion in agar gel. ELISA titers of the antibodies against purified agglutinin varied from 10,240 to 40,960 (see Table 6), and the ELISA reactivities of all the monoclonal antibodies were inhibited by purified agglutinin.

By using monoclonal antibody IB9 in an ELISA inhibition assay, we found that the concentration of the streptococcal agglutinin in saliva samples collected from 10 different donors ranged from 16 to 120 μg/ml (Table 5). Western blot analysis of saliva samples electrophoresed in SDS gel demonstrated reactivity with a single band in expectorated saliva with a mobility identical to that of the isolated agglutinin (Fig. 6, lanes A and B). Similar analysis showed that the agglutinin is a component of the salivary material adsorbed to hydroxyapatite beads (2) (Fig. 6, lane C). Western blot analysis of parotid saliva demonstrated the same agglutinin band plus three additional bands migrating at lower molecular weight ranges (Fig. 6, lane D). However, when the parotid saliva was treated with trypsin before the Western blot analysis, only the 60-kilodalton band was visible; the remaining three bands were susceptible to trypsin. Recent work by Kauffman and Keller (20) indicates that the relative proportions of nonglycosylated and glycosylated amylase in parotid secretions did not vary with stimulation. Our finding that the cross-reactive parotid proteins are more susceptible to trypsin digestion may suggest that these proteins were degraded before we collected the whole saliva. Alternatively, these cross-reactive proteins may be removed by absorption as the parotid secretions pass over oral surfaces or come into contact with the microbial flora of the oral cavity. The three additional bands have not been further identified. None of the bands reacted with antisera directed against whole human plasma or with antisera against components of serum (data not shown), suggesting that the immunoreactive salivary material is not a component of blood.

Because of the high content of carbohydrates in the agglutinin, we performed experiments to determine whether any of the monoclonal antibodies were directed against the oligosaccharide moiety. In the first experiment, the monoclonal antibodies were first incubated with agglutinin samples that had been treated with $NaIO_4$ to chemically modify

TABLE 4. Effect of $NaIO_4$ and trypsin treatment on the ability of the agglutinin to aggregate *S. mutans* 6715

Agglutinin treated with:	% Aggregation
None	35
$NaIO_4$[a]	34
Trypsin[b]	4
Trypsin, heat-inactivated (control)	31

[a] Two milligrams of agglutinin was digested for 24 h at ambient temperature with 0.2 M $NaIO_4$ in a total volume of 2 ml in 0.05 M acetate buffer at pH 4.5.

[b] Two milligrams of agglutinin was digested for 1 h at 37°C with 1 mg of trypsin in a total volume of 2 ml of 0.5 M Tris buffer at pH 8.2. A control sample was treated in the same way with heat-inactivated (60°C for 1 h) trypsin. Both the $NaIO_4$-treated and the trypsin-treated samples were dialyzed against PBS before being used in the aggregation assay.

TABLE 5. Quantitation of streptococcal agglutinin in samples of saliva from different donors by the ELISA inhibition assay

Donor[a] (sex, age)	Agglutinin concn[b] (μg/ml)
1 (M, 34)	60
2 (M, 31)	45
3 (F, 26)	120
4 (M, 28)	56
5 (F, 29)	60
6 (M, 33)	54
7 (F, 32)	50
8 (M, 36)	118
9 (M, 36)	16
10 (M, 34)	29

[a] All donors were healthy laboratory personnel with no unusual caries activity.

[b] Hybridoma ascites fluid IB9, diluted 1:1,000 in PBS, was preincubated for 60 min at 37°C with serial twofold dilutions of saliva before being added to ELISA plates coated with purified agglutinin. The concentration was then estimated from a standard curve as described in the text.

TABLE 6. Immunological characteristics of monoclonal antibodies prepared against the purified agglutinin

Hybridoma antibody	IgG class	ELISA titers against purified agglutinin	Inhibition of ELISA[a] by:			
			Untreated agglutinin	$NaIO_4$-treated agglutinin	Pronase-treated agglutinin	Sugars[b]
IB9	1	20,480	+	+	−	−
D6	1	10,240	+	+	−	−
C7	2a	40,960	+	+	−	−
FA12	3	10,240	+	−	+	+[c]
AO7	3	10,240	+	−	+	+[d]

[a] For inhibition of ELISA, each of the ascites fluids containing the respective monoclonal antibodies was first diluted to give an A_{405} of 0.4 to 0.8 against the purified agglutinin as the solid-phase antigen. The diluted fluid was then preincubated with a 1-mg/ml solution of untreated, $NaIO_4$-treated, or pronase-treated agglutinin or various sugars (see below) for 60 min at 37°C before being added to the ELISA cuvettes coated with the purified agglutinin.

[b] The sugars tested included D-mannose, α-*methyl*-D-mannoside, D-glucose, α-*methyl*-D-glucoside, *N*-acetylglucosamine, *N*-acetylgalactosamine, arabinose, fucose, D-galactose, and sialic acid.

[c] Inhibited only by *N*-acetylgalactosamine.

[d] Inhibited only by α-*methyl*-D-mannoside and D-mannose.

the sugar moieties and then tested for their ability to react with the intact agglutinin. The $NaIO_4$-treated agglutinin inhibited the reactivity of monoclonal antibodies IB9, D6, and C7 but not that of AO7 or F12 with native agglutinin (Table 6). In the second experiment, the monoclonal antibodies were preincubated with pronase-digested agglutinin. In contrast to results obtained with the $NaIO_4$-treated agglutinin, the pronase-treated agglutinin inhibited the reactivities of FA12 and AO7 but not those of IB9, D6, or C7. In the third experiment, assays were performed to determine whether the reactivities of FA12 or AO7 could be inhibited by specific sugars. The reactivity of FA12 antibody was abolished only by *N*-acetylgalactosamine, whereas that of AO7 was abolished only by α-*methyl*-D-mannoside, among a number of different monosaccharides tested (Table 6). These sugars had no inhibitory effect on the reactivities of monoclonal antibodies IB9, D6, or C7 with the purified agglutinin. These results indicate that our set of monoclonal antibodies distinguish epitopes formed by oligosaccharide as opposed to polypeptide moieties.

Inhibition of agglutinin- or whole saliva-mediated aggregation of *S. mutans* by monoclonal antibodies. To test the effect of the monoclonal antibodies on the functional properties of the agglutinin, samples of the agglutinin or whole saliva were preincubated with each of the hybridoma ascites fluids, then added to aggregation mixtures. Two of the antibodies, C7 and D6, blocked both the agglutinin-mediated and the whole saliva-mediated aggregation of *S. mutans* 6715 (Table 7). The other three antibodies had no effect on aggregation. Since antibodies C7 and D6 recognize pronase-sensitive epitopes, these results further indicate that the agglutination reaction is mediated by a polypeptide rather than a carbohydrate determinant of the streptococcal agglutinin.

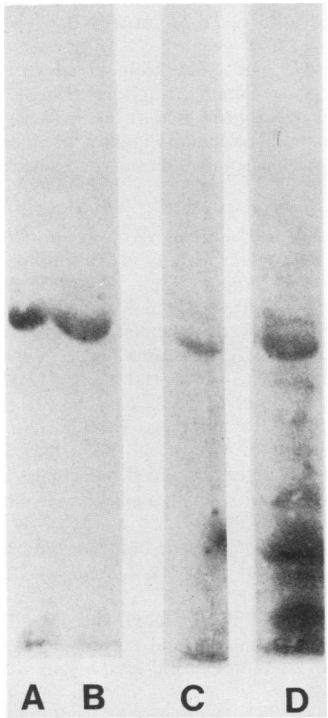

FIG. 6. Western blot analysis of (A) purified agglutinin, (B) whole saliva, (C) artificial tooth pellicle (material adsorbed from saliva onto hydroxyapatite beads), and (D) parotid saliva. Each of the materials was electrophoresed in SDS-gel, transferred to nitrocellulose paper, and then reacted with monoclonal antibody (IB9) as indicated in the text.

TABLE 7. Inhibition of agglutinin- or saliva-mediated aggregation of *S. mutans* by monoclonal antibodies prepared against the purified agglutinin

Hybridoma antibody	% Inhibition of bacterial aggregation mediated by:	
	Purified agglutinin	Whole saliva
IB9	0	0
D6	89.0	44.0
C7	83.0	30.0
FA12	0	0
AO7	3.0	2.3

DISCUSSION

In these studies, we have demonstrated that clarified human saliva contains a relatively low-molecular-weight (60,000 M_r) glycoprotein that agglutinates certain strains of *S. mutans* bacterial cells. The glycoprotein could be purified to homogeneity by ultrafiltration, gel filtration, ion-exchange chromatography, and HPLC. The evidence that the agglutinin was of salivary gland origin was that (i) the agglutinin could be detected not only in whole, expectorated saliva, but also in saliva collected directly from the parotid ducts, and (ii) that antisera against various plasma and serum components failed to react with the agglutinin in Western blot analyses of whole or parotid saliva. The possibility that the agglutinin may have originated from the blood was considered because it is well known that various components of blood may reach the oral cavity by the passive transudation of dental crevicular fluid (4, 6). Such an explanation, however, is unlikely because of the presence of the agglutinin in parotid secretions and its lack of immunologic cross-reactivity with serum proteins. It should be pointed out that these studies have concentrated on the isolation of a salivary agglutinin from clarified saliva, which is in essence a final end-stage fluid in the oral cavity. Therefore, the relative proportion of this material in glandular secretions may not be strictly related by these studies.

The finding of yet lower molecular-weight immunoreactive material in the parotid secretions was puzzling since such material was not found in expectorated saliva. It is possible that the lower-molecular-weight forms are less glycosylated and, therefore, may be more susceptible to degradation by proteolytic enzymes present in saliva. This observation was found to be in agreement with the earlier findings of Oppenheim et al. (29), who reported decreased carbohydrate content in the proteins isolated from stimulated parotid saliva. The role of these smaller molecules in the interaction with bacteria remains to be determined once sufficient material is purified for further study.

The quantitative amino acid analysis of the agglutinin resembles that of the agglutinin characterized by Ericson and Rundegren (8) in the amounts of proline, serine, and threonine. The activities are similar in that both their agglutinin and our agglutinin aggregate serotype C strains of *S. mutans*. Our agglutinin was shown to interact as well with other strains of *S. mutans*. The two agglutinins differ, however, as to molecular weight and contents of hexosamines, hexoses, fucose, and sialic acid. It is unlikely that our agglutinin represents a functional fragment of the molecule described by Ericson and Rundegren (8) because we could not detect any high-molecular-weight material with our monoclonal antibodies against the purified agglutinin. It

is still possible that the epitopes of the hypothetical fragment may be less exposed in the parent molecule because of differences in degree of glycosylation or in molecular conformation.

Of the oral streptococci tested, the agglutinating activity of our salivary glycoprotein was restricted to certain strains of *S. mutans*. No activity was found against strains of *S. salivarius*, *S. mitis*, *S. sanguis*, or *S. mutans* BHT, B14, B421, and OMZ176 strains. The aggregation of *S. mutans* appears to be mediated by the binding of the agglutinin to the streptococcal cell surface; the degree of binding correlated well with the degree of aggregation.

Of particular interest was the finding that the aggregating activity of the agglutinin resides in the polypeptide and not in the carbohydrate components of the agglutinin molecule. $NaIO_4$-treated agglutinin, which lost its reactivity with monoclonal antibodies directed specifically against D-mannose or *N*-acetylgalactosamine residues, nevertheless retained its ability to bind to and aggregate cells of *S. mutans*. Trypsin digestion of the agglutinin abolished aggregating activity. Furthermore, the agglutination was blocked by the polypeptide-specific but not by the carbohydrate-specific monoclonal antibodies. Perhaps the sugar residues serve to protect the agglutinin molecule against proteolytic and chemical degradation by various components of saliva.

As with other salivary agglutinins described by Hay et al. (16), our agglutinin was found to be present in artificial tooth pellicles formed on hydroxyapatite beads treated with saliva. This finding is of importance with respect to the possible role played by the agglutinin in the *S. mutans* ecology of dental tissues. In the soluble form, the agglutinin may serve to aggregate and thereby eliminate the bacteria, whereas in the immobilized state in tooth pellicles, it may serve to bind cells of *S. mutans* to tooth surfaces. That the agglutinin may play a role in this respect was supported by the findings of varying amounts of immunoreactive agglutinin in saliva of all donors tested.

The surface component(s) of *S. mutans* that recognizes and binds this salivary agglutinin is not known. With the purified agglutinin in hand, however, the identification and characterization of the streptococcal binding site should be feasible.

In conclusion, we have isolated and purified a salivary glycoprotein to homogeneity. It binds to and specifically aggregates four of the eight strains of *S. mutans* tested. It may exist both in a soluble form in saliva and in an immobilized form on tooth pellicles. A balance between these two forms of the glycoprotein may serve to modulate the bacterial ecology of the oral cavity and dental tissues.

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