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Human Common Salivary Protein 1 (CSP-1) Promotes Binding of *Streptococcus mutans* to Experimental Salivary Pellicle and Glucans Formed on Hydroxyapatite Surface

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Summary

The saliva proteome includes host defense factors and specific bacterial-binding proteins that modulate microbial growth and colonization of tooth surface in the oral cavity. A multidimensional mass spectrometry approach identified the major host-derived salivary proteins which interacted with *Streptococcus mutans* (strain UA159), the primary microorganism associated with the pathogenesis of dental caries. Two abundant host proteins were found to tightly bind to *S. mutans* cells, common salivary protein-1 (CSP-1) and deleted in malignant brain tumor 1 (DMBT1, also known as salivary agglutinin or gp340). In contrast to gp340, limited functional information is available on CSP-1. The sequence of CSP-1 shares 38.1% similarity with rat CSP-1. Recombinant CSP-1 (rCSP-1) protein did not cause aggregation of *S. mutans* cells and was devoid of any significant biocidal activity (2.5 to 10 µg/ml). However, *S. mutans* cells exposed to rCSP-1 (10 µg/ml) in saliva displayed enhanced adherence to experimental salivary pellicle and to glucans in the pellicle formed on hydroxyapatite surfaces. Thus, our data demonstrate that the host salivary protein CSP-1 binds to *S. mutans* cells and may influence the initial colonization of this pathogenic bacterium onto tooth surface.

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Supplementary data. Table 1, 2, 3, 4 are provided.

Keywords

Common Salivary Protein-1; Human Saliva; Saliva-Microbial Interaction; Affinity and Lectin Chromatography; Mass Spectrometry

Introduction

Saliva is a complex fluid containing host- and microbial-derived proteins secreted into the oral cavity by numerous major and minor salivary glands and a diverse oral microbiome, respectively ^{1, 2}. It has long been recognized that many microorganisms in the mouth have the capacity to adsorb specific salivary proteins ³ and extracellular bacterial products ⁴, which can modulate the composition of the oral microflora by causing aggregation, direct killing and/or mediating adherence to tooth surface ⁵. Therefore, perturbations in the salivary ecosystem by extrinsic or intrinsic factors may alter homeostasis leading to oral disease ⁶.

Dental caries is infectious in character and continues to be the single most prevalent and costly oral disease worldwide ⁷. The microorganisms responsible for this disease are acquired from other humans ⁸. Thus, when one human is infected by oral bacteria from another, the organisms entering the new host are coated with saliva and/or microbial products from the donor host. The initial step in the formation of a biofilm on the tooth surface (also known as plaque) is the deposition of a thin layer of salivary glycoproteins termed pellicle. Subsequently, the successful attachment and further colonization of the tooth surface by pathogenic organisms occurs, which may ultimately lead to the development of dental caries ^{9, 10}.

Available evidence strongly suggests, for example, that the adsorption of bacterial glucosyltransferases (Gtf) to the surface of *Streptococcus mutans* and non-Gtf producing oral bacteria (*Lactobacillus casei* and *Actinomyces naeslundii*) facilitates their adherence to tooth surfaces ¹¹. Similarly, adsorption of salivary amylase increases the colonization by *S. sanguinis* and other oral streptococci ¹². Furthermore, other secreted salivary host proteins such as lysozyme, lactoferrin and lactoperoxidase bind to the microbial cells causing aggregation and/or direct killing ⁵. Nevertheless, the exact identities and biological functions of salivary and/or bacterial proteins that interact with specific oral bacteria associated with oral diseases, such as dental caries, remains to be fully explored.

In this study, we conducted a comprehensive, large-scale proteomic study to identify which host salivary proteins bind to *Streptococcus mutans* UA159 cells, and whether the identified proteins (i) influence *S. mutans* adhesion to salivary pellicle and glucans formed on apatitic surfaces, (ii) affect viability, or (iii) cause aggregation of bacterial cells. *S. mutans* UA159 is a proven virulent cariogenic dental pathogen associated with caries disease, and consequently, this strain was selected for genomic sequencing ¹³. Among different host salivary proteins tightly bound to *S. mutans* cells, gp340 and common salivary protein-1 (CSP-1; detected for the first time) were the most abundant. Salivary agglutinin has been well characterized for its biological functions against pathogens by triggering the aggregation and clearance of streptococci from the oral cavity ¹⁴, while limited information is available on CSP-1. Thus, CSP-1 was cloned and expressed for functional studies. Results demonstrate that *S. mutans* exposed to rCSP-1 in saliva displayed enhanced binding to experimental salivary pellicle and to glucans synthesized *in situ* in the pellicle formed on hydroxyapatite surfaces, suggesting that such interactions may contribute to the initial colonization of this pathogen on tooth surface.

Materials and Methods

Saliva Collection

The protocol for the collection of human saliva and parotid gland tissue by informed consent was approved by the University of Rochester Institutional Review Board. Saliva collection was performed under standard conditions after overnight fasting at the University of Rochester Medical Center. Whole saliva was collected from a healthy, non-medicated and non-smoking Asian male donor (37 years old). Whole saliva was collected on ice after chewing on paraffin and then clarified by centrifugation at 7,200 rpm ($8,500 \times g$ at 4°C for 10 min) as detailed previously¹⁵. Parotid and submandibular/sublingual (SM/SL) salivas were obtained from a healthy, non-medicated and non-smoking Caucasian male donor (48 years old). Ductal secretions were collected on ice after stimulation with 0.4% citric acid using a Lashley cup-like device¹⁶ for parotid secretions and a Block and Brotman collector for SM/SL secretions¹⁷ centrifuged at 4300 rpm ($3820 \times g$) for 20 min.

Bacterial Strains and Growth Conditions

The bacterial strain used in this study was *Streptococcus mutans* UA159 (ATCC 700610), a well-characterized cariogenic bacterium (13). The cultures were stored at -80°C in tryptic soy broth (TSB) containing 20% glycerol. The *S. mutans* cells were cultured in ultrafiltered (Prep/Scale, Millipore, Billerica, MA) tryptone-yeast extract broth (2.5% tryptone and 1.5% yeast extract, pH 7.0) supplemented with 0.3% glucose at 37°C and 5% CO_2 . Growth was assessed in terms of optical density of the culture at 600 nm. Cultures were harvested at mid-exponential growth phase ($\text{OD}_{600\text{nm}}$ 0.5) by centrifugation at 8,000 rpm ($10,000 \times g$ for 10 min at 4°C).

Interaction of Salivary Proteins with *S. mutans*

Forty five ml of ductal saliva (equal volumes of parotid and SM/SL saliva) was incubated with 6.6×10^{11} *S. mutans* cells for 20 min at room temperature. Following centrifugation at 4300 rpm ($3820 \times g$) for 20 min, the supernatant was discarded and cells were resuspended in 30 ml of buffer (10 mM NaHCO_3 ; 5 mM KH_2PO_4 ; 17 mM KCl; 0.3 mM CaCl_2 , pH 6.75). The resuspended bacterial pellet was sonicated 5 times for 10 s at 10 watts (Branson Ultrasonics Corporation, Danbury, CT, USA), centrifuged, and the supernatant discarded. The above washing step was repeated three times. Control experiments were performed by incubating *S. mutans* in buffer (10 mM NaHCO_3 ; 5 mM KH_2PO_4 ; 17 mM KCl; 0.3 mM CaCl_2 , pH 6.75) instead of saliva. Cell pellet was washed and the supernatant collected for protein identification.

Elution of Proteins Bound to *S. mutans*

The washed bacterial pellet from above was resuspended in 50 ml of 0.85% saline, sonicated and centrifuged as above, and the supernatant saved (pool A, weakly bound proteins). Subsequently, the bacterial pellet was resuspended in 50 ml of high salt/EDTA buffer (1 M NaCl, 20 mM EDTA, 100 mM KH_2PO_4 , pH 6.5–7.5), followed by sonication 5 times, centrifugation, and the supernatant saved (pool B, moderately bound proteins). Finally, the pellet was resuspended in 50 ml of urea elution buffer (2 M urea, 500 mM NaCl, 50 mM KH_2PO_4 , pH 6.5–7.5), followed by sonication 5 times, centrifugation, and the supernatant saved (pool C, tightly bound proteins). Protease cocktail inhibitor was added to the supernatants (pools A, B and C) and dialyzed against 40 mM ammonium bicarbonate at 4°C using a 3.5 kDa molecular weight cut-off membrane and stored at -80°C . All pools were lyophilized prior to characterization by mass spectrometry.

Processing, Trypsin Digestion and Analyses of Salivary Proteins by Multi-Dimensional Protein Identification Technology (MudPIT)

The lyophilized fractions were suspended in 100 mM Tris-HCl buffer pH 8.5. The protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Bio-Rad) per manufacturer's instructions, followed by addition of 100 mM Tris-8 M urea buffer, reduced by 25 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and cysteine alkylated by 30 mM iodoacetamide. The samples were digested by Lys-C (Roche, Mannheim, Germany; enzyme to substrate ratio 1:200) at 37 °C for 4 hr. Subsequently, the sample solutions were diluted to a final urea concentration of 2 M, and digested again with trypsin (Promega, Madison, WI; enzyme to substrate ratio 1:50) at 37 °C overnight. The enzyme reaction was terminated by adding 90% formic acid to a final concentration of 3–5%.

Digested proteins were analyzed by MudPIT as previously described^{2, 18}. In brief, 50–100 µg of protein was pressure loaded onto a biphasic microcapillary column packed with a strong cation exchanger (SCX, Whitman, Clifton, NJ) and RP resin (Aqua C18, Phenomenex, Ventura, CA). The column was attached to an analytical microcapillary column packed with RP resin and placed in line with an Agilent 1100 quaternary HPLC (Agilent, Palo Alto, CA). Samples were analyzed using a modified 8 or 12-step separation. As peptides eluted from the microcapillary column they were electrosprayed directly into either an LTQ 2-dimensional ion trap (ThermoFisher Scientific San Jose, CA, USA) or an Orbitrap (ThermoFisher Scientific) mass spectrometer with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan mass spectrum (400–1400 m/z) followed by 7 data-dependent MS/MS spectra of all charge states were sequentially isolated and fragmented at a 35% normalized collision energy repeating continuously throughout each step of the multidimensional separation. The m/z ratios selected for MS/MS were dynamically excluded for 75s. Application of mass spectrometer scan functions and HPLC solvent gradients was controlled by the Xcalibur data system (Thermo Fisher Scientific)

MS Data Analysis

Poor quality spectra were removed from the dataset using an automated spectra quality assessment algorithm¹⁹. MS/MS spectra remaining after filtering were searched with the ProLuCID²⁰ algorithm (version 1.1.2) against the EBI human IPI database (<ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/>, version 3.01, release date November 1, 2004) concatenated to a decoy database in which the sequence for each entry in the original database was reversed²¹. MS/MS database search parameters were: candidate peptides could be of any tryptic status; unlimited missed cleavages were permitted, and carbamidomethylation of Cys residues (C+57) is considered. The mass tolerance for database searching was +/- 4.5 Da. ProLuCID results were assembled and filtered using the DTASelect program²² (version 2.0) DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false positive rate assessed by the reverse hits (1% at the protein level in this analysis, corresponding to a ≥ 99% confidence level at the MS/MS level). The DTASelect 2.0 program assembles identified peptides into proteins and protein groups by using a parsimony principle in which the minimum set of proteins accounts for all observed peptides. Only proteins with at least two unique peptide hits were accepted. All peptides identified are at least half tryptic.

PCR Amplification, Cloning, Design of Expression Vector and Expression of Recombinant Common Salivary Protein-1 (rCSP-1)

Total RNA was isolated from the parotid tissue of an adult human male and first strand cDNA synthesis was conducted using Super Script III (Invitrogen) using an oligo (dT)

primer. For RT-PCR, first strand cDNA was added to a GoTaq polymerase chain reaction (PCR) mixture, according to the manufacturer's instruction (Promega), containing primers: CSP-1S-1023: [CCACACGCGTCTGAAGATGTATGGCCCTGGAGGAG] and CSP-1AS-1024: [GAGAAGCGGCCGCAGTACCATCAGCCACCACCACACAG]. The PCR products were inserted into the MluI and NotI sites of the *Drosophila* S2 insect vector pS2-SP in a position downstream from a metallothionein promoter, and after an N-terminal secretory signal peptide and a high affinity metal chelate binding site (HHWHHH)²³. Although proteins heterologously expressed in *Drosophila* Schneider (S2) cells are known to be glycosylated²⁴, the glycosylation of rCSP-1 may not be identical to that in mammalian cells. The high affinity metal chelate binding site allows purification of recombinant protein CSP-1 using affinity chromatography with TALON resin. Ligated plasmids were transformed into *Escherichia coli* and isolated by plasmid mini-preparation (Qiagen Turbo 8). DNA sequencing validated the insert.

S2 cells were cultured in SDM medium (Gibco) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin at 27 °C. *Drosophila* Schneider (S2) cells were transfected using the calcium phosphate method, as described by the supplier (Invitrogen, DES system). S2 cells were co-transfected with pCo-BLAST and placed under blastocidin selection for 4–12 weeks (Invitrogen). Expression of the recombinant rCSP-1 protein was induced from the metallothionein promoter by adding 650 µM copper sulfate. After 5 days, the culture medium was harvested and buffer exchanged, using a 10-kDa molecular mass cut-off membrane filter and a pellicon tangential flow system (Millipore, Billerica, MA, USA).

Purification of Recombinant Common Salivary Protein-1 (Rcsp-1) by Metal Affinity Followed by Con A Lectin Affinity Chromatography

The secreted rCSP-1 protein was purified at 4 °C by metal chelate chromatography using TALON Superflow metal affinity resin (Clontech, Palo Alto, CA, USA) on a Biologic DuoFlow Maximizer purification system (Bio-Rad, Hercules, CA, USA) as described in Ambatipudi et. al.²⁵. In briefly, the column was equilibrated with 50 mM sodium phosphate and 300 mM sodium chloride pH 8. After the sample was loaded, the column was washed with 50 mM sodium phosphate and 300 mM sodium chloride pH 7.5. For elution of proteins, 150 mM imidazole pH 7 was applied to the column and 1 ml fractions were collected and monitored at 254.

Following TALON affinity chromatography, rCSP-1 was further purified by lectin affinity chromatography on a concanavalin A (con A)-agarose column (Sigma). In brief, the con A column was equilibrated with Tris buffered saline (TBS: 0.05M Tris & 0.15 M NaCl, pH 7.2) followed by sample loading. The column was washed with TBS buffer and then rCSP-1 was eluted by applying α-D-mannose (0.1 M; Sigma, St Louis, MO, USA). Flow-through and elutions were collected as 300 µl fractions. Protein yield and purity of rCSP-1 was determined by staining SDS-PAGE gels with Simply Blue stain (Invitrogen).

Confirmation of Recombinant Common Salivary Protein-1 (rCSP-1) by Mass Spectrometry

Protein migrating at approximately 30 kDa, the expected molecular weight of rCSP-1, was excised from the gel and washed three times in 50% (v/v) acetonitrile and 25 mM NH₄HCO₃, pH 7.8 at 37 °C for 10 min, and then dried at room temperature (Speedy-Vac; Savant, Farmingdale, NY, USA). The resulting gel pellet was digested overnight at 37 °C with trypsin (15 ng/µL). Products were recovered from the gel pellet by sequential extractions with 10% acetonitrile and 1% (v/v) formic acid. Samples (2 µL) were loaded into a filled loop injector (Dionex, Houston, TX, USA) in line with a fritless nano column. Digested peptides were eluted using a linear gradient of H₂O:CH₃CN (98:2, 0.1 % formic

acid) to H₂O:CH₃CN (50:50, 0.1 % formic acid) at ~300 nL/min over 30 min. The capillary temperature and spray voltage were 200 °C and 2.5 kV, respectively, for the LCQ Deca XP Max mass spectrometer (Thermo Electron, San Jose, USA). Samples were analyzed over a mass (m/z) range of 400–2000 followed by sequential isolation and fragmentation of the three most intense ions in the full MS with a 35% normalized collision energy. The LCQ was operated in positive ion mode with activation q = 0.25 and activation time of 30 ms.

The acquired MS/MS spectral data were processed for automated interpretation using Sequest algorithm (Bioworks 3.2 EF2 Thermo Electron) against theoretical entries in the NCBI nr database (June 2009). Ion search criteria were as follows: taxonomy – human, trypsin digestion allowing up to one miscleavage, variable modification – oxidation of methionine, cysteine as carboxyamidomethylation or propionamide, precursor tolerance 2 Da and product ion tolerances ± 0.8 Da. Only the peptides passing the Xcorr defined by Washburn et al (18) were considered. Only spectra corresponding to CSP-1 were detected.

Bacterial Growth and Aggregation Assays

The effects of rCSP-1 on *S. mutans* viability and growth rate were investigated using an automated microbiological growth analyzer Bioscreen C system (Labsystems, Helsinki, Finland) ²⁶. The bacterial cells were grown to mid-exponential phase, and then diluted 1:40 in ultrafiltered (10 kDa molecular-weight cut-off) buffered tryptone yeast-extract broth (2.5% tryptone and 1.5% yeast extract, pH 7.0) containing 0.3% or 1% (w/v) glucose (with or without clarified human whole saliva) and rCSP-1 (final concentrations ranging from 2.5 to 10 µg/ml). The physiological concentration of CSP-1 in human parotid saliva was estimated to be approximately 10µg/ml by densitometric analysis using an AlphaImager and AlphaEase FC software (Alpha Innotech, San Leandro, CA). The intensity of the band corresponding to CSP-1 in 10 µg parotid saliva protein was compared to bands generated by known amounts of BSA (5, 10 and 15 µg) on a SDS-PAGE stained with Simply Blue. Given that proteins will stain differentially based on their composition, this value is considered an estimate. The mixture was transferred to 100-well plates and placed in the Bioscreen. The plates were incubated at 37 °C for 24 h and absorbance measurements (OD_{600 nm}) of each well were recorded every 15 min after 60 s shaking. Growth curves were generated and analyzed using the Bioscreen C Reader software (Research Express, version 1.00). Bacterial cells were also grown in the presence of chlorhexidine (a broad-spectrum antimicrobial agent) at 1 µg/ml (bactericidal effect against *S. mutans*) or 0.1 µg/ml (bacteriostatic effect), as experimental control ²⁷.

The aggregation activity of rCSP-1 was measured spectrophotometrically at 700 nm as detailed by Ericson and Rundegren (1983) ²⁸ and by microscopic observation ²⁸. Briefly, bacterial cells (1 × 10¹⁰ cells/ml) were mixed with rCSP-1 (final concentrations ranging from 2.5 to 10 µg/ml). The mixture was immediately transferred to a Beckman DU-800 spectrophotometer at 37 °C and aggregation was measured by a continuous recording of decrease in absorbance with time over 2 h incubation ²⁸; aliquots were also taken to check for aggregation at 40x magnification. Concanavalin A (con A; at 250 µg/ml), a well-characterized lectin known for causing aggregation of *S. mutans* cells ²⁹, was run in parallel as an experimental control; con A at lower concentrations (2.5 to 10 µg/ml) did not agglutinate the bacterial cells. Triplicates from two separate experiments were conducted in each assay.

Bacterial Adherence to Experimental Salivary Pellicle and to Glucans in the Pellicle Formed on Hydroxyapatite Surface

Bacterial adherence assays were conducted using *S. mutans* UA159 grown in ultrafiltered (10-kDa molecular weight cutoff membrane) tryptone-yeast extract broth containing 185

kBq/ml ^3H -thymidine (Perkin-Elmer Life and Analytical Sciences, Boston, Mass., USA) as detailed previously³⁰. Samples of bacteria were sonicated using a Branson Sonifier 450 (six 10-second pulses with 5-second intervals at 20 W; Branson Ultrasonics Co., Conn., USA) to obtain single-cell suspension. Approximately 1.0×10^{10} cells were used in each of the binding assays.

Whole saliva was collected on ice from one donor after chewing on paraffin. Afterwards, the saliva was diluted 1:1 with adsorption buffer (50 mM KCl, 1.0 mM KPO_4 , 1.0 mM CaCl_2 , 0.1 mM MgCl_2 , pH 6.5), supplemented with sodium azide (0.02%, final concentration) and protease inhibitor PMSF (1.0 mM, final concentration), and then clarified by centrifugation at 7,200 rpm ($8,500 \times g$ at 4°C for 10 min.). The resulting clarified whole saliva (CHWS), which is also Gtfs-free, is optimum to form experimental salivary pellicle on the surface of hydroxyapatite beads for bacterial binding as detailed elsewhere^{25, 30}. Similarly, to study the bacterial adherence to glucans formed *in situ* in the salivary pellicle (gsHA), the sHA was exposed to saturating amounts of purified streptococcal glucosyltransferase B (GtfB), and incubated with sucrose (100mmol/L, final concentration) at 37°C for 4 h to allow glucan formation on the surface³⁰.

The functional activities of rCSP-1 (final concentrations ranging from 2.5 to 10 $\mu\text{g}/\text{ml}$ in CHWS or in PBS) or vehicle control (CHWS or PBS solution) were tested in two distinct experiments. Albumin (2.5 to 10 $\mu\text{g}/\text{ml}$) was run in parallel as an experimental control to check for non-specific protein interactions; albumin does not affect *S. mutans* binding to apatitic surfaces coated with saliva or with glucans³¹ (Koo et al., unpublished results).

First, 1.0×10^{10} cells of radiolabeled *S. mutans* were incubated with rCSP-1 (in CHWS or in PBS), albumin (in CHWS or in PBS) or vehicle control (CHWS or PBS alone) for 40 min at 37°C (total volume 1 ml). The cells were washed 3 times with adsorption buffer (50 mM KCl, 1.0 mM KPO_4 , 1.0 mM CaCl_2 , 0.1 mM MgCl_2 , pH 6.5) to remove excess, unbound rCSP-1, and resuspended in 1 ml of adsorption buffer. The bacterial suspension (1.0×10^{10} cells/ml) was then incubated with either sHA or gsHA. After 60 min incubation, the beads were washed to remove unbound cells and the number of adherent bacteria was determined by liquid scintillation counting³⁰.

In the second experiment, sHA or gsHA was exposed to rCSP-1 (in CHWS or in PBS), albumin (in CHWS or in PBS) or vehicle control (CHWS or PBS alone) for 40 min at 37°C . The beads were washed 3 times with adsorption buffer, and then incubated with 1.0×10^{10} cells of radiolabeled *S. mutans* UA159 for 60 min (total volume 1 ml). After incubation, the beads were washed and the number of cells adherent to the beads was determined by liquid scintillation counting.

Triplicates from two separate experiments were conducted in each assay. The data were analyzed by ANOVA, and the F-test was used to test for differences among the groups. When significant differences were detected, pair-wise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. Statistical software JMP version 3.1 (SAS Institute, Cary, NC, USA) was used to perform the analyses. The level of significance was set at $P < 0.05$.

Results

Protein Identification

The identification of proteins by tandem liquid chromatography-mass spectrometry is significantly enhanced using Multidimensional Protein Identification technology (MudPIT)¹⁸. The total number of tandem mass spectra that were confidently assigned to any peptide

belonging to a protein is known as spectral count³² and those spectra passing through the DTASelect filtering criteria with a false positive rate of less than 5% were considered a confident match and used for further analysis. Additionally, to increase confidence, positive identifications were deemed acceptable only when two unique peptides were detected for each protein. Furthermore, a manual cut-off score of 10 for spectral counts was set to eliminate low abundance proteins. Using this selection strategy, 90 unique host salivary proteins were eluted from the surface of *S. mutans* under three conditions: isotonic (pool A, 43 weakly bound proteins), high salt (pool B, 68 moderately bound proteins) or urea elution (pool C, 31 strongly bound proteins). This data clearly indicates that the host proteins identified are indeed bound to *S. mutans*. Furthermore, studies with other relevant organisms (i.e. *Lactobacillus* spp, *Fusobacterium* spp, and *Candida* spp) are currently being carried out to determine whether CSP-1 and other strongly bound proteins are uniquely adsorbed to *S. mutans*. These human salivary proteins eluted from the surface of *S. mutans* by these conditions are listed in Supplementary Table 1–3, respectively. In contrast no human proteins were identified from control experiments.

Figure 1 shows a comparison of the total number and overlap of non-redundant proteins identified across the three different pools (pools A–C) and are listed in Supplementary Table 4. Although spectral count is not an absolute measure of the relative amount of protein in samples, it is a widely used and semi-quantitative measure of abundance (30). It is also important to note that if a protein is identified by a set of peptides that is a proper subset of another protein then the subset protein is eliminated from the final dataset. Of the 90 non-redundant host proteins bound to *S. mutans*, 10, 34 and 9 were detected exclusively in pool A (~11%), B (~37%) or C (10%), respectively, while 15 proteins were common to all three pools (~16%). The apparent binding properties of these 15 common proteins are displayed in Figures 2A (high abundance, >160 spectral counts) and 2B (low abundance, <160 spectral counts) based on the number of spectral counts (roughly corresponding with protein abundance): i.e. weakly (0.85% normal saline, pool A), moderately (1 M salt elution, pool B) and strongly (2 M urea elution, pool C) bound to *S. mutans* surface. Figure 2A shows that alpha-amylase precursor was highly abundant in both pools A and B (spectral counts = 1055 & 1088, respectively), with comparatively few spectral counts in pool C (spectral counts = 71) suggesting that it has a low to moderate affinity for *S. mutans* cells. Similarly, cystatin SN and S precursor proteins, prolactin inducible protein and basic proline-rich protein 1 have low to moderate affinities for *S. mutans* cells (pools A and B). In contrast, polymeric immunoglobulin precursor, Ig kappa chain V–II, Ig lambda chain C, and carbonic anhydrase VI precursor were identified in all three pools in similar abundance as shown Fig. 2B. Lysozyme C was one of three abundant proteins in pool C (spectral counts 322), but it was >2.5-fold more abundant in pools A and B (spectral counts 763 and 887, respectively). The other two proteins strongly bound to *S. mutans* (pool C) were gp340 and common salivary protein-1 (CSP-1). Salivary agglutinin was considerably more abundant in pool C (spectral counts 587) than in pools A (186) and B (127). Salivary agglutinin has been previously shown to protect against bacterial infections and to inhibit tumorigenesis in different tissues³³. A hypothetical protein, CSP-1, was detected in comparable abundance in pools A (563), B (400) and C (465).

A sequence similarity search was performed on the peptides identified as a hypothetical protein (CSP-1, Accession number IPI00060800.3). A BLAST search identified HRPE773 (GenBank AA89380.1), which displays a moderate degree of similarity with rat CSP-1 (38.1%) and mouse Demilune cell and parotid protein (Dcpp, 37.4%; GenBank ABB59012.1). Such a divergent evolution between species was previously noted for CSP-1³⁴ and for other salivary proteins³⁵. The mass spectra of peptides identified from three elutions (low salt, 1M salt and 2MUrea) that corresponded to a hypothetical protein was mapped against the NCBI_{nr} sequence of human CSP-1 shown in Fig. 3 (bold italics)

suggesting a wide coverage (51.1%). This confirms that this hypothetical protein is a human ortholog of rat CSP-1 and mouse Dcpp.

Expression, Purification and Confirmation of Recombinant Common Salivary Protein-1 (rCSP-1)

To explore the functional importance of the interaction of CSP-1 with *S. mutans*, total parotid RNA/cDNA was amplified and expressed in Drosophila S2 Schneider Cells. Expressed recombinant CSP-1 from saliva had 178 amino acids without the predicted signal sequence on the N-terminus as compared to the full-length sequence of the same protein also known as Zymogen granule 16B homolog or Jacalin-like lectin with 208 amino acids. This difference in length suggests that the processed, mature and secreted form of CSP-1 is an alternate splice variant found in saliva. This is also consistent with the lack of peptides identified from the N-terminal region of the protein by mass spectrometry.

The metal binding site of the recombinant protein (Fig. 4A) was used to isolate rCSP-1 (Fig. 4B, lane 1). The sample was further purified on a con A lectin affinity column (Fig. 4B, lane 3). The mobility of rCSP-1 was consistent with its estimated molecular weight (~30 kDa). The predicted MW of the rCSP-1 is 23kDa, somewhat less than predicted from the SDS-PAGE gel (MW = 30kDa). This observation, along with its ability to bind a lectin column, suggests that rCSP-1 is glycosylated. The purity of rCSP-1 was confirmed by excising a purified rCSP-1 protein band from the gel and analyzing by nanospray liquid chromatography coupled tandem mass spectrometry (LC-MS). Recombinant CSP-1 was identified by LC/MS/MS with 19.1% sequence coverage, indicating a high level of sample purity. No other proteins were identified from the gel band.

Functional Characterization of rCSP-1

The interaction of human CSP-1 with *S. mutans* might be expected to modulate the biological function of this bacterium by inhibiting its growth, aggregation and/or adherence to the tooth surface. However, rCSP-1 did not cause aggregation of the bacterial cells and was devoid of any significant effects on viability (biocidal activity) or growth rate of *S. mutans* at the concentrations tested in this study (2.5 to 10 µg/ml); consistent with previous studies^{25, 29}, chlorhexidine (at 0.1 and 1 µg/ml) and con A (at 250 µg/ml) displayed antibacterial activity and caused aggregation of *S. mutans* cells, respectively (data not shown).

In contrast, rCSP-1 was found to increase the adherence of *S. mutans* to experimental salivary pellicle formed on hydroxyapatite surface (sHA) and to glucans formed *in situ* in the pellicle (gsHA). Pellicle was formed by treatment of HA with clarified human whole saliva (CHWS). Glucans were formed in pellicle by exposing sHA to purified streptococcal GtFB; the samples were then incubated with sucrose to permit *in situ* glucan synthesis by the adsorbed enzyme. Two distinct experiments were conducted. First, radiolabeled *S. mutans* cells were exposed to rCSP-1 (10 µg/ml in CHWS or in PBS), and then incubated with sHA or gsHA. Figure 5 shows that cells exposed to 10µg/ml rCSP-1 in CHWS (on the right) displayed enhanced binding to sHA (panel A) and gsHA (panel B) compared with the number of adherent *S. mutans* cells exposed to saliva alone ($P<0.05$). Although a similar trend was observed with cells incubated with rCSP-1 in PBS (on the left, panels A and B), the influences on bacterial binding were less pronounced and did not reach statistical significance (vs. PBS or saliva alone; $P>0.05$). As previously shown³⁰, the presence of glucans formed on sHA surface promoted a large increase in the binding of *S. mutans* cells compared with the bacterial adherence observed to the salivary pellicle alone (compare Figs. 5A to 5B, more than 0.5 log order greater adherence). The glucans form an amorphous polymeric layer covering the HA surface likely masking the host-derived components in the

pellicle³⁰, thus, the bacterial binding to the apatite surface is mediated mostly by *in situ* formed glucans.

In the second experiment, sHA or gsHA was exposed to rCSP-1 (in CHWS or in PBS) prior to incubation with *S. mutans* cells. In contrast to the first assay, treatments with rCSP-1 did not cause any significant changes in the number of adherent cells to either sHA or gsHA compared to those exposed to buffer or saliva alone (not shown). rCSP-1 at lower concentrations (2.5 and 5 µg/ml) and albumin (2.5 to 10 µg/ml) was devoid of any significant biological effects on bacterial binding to salivary pellicle or surface-adsorbed glucans in both experiments (not shown).

Discussion

It has been suggested that host-derived salivary proteins such as lysozyme, lactoferrin and lactoperoxidase modulate the composition of the oral microflora by binding to the microbial cells and causing aggregation and/or direct killing, while other salivary proteins including amylase enhance bacterial binding to tooth surfaces⁵. In parallel, salivary and microbial proteins selectively bind onto tooth enamel forming the acquired enamel pellicle^{36–38}. The glucosyltransferases (Gtfs) secreted by *S. mutans*, for example, bind avidly to the pellicle formed on the tooth surface where they are highly active; i.e. when exposed to sucrose, the adsorbed Gtfs form a layer of glucans on the surface within minutes^{39, 40}. The polysaccharides on the pellicle provide specific binding sites for bacterial colonization, particularly mutans streptococci^{30, 41}. Thus, specific host receptors (e.g. agglutinins, proline-rich proteins, amylases) and glucans synthesized *in situ* by bacterial Gtfs bound to the pellicle act as bacterial anchor sites, and along with cell-surface proteins dictate the composition of initial microbial tooth colonizers^{9, 30, 39, 42}.

Streptococcus mutans cells attach initially to saliva coated surfaces through sucrose-independent mechanisms mediated primarily by lectin-like interactions between specific pellicle proteins (e.g. agglutinins) and adhesins (e.g. P1) present on the bacterial cell surface^{9, 42}. Furthermore, *S. mutans* cells also bind to the glucan-coated surfaces, and more importantly, in larger numbers and with higher adhesion strength than to saliva-coated surfaces^{30, 43} through expression of several glucan-binding proteins⁴⁴. Binding of *S. mutans* to the tooth surface is critical for its establishment and initiation of pathogenic biofilm formation. Thus, any molecule that modulates adherence of *S. mutans*, especially glucan-mediated binding, may influence colonization and further accumulation on the tooth surface. The majority of the studies conducted thus far have been focused on identification of streptococcal surface proteins responsible for mediating bacterial binding to the salivary pellicle⁴². Considering that *S. mutans* cells (and, in fact, all other oral pathogens) are present in and coated with whole saliva in the mouth, it is critical to identify which salivary proteins bind to this pathogenic organism and whether the surface-bound host protein mediates their initial attachment to the tooth surface, affect bacterial viability and/or cause aggregation.

The present study identified by multi-dimensional protein identification technology 90 non-redundant salivary proteins bound to *S. mutans*, suggesting their potential significance in oral defense and colonization in the oral cavity. Different conditions (low to high salt and urea) were used to differentiate between proteins with weak, moderate and strong binding affinities to *S. mutans* surface. Of the proteins identified, gp340 and CSP-1 were identified as the most abundant, tightly bound proteins on the microbial surface. Salivary agglutinin is a well known bioactive (defense) salivary molecule associated with aggregation capacity of saliva, which may modulate implantation and colonization of cariogenic bacteria (such as *S. mutans*) on tooth surfaces¹⁴. It is noteworthy that calgranulin B, a component of the

acquired pellicle³⁸, was also detected in high abundance on *S. mutans* surface. Although there is no evidence showing this protein mediates *S. mutans* binding, calgranulin family contains a calcium-binding domain possibly involved in enamel deposition³⁸ which may have a potential significance for caries disease.

CSP-1 appears to be expressed mainly in human salivary tissue and to a lesser extent in trachea and prostate gland, based on large-scale analysis of the transcriptome of 79 human tissues³⁴. CSP-1 was identified as hypothetical protein (IPI00060800.3) in this study, and is the human ortholog of rat CSP-1 and mouse Dcpp (Demilune cell and parotid proteins). Human CSP-1 was also reported as hypothetical protein (accession number IPI00060800) by Denny et al.², while it was reported as “similar” to common salivary protein (accession number gi3 21687060) by Wilmarth et al.⁴⁵. This difference in terminology and accession numbers was due to the use of different protein databases; Denny et al. used the EBI protein databases, while the NCBI non-redundant database was used by Wilmarth et al. for protein identifications. As compared to other known salivary proteins such as lysozyme (~21 µg/ml)⁴⁶, IgA (109 µg/ml)⁴⁷, CSP-1 is a moderately abundant proteins with its concentration estimated as 10 µg/ml in parotid saliva. CSP-1 shares a similarity of 38.1% with rat CSP-1 and 37.4% with mouse sublingual demilune protein (also called as SPT2)³². The similarities between human CSP-1 and these related proteins include a classical NH₂-terminal signal sequence, a putative jacalin-related lectin (JRL) domain, and potential N-linked glycosylation sites⁴⁸. Members of the JRL protein family bind to glycoproteins, are ubiquitously expressed throughout the plant and animal kingdom⁴⁹, and perform functions such as cell agglutination and antimicrobial activity³⁴. Future studies are required to determine if the strong binding of CSP-1 to *S. mutans* cells is mediated by the JRL domain in CSP-1, or if other functional domains in CSP-1 bind to *S. mutans* either directly or as part of a protein complex.

Considering that CSP-1 binds tightly to *S. mutans*, we examined whether bacterial cells exposed to CSP-1 display (i) changes in their binding activity to experimental pellicle (sHA) and glucans (gsHA) formed on apatitic surface, (ii) altered viability/growth rate and/or (iii) enhanced cell aggregation. This was the first step toward identifying additional novel salivary proteins that potentially modulate the functional and biological activities of *S. mutans*.

Our data indicate that *S. mutans* cells exposed to rCSP-1 (10 µg/ml) in saliva significantly increased bacterial binding to sHA and gsHA compared to bacterial cells exposed to saliva alone. In contrast, sHA or gsHA exposed to rCSP-1 prior to incubation with *S. mutans* cells (which had not been pre-treated with rCSP-1) displayed negligible effects on bacterial adherence on these surfaces. These results demonstrate that the presence of rCSP-1 in saliva may contribute to the initial binding of *S. mutans* to both salivary pellicle and glucans formed in the pellicle by interacting directly with bacterial cell surface rather than affecting binding sites on the apatitic surface (i.e. receptor). It is noteworthy that our CHWS preparation is free of significant levels of native CSP-1 and also glucosyltransferases-Gtfs which could interfere with the interpretation of the data. The concentration of native CSP-1 in our CHWS preparation (which is diluted in adsorption buffer and clarified by centrifugation) is negligible in causing effects on bacterial binding in vitro; which may indicate CSP-1 association with high-molecular weight salivary proteins (during clarification process); *S. mutans* cells exposed to CHWS (no added rCSP-1) showed no difference on bacterial binding compared to those exposed to PBS only (no added rCSP1).

The adhesion of this bacterium to dental surfaces involves multiple potential mechanisms, including those mediated by hydrophobic or electrostatic forces (low affinity and non-specific) and highly-specific adhesion-receptor interactions between bacterial cell and

acquired enamel pellicle formed on tooth enamel surfaces⁴². *S. mutans* expresses multiple highly specific surface adhesins that are able to mediate attachment of the bacteria to host-derived and bacterium-derived binding sites on tooth surfaces. For example, the bacterial surface-protein P1 recognizes and attaches to saccharide receptors in the salivary glycoproteins constituents of the pellicle (e.g. agglutinins)⁴², whereas glucan-binding proteins on *S. mutans* membrane bind glucans synthesized by surface adsorbed Gtfs, such as those from GtfB and GtfC activity⁴⁴. Our data suggest that the enhanced binding of *S. mutans* to sHA and gsHA are mediated by distinct but complementary mechanisms where a host-derived protein bound to microbial membrane could (1) act as an additional adhesin-like component recognizing and attaching to salivary proteins and glucans present within salivary pellicle and/or (2) modify the properties of the microbial surface influencing the non-specific adherence forces between bacterial and pellicle surfaces⁵⁰.

It is noteworthy, however, that the enhancement of bacterial adherence was most robust when *S. mutans* cells were exposed to rCSP-1 in saliva indicating that the presence of other salivary proteins (as occurs in the mouth) may be mediating the attachment of rCSP-1 to the cell-surface and/or between the bacterium and pellicle/glucan surfaces, possibly by forming protein complexes, thereby influencing adhesion. For example, it is well-known that mucins are highly glycosylated and form heterotypic complexes with specific salivary proteins, i.e. immunoglobulin A, lactoferrin agglutinin, cystatin, PRPs, histatins^{51–54}. These complexes may influence the biological property of individual molecules⁵⁵ or may serve as a bridge between *S. mutans* and other salivary proteins, including those in the pellicle⁵⁶. Similarly α -enolase interacts with mucin either for the microbial attachment to oral tissues or successful removal from the oral cavity⁵¹. Further studies are needed to elucidate whether CSP-1 form complexes with specific proteins in saliva in the fluid phase or adsorbed state, and how these interactions enhance its ability to promote adherence of *S. mutans* cells to saliva and glucan-coated apatitic surfaces. In addition, it is possible that glycosylation in insect cell line may be different from native form of the protein found in saliva which may affect formation of protein complexes.

Although CSP-1 shares a lectin-like domain, which suggests a possible effect on cell agglutination and microbial growth^{34, 57}, rCSP-1 at concentrations tested in this study did not show any detectable effects on these parameters. Thus, it appears that CSP-1 may not participate directly in host defense in the oral cavity but rather on modulation of bacterial binding to apatitic surfaces. Consequently, the biological importance of such differences highlights that saliva-induced aggregation and saliva-mediated adhesion of bacterial cells may be independent processes, and presumably mediated by different salivary proteins⁵⁸.

Collectively, CSP-1 and gp340 were identified as major proteins in parotid: SM/SL saliva that strongly binds to *S. mutans* cells, each with distinctive biological functions that could influence the survival and colonization of this ubiquitous pathogen in the oral cavity. Furthermore, we demonstrated that host-derived CSP-1 may play a role in modulating *S. mutans* adherence to tooth surface, which is critical for initial microbial colonization and further development into pathogenic biofilms. Additional studies are warranted to determine how CSP-1 bridges the specific interaction(s) between the bacterial and pellicle/glucan surfaces by (i) identifying the specific proteins and/or sugars on the surface of *S. mutans* that recognize and bind CSP-1, (ii) identifying the pellicle and glucan component/structure that bind cell surface-adsorbed CSP-1, and (iii) examining whether CSP-1 form complexes with other salivary proteins. The overall results of this study should encourage future research to consider the importance and to elucidate the exact mechanisms involved in the complex interplay between specific proteins in saliva and microbial surfaces which will advance our current understanding of the pathogenesis of dental caries and other oral infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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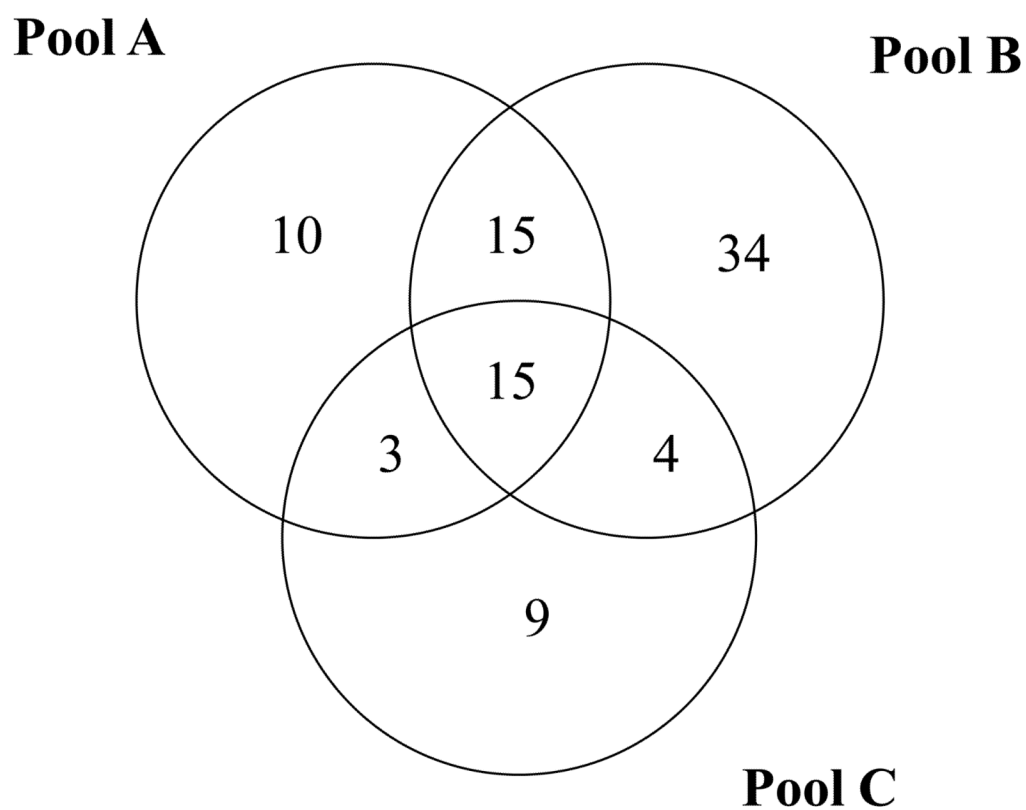


Figure 1. Human salivary proteins eluted from the surface of *S. mutans*
Venn diagram showing the human salivary proteins differentially bound to the surface of *S. mutans*, as grouped by the number of overlapping and non-overlapping proteins in the different affinity pools: 0.85% salt (weakly bound, pool A), 1 M salt (moderately bound, pool B) and 2M urea (tightly bound, pool C) solutions.

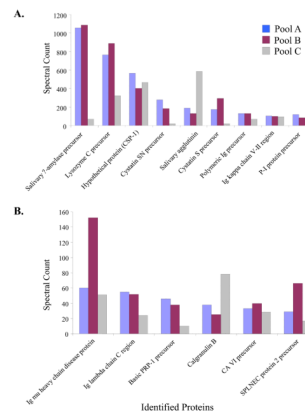


Figure 2. Comparison of overlapping human salivary proteins eluted from the surface of *S. mutans*

A comparison of the fifteen human salivary proteins bound to the surface of *S. mutans* present in all three affinity pools was performed based on their spectral counts: 0.85% salt (weakly bound, pool A), 1 M salt (moderately bound, pool B) and 2M urea (strongly bound, pool C) solutions. (A) Comparison of the nine most abundant proteins (spectral counts >160) identified in pools A, B and C. (B) Comparison of the six least abundant proteins (spectral counts <160) detected in pools A, B and C. CSP-1 = common salivary protein-1; Salivary agglutinin (gp340) = deleted in malignant brain tumor 1; Polymeric-Ig precursor = Polymeric-immunoglobulin precursor; P-I protein precursor = Prolactin-inducible protein precursor; Basic salivary PRP 1 precursor = Basic salivary proline rich protein 1 precursor; CA VI precursor = Carbonic anhydrase VI precursor; SPLNEC = short palate, lung & nasal epithelium carcinoma.

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HRPE773:      1      23
M-----K*YFSTTEDIYDRIETGLRVSNGLLLVKSVQV*KLGDSDVKL*GALGNTQEVTLQPGENTKVFVAFQFLRG
Peptide 4-6:      KYFSTTEDIYDRIETG      KLGDSWDVKL*GALGNTQEVTLQPGENTKVFVAFQFLRG
Peptide 4-6:
HRPE773:      148 178
MVMYTSKDRYFYFKLLDGGSSAYPSQEGQVLVGIYGGYQLLGIK*-----
Peptide 10-18:      KDRYFYFKLLDGGSSAYPSQEGQVLVGIYGGYQLLGIK*

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Figure 3. Multiple sequence alignment

The amino acid sequences were aligned for HRPE773 (also known as human CSP-1, GenBank AAQ89380.1) and rat CSP-1 (Accession no. NP598306.1) sourced from from NCBI_{nr}. Amino acid identities across both species are indicated by an asterisk. Individual peptides identified through MS analysis are mapped to the HRPE773 protein sequence (amino acids in bold italics) and provide 51% sequence coverage, validating the identification of the protein as CSP-1.

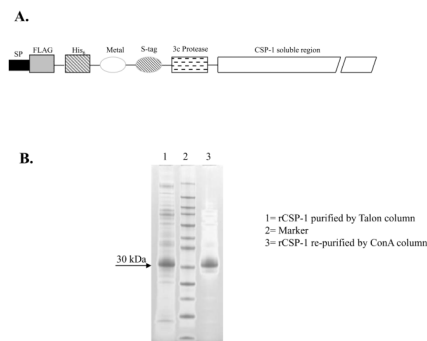


Figure 4. Structure, purification and validation of the recombinant human CSP-1 protein (rCSP-1)

Human CSP-1 was cloned and the recombinant protein expressed in S2 cells. (A) The rCSP-1 protein contains an N-terminal secretory signal peptide (SP; black box), FLAG epitope tags (grey box), six histidine residues (dotted black box), metal chelate tag (empty oval), S-peptide tag (striped oval), 3c protease cleavage site (dotted box) and soluble region (white box). Note that the CSP-1 portion of the recombinant protein is not to scale so that the N-terminal region can be more easily visualized. (B) SDS-PAGE (4–12%) analysis of rCSP-1 stained with Simply Blue. Lane 1, metal chelate affinity chromatography of rCSP-1; lane 2, molecular weight markers; lane 3, rCSP-1 re-purified using con A lectin affinity chromatography.

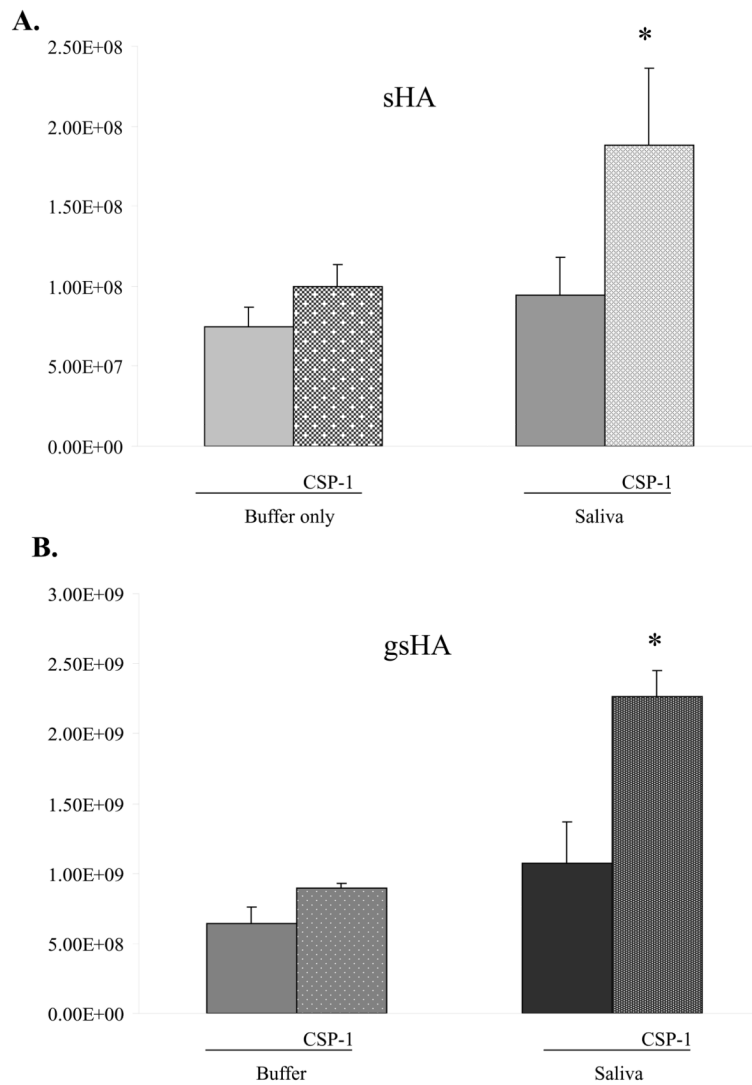


Figure 5. Recombinant human CSP-1 protein (rCSP-1) enhances bacterial adherence to experimental salivary pellicle (sHA) and to glucans synthesized in the pellicle (gsHA) formed on hydroxyapatite surface

Modulation of bacterial adherence to sHA (panel A) and gsHA (panel B) by *S. mutans* exposed to rCSP-1 (in PBS or in clarified human whole saliva, left and right, respectively). The data shown are mean values \pm standard deviations (n=6). Values marked with an asterisk are significantly different from each other ($P < 0.05$, ANOVA, comparison for all pairs using Tukey's test).