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STATHERIN: A MAJOR BOUNDARY LUBRICANT OF HUMAN SALIVA

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The lubricating properties of human submandibular-sublingual salivary fractions were examined using a servohydraulic model of mandibular movement. Fractions containing statherin exhibited a strong tendency to boundary lubrication. The lubricity of purified statherin was confirmed and compared to the amphipathic molecules gramacidin S and sodium dodecyl sulfate. Contact angle measurements of statherin paralleled the other amphipathic molecules. The helical content of statherin increased in trifluoroethanol indicating the presence of amphipathic helical regions. CD studies and hydrophobic moment calculations indicated that statherin adopts an amphipathic helical conformation at the N-terminus. An energy-minimized model of the polar N-terminal residues 1-15 suggested that this domain could be positioned in space to interact with a hydroxyapatite substrate. These data imply that under appropriate conditions statherin may display an amphipathic nature which enables it to function as a boundary lubricant on enamel.

Human saliva, in common with other humoral fluids of the body, plays a major role in lubrication (1-4). Reeh and coworkers (5) suggest that human saliva is a tribologically complex fluid operating between a variety of hard and soft tissue interfaces under differing oral conditions, including dental incisal function, light and heavy mastication, and dental bruxing. There is evidence that the tribological behavior of human saliva is expressed in different lubricity mechanisms, which is a manifestation of the structural properties of different salivary macromolecules (5,6). Of the major salivary secretions, submandibular-sublingual saliva (HSMSL) had the greatest potential to vary these mechanisms (5). Several studies have shown that the salivary glycoproteins, including mucins, are effective lubricants under light masticatory conditions (3,4,7). These glycoproteins and their complexes provide thin film lubrication under dental conditions of low occlusal load and high sliding speed. Physicochemical factors that contribute to this lubrication may be water retention due to solvation of carbohydrate residues with consequent effects on colligative properties and viscosity modification of saliva (5). Conversely, so-called boundary lubrication under heavy masticatory conditions depends on the maintenance of an adsorbed amphipathic film, as exemplified by sodium dodecyl sulfate (SDS) on enamel which may be used as a positive control (8).

The object of the present study was to investigate the lubricity of HSMSL fractions and identify those components responsible for boundary lubrication under conditions that model the

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approximate movements and forces generated by the human mandible in the oral environment (3,4,7).

MATERIALS AND METHODS

Preparation of salivary fractions and purification of statherin.

Human submandibular-sublingual saliva (HSMSL) from a 30-year-old healthy female donor was collected and processed as previously described (9). The proteins and glycoproteins of HSMSL were initially fractionated into four pools (A-D) according to size by gel filtration on Sephadex G-200 using 100 mM Tris-HCl, pH 7.5, with guanidine HCl (10). Pool A contained high and low molecular weight salivary mucins (MG1 and MG2, respectively), sIgA and amylase. Pool B contained unidentified 43 and 37 kDa components and small amounts of acidic proline-rich proteins. Pool C contained predominantly cystatins and acidic proline-rich proteins; while Pool D contained mostly statherin and lesser amounts of basic proline-rich peptides. Statherin was purified from Pool D as previously described (10). Briefly, ~110 mg of Pool D in 10 ml of 5 mM Tris-HCl, pH 7.6, was subjected to chromatography on DE-52 cellulose. Basic proline-rich proteins were eluted from the column (2.5 x 50 cm) with 500 ml of 5 mM Tris-HCl, pH 7.6. After washing with an another 500 ml of 100 mM Tris-HCl, pH 7.6, statherin was eluted with a linear salt gradient consisting of 500 ml of 100 mM Tris-HCl, pH 7.6 and 500 ml of 100 mM Tris-HCl, pH 7.6, with 1 M NaCl. Fractions were collected at 6°C at a flow rate of 15-20 ml/h. After dialysis and lyophilization, statherin was further desalted using Sephadex G-25 with 0.02 M acetic acid.

Structure prediction.

Based upon the amino acid sequence of statherin (Figure 1), the secondary structure predictions were performed according to Chou-Fasman (11) and Garnier-Osguthorpe-Robson (12) methods. The hydrophobic moment calculations were carried out using Eisenberg's algorithm (13).

Circular dichroism (CD) studies were performed on a Jasco J-600 spectropolarimeter interfaced to an IBM Model 30 Computer. The instrument was calibrated using an aqueous solution of 0.06% (w/v) ammonium d-10 camphorsulfonate. Spectral data were recorded at 0.2 nm intervals in the range 240-190 nm at a sample concentration of 1 mM using a cell pathlength of 0.1 cm. The molar ellipticity, $[\Theta]$, was calculated with a mean residue molecular weight of 128.1. The percent helix content was calculated as described by Chen et al. (14). All the CD spectra were corrected for solvent refractive index and represent the average of 5 scans at a scan speed of 10 nm/min.

Molecular model building studies were carried out using the program SYBYL 5.3 (Tripos Associates, St. Louis, MO). The residues 1-4 were built as a β-turn followed by a helical segment for residues 5-15. The molecular model was energy minimized using conjugate-gradient algorithm and the parameters from the Tripos force field.

Measurement of lubricity.

The apparatus for the assessment of lubricity effects is based on a servohydraulic model of mandibular movement. This system has been extensively described in the literature under the terminology of artificial mouth (15-19) and is capable of measuring wear and friction simultaneously (5,8). Based upon experience (5), Pools A-D were prepared at a concentration of 1 mg dry weight/ml deionized water.

Maxillary and mandibular elements were obtained from flat slabs of bovine enamel which were cut from teeth using a diamond saw under light pressure and copious amounts of water. A chemically clean and reproducible enamel surface was achieved at the start of each experiment by the acid etch technique (5), and polished by ultrafine alumina, followed by washing with copious amounts of water. The experiments were carried out in a micro-chamber (capacity of 3 ml) with a double circulation maintaining the environment of the enamel and the circulating salivary fluids at 37°C as monitored by a thermocouple. With at least 3 ml of the sample circulating, the enamel surfaces were brought into contact under load control and a reciprocating action of the lower enamel surface against the upper was initiated. Individual experiments involving salivary fractions were of short duration, typically less than 15 minutes. The frictional force reached after equilibrium was measured by comparison with a calibration curve. The following biomechanical conditions were imposed on the experimental setup: four sliding speeds were used from 1.99 mm/s to 7.84 mm/s and five vertical forces from 3.8 N to 19.5 N; providing 20 biomechanical conditions for each experiment.

Using this protocol, lubricity experiments were carried out on purified statherin and the amphipathic antibiotic gramicidin S (G-5127, Sigma Chem. Co., St. Louis, MO). Contact angles

Table 1. Means for coefficients of friction for Pools A-D under 19.5 N occlusal load and varying sliding speeds

Speed (mm/s)	Water (11)#	Pool A (11)	Pool B (6)	Pool C (6)	Pool D (11)	p*
1.99	0.79	0.74	0.62	0.65	0.47	0.0003
3.53	0.73	0.69	0.64	0.67	0.48	0.0065
5.67	0.69	0.68	0.65	0.66	0.51	0.0628
7.84	0.67	0.67	0.64	0.68	0.53	0.1777

Numbers in parentheses indicate the number of trials carried out for each sample.

* The p value is the statistical significance obtained for simple effects using the means of coefficient of friction for all the pools and water. A value less than 0.05 is significant. For example, at the conditions of 1.99 mm/s speed and 19.5 N occlusal load, the coefficient of friction for water and Pool D were significantly different whereas at 7.84 mm/s and 19.5 N, they were not.

were determined on bovine enamel (30° incline) for water, 1 mg dry weight/ml aqueous solutions of statherin and gramicidin S, and 0.0025-0.25% solutions of SDS.

RESULTS AND DISCUSSION

The means for the coefficients of friction for each of the biomechanical conditions show the order of increasing lubricity to be: distilled water (negative control) < Pool A < Pool B ~ Pool C < Pool D (Table 1). The higher lubricity of Pool D over Pool A is due to the fact that the chosen biomechanical conditions (19.5 N) select for boundary lubrication (5,20). Tests for simple effects show strong statistical significance at the lowest speeds (Table 1). By reference to differences between means, the statistical significance is attributed to the difference between Pool A and Pool D. Associated with these lubricity differences, Table 2 shows a change in the velocity gradient of the coefficient of friction, which has been linked both experimentally and theoretically with intermittent motions due to slip-stick friction (21-23). The negative velocity gradient, which is markedly present in water and Pool A, is reduced with Pool B and C, and is almost completely absent with Pool D. Thus, in changing from Pool A to Pool D, a transition takes place from slip-stick friction to a smoother friction between the opposing enamel surfaces. Spikes and Cameron (20), Frewing (24), and others have shown that the elimination of slip-stick friction on susceptible surfaces is associated with the development of a boundary lubrication following the deposition of

Table 2. Velocity gradient of the coefficient of friction for Pools A-D. The higher negative velocity gradients are closely associated with the phenomenon of slip-stick friction (20,22) and the transition from slip-stick to smooth friction is associated with the development of boundary lubrication (20,24)

	3.8 N	6.7 N	11.5 N	15.0 N	19.5 N	
Water	-0.0375	-0.0336	-0.021	-0.021	-0.020	Slip-stick
Pool A	-0.037	-0.021	-0.014	-0.017	-0.011	I
Pool B	-0.043	-0.022	-0.007	-0.004	+0.003	I
Pool C	-0.032	-0.003	-0.0006	+0.005	+0.004	I
Pool D	-0.016	-0.019	-0.006	-0.0002	+0.011	Boundary lubrication

Table 3. Means of coefficients of friction of various amphipaths under 19.5 N occlusal load and varying sliding speeds

Speed (mm/s)	Water (2)#	Gramicidin S (2)	Statherin (2)	SDS (2)
1.99	0.89	0.57	0.47	0.13
3.53	0.78	0.55	0.44	0.11
5.67	0.72	0.56	0.43	0.11
7.84	0.72	0.59	0.43	0.11
Average velocity gradient	-0.026	0.0038	-0.0066	-0.0031

Numbers in parentheses indicate the number of trials carried out for each sample.

an adsorbed oriented monolayer on the substrate surfaces. The strong inference from the present work is that one or more components in Pool D can be considered a boundary lubricant.

Statherin is the major component in Pool D (10) and has been shown to strongly adsorb to enamel surfaces (25, for review). Therefore, single blind lubricity experiments were carried out on purified statherin, the amphipathic antibiotic gramicidin S, and SDS. The strong tendency to boundary lubricity by statherin was confirmed by the low coefficient of friction across all conditions (Table 3). Typically solutions of amphipaths up to the critical micelle concentration will increase the spreading power of water on substrates amenable to the formation of oriented adsorbed films (26,27). This phenomenon is demonstrated in Figure 2 showing the decrease in receding contact angle by solutions of purified statherin which paralleled the other amphipathic molecules, gramicidin S and SDS.

The primary structure of statherin is highly asymmetric with almost all of the charged residues clustered in the N-terminal region (Figure 1). This provides statherin with a polar end (residues 1-15) and a less polar C-terminal tail. From the secondary structure prediction and hydrophobic moment calculations, an amphipathic helical segment corresponding to residues 5-15 was identified (Figure 3). The peak hydrophobic moment value for this segment was 0.55. The helical model built for this segment showed the segregation of hydrophilic and hydrophobic residues to opposite faces of the helix. The CD spectrum of statherin (1 mM concentration in 20 mM phosphate buffer, pH 7.2) indicated about 15% helical content. Spectra of samples dissolved in 50% (v/v) trifluoroethanol showed an increase of helicity to approximately 25% (Figure 4). An increase in helicity in the presence of trifluoroethanol can be indicative of potentially amphipathic helical regions (28).

Hauschka and Wians (29) demonstrated that osteocalcin adopts an amphipathic helical structure to interact with hydroxyapatite in the extracellular organic matrix of bone. From the CD studies and hydrophobic moment calculations, it appears that statherin may adopt an amphipathic

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     5        10        15        20        25        30        35        40
D-S-S-E-E-K-F-L-R-R-I-G-R-F-G-Y-G-Y-G-P-Y-Q-P-V-P-E-Q- P-L-Y-P-Q-P-Y-Q-P-Q-Y-Q-Q-Y-T-F
  | |
  P P
- - - - -+    ++   +
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Figure 1. The primary structure (43 residues) of statherin (34).

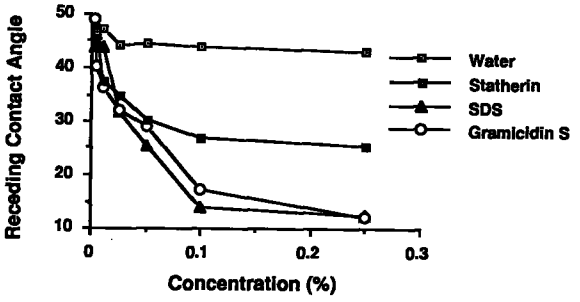
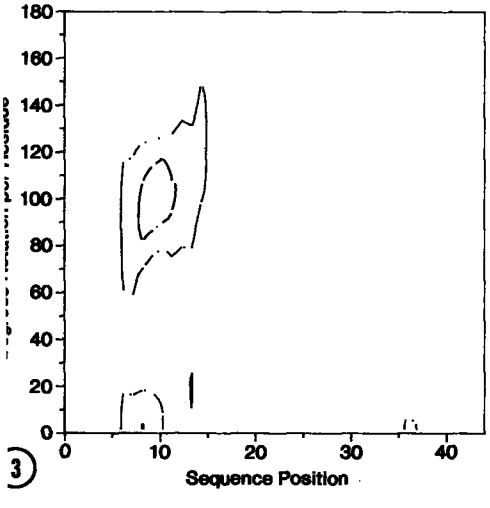


Figure 2. The plot of contact angle versus concentration of lubricant. A drop of the sample was measured using an incline angle of 30° (receding contact angle). The similarity between statherin and the other amphipaths gramicidin S (35) and SDS is conspicuous up to a concentration of 0.05%.

helical conformation at the N-terminus. Further, in the energy-minimized model, the N-terminal region (e.g. residues 1-15) appears to be suitably positioned in space to interact with a solid hydroxyapatite substrate. The distance between two phosphate groups is 5.4 Å in the model which corresponds to the distance between two calcium ions in the crystal structure of hydroxyapatite. Also, the other hydrophilic side chains at one side of the helix may interact with the surface. Other molecules that act as lubricants at interfaces include the lung surfactant proteins, SP-1 and SP-2, which have also been shown to contain amphipathic helical regions in their structure (30).



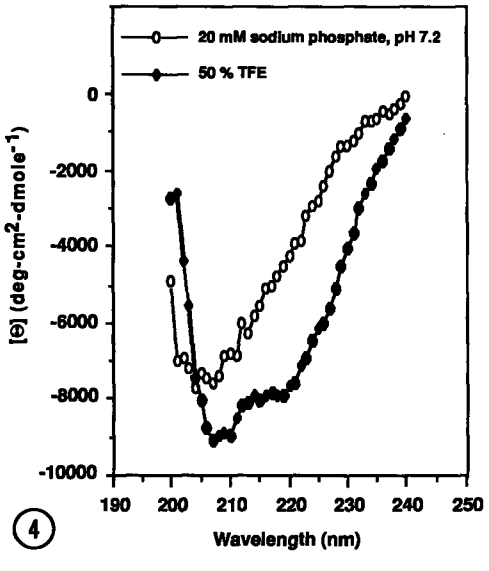


Figure 3. The hydrophobic moments calculated for varying degrees of rotation are shown as a contour plot in which the residue position is plotted against the degree of rotation. The contours are at an interval of 0.20 starting at 0.35. The window corresponding to residues 5-15 show a peak moment value of ~0.6.

Figure 4. CD spectra of statherin. The increase in helicity is seen from the increase in the intensity of the negative band at 222 nm. In the presence of trifluoroethanol, the amount of helicity present is 25%.

Moreno et al. (31) have calculated the cross-sectional area of statherin based on a globular form to be 4.37 nm^2 per molecule. Using their value for the maximum number of adsorption sites and Avogadro's Number, it can be shown that the experimental value for the cross-sectional area of the adsorption site of statherin to be 3.29 nm^2. This difference points to an asymmetry in the adsorbed molecule which may be compatible with its conformation as an amphipathic species. The role of amphipathic oriented films in the study of boundary lubrication is exemplified by simple amphipathic molecules such as fatty acids and their analogs (20,24,32,33). In the case of statherin, the polar end (residues 1-15) is essential for the adsorption of the molecule such that it is not easily desorbed or swept aside from the hydroxyapatite surface by the antagonistic tooth cusp. The oriented hydrophobic residues may further stabilize the film by London dispersive forces (20,32) and offer reduced resistance to horizontal sliding. Such boundary lubrication is in contrast to that of thick film lubrication where other physicochemical parameters such as viscosity, are important. In preliminary experiments, synthetic fragments of statherin that correspond to residues 1-15, 5-15, 15-29, 29-43, 19-43 did not display boundary lubricity as compared to the intact molecule (data not shown). These results suggest that the lubricity exhibited by statherin requires both a polar end and a less polar tail to form an oriented film at the enamel surface.

In summary, statherin has been previously shown to play an important role in mineralization processes in the oral cavity (25). First, it inhibits the primary precipitation of calcium phosphate salts which prevents spontaneous formation of calculi within the salivary ducts and also prevents extracoronal crystal growth on teeth. Second, it controls secondary crystal growth intracoronally by providing epitaxial repair of incipient caries lesions and the maintenence of integrity of the tooth's mineralized matrix. The present report has shown that under appropriate conditions, statherin may display an amphipathic nature which permits it to function as a boundary lubricant on enamel. The provision of boundary lubrication provides a defense against slow sliding and high occlusal loads that occur during mastication and bruxing.

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