Published in final edited form as:

Arch Oral Biol. 2009 April ; 54(4): 345–353. doi:10.1016/j.archoralbio.2008.11.010.

Concentration and Fate of Histatins and Acidic Proline-rich Proteins in the Oral Environment

Melanie Campese, Xiuli Sun, Jos A. Bosch, Frank G. Oppenheim, and Eva J. Helmerhorst*

Abstract

Saliva plays a critical role in the protection of oral hard and soft tissues and contains a multitude of constituents with well-characterized biological activities *in vitro*. Among these are histatins and acidic proline-rich proteins (PRPs). Nevertheless, few functional studies have recognized the structural instability of these proteins in the proteolytic environment of whole saliva. The aim of this investigation was to determine histatin and acidic PRP levels in parotid secretion (PS) and in whole saliva (WS) as well as to establish their susceptibility to proteolysis in these salivary fluids. Using cationic polyacrylamide gel electrophoresis and densitometric analysis the average total histatin concentration (histatin 1+3+5) in WS was determined to be 33.3 ± 16.7 $\mu\text{g/ml}$ ($n=22$) and the average total acidic PRP concentration (PRP1/PIF-s+PRP3/PIF-f) was 427.9 ± 123.3 $\mu\text{g/ml}$ ($n=22$). Histatin and acidic PRP concentrations in PS were 6 and 1.5 times higher than in WS ($n=7$), respectively. WS histatin and acidic PRP levels each correlated significantly with WS total protein concentrations ($P < 0.01$ and $P < 0.05$, respectively), as well as with each other ($P < 0.01$). Stability studies of histatin 3 and PRP1/Pif-s in PS revealed $t_{1/2}$ times of 7.2 ± 5.5 hr and 50.3 ± 24.8 hr, respectively ($n=7$). Histatin 3 (40 $\mu\text{g/ml}$) and PRP1 (400 $\mu\text{g/ml}$), added to WS in concentrations equivalent to their concentrations in PS, disappeared at a much faster rate, with $t_{1/2}$ values of 1.7 ± 1.6 min and 29.3 ± 15.3 min, respectively ($n=7$). The data indicate that proteolysis in WS is an important factor in explaining the substantially lower concentrations of histatins and acidic PRPs in WS as compared to in glandular secretions.

Keywords

saliva; proteolysis; histatin; proline-rich proteins; enzymes; oral; peptides

Introduction

Several of the biochemical constituents of saliva, such as acidic and basic proline-rich proteins (PRPs), lysozyme, lactoferrin, peroxidase, histatins, cystatins, mucins and secretory immunoglobulins A (s-IgA), have been isolated in sufficient quantities, mostly from pure glandular secretions, enabling studies to investigate their functional roles (1). Major groups of these salivary proteins, including acidic PRPs, statherin and histatins, have characteristics deemed important for oral health (2–6). Their beneficial properties comprise the inhibition of primary and secondary calcium phosphate precipitation which is essential for maintaining tooth enamel integrity (statherin, PRPs) and antimicrobial activities critical for the host defense

*Author for correspondence: Eva J. Helmerhorst, Ph.D., Boston University, Goldman School of Dental Medicine, 700 Albany Street, W-201, Boston, MA 02118, USA, Phone: 617-414-1119, Fax: 617-638-4924, Email: E-mail: helmer@bu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

against oral infections (histatins). More recently, histatins were found to increase the migration of transformed oral buccal epithelial cells in an in vitro model for wound healing (7). Based on the biological activities of these and other salivary proteins, saliva is believed to represent an early, principal barrier to oral infections, including dental caries and periodontal disease, and to facilitate healing.

It is frequently overlooked that oral fluid and the pure major glandular secretions are distinctly different in terms of their protein/peptide compositions, despite the fact that contributions from the major glands make up the bulk of the oral fluid volume. The release of glandular salivary proteins into the oral cavity, being the port of entry to the gastro-intestinal tract, involves the transition from a sterile to a non-sterile environment. This transition is concomitant with the mixing of the pure secretions with a variety of non-exocrine components present in whole saliva including multiple of host and bacteria-derived proteases. Two-dimensional PAGE patterns of parotid secretion (PS) and submandibular/sublingual secretion (SMSL) on the one hand and WS on the other hand have been shown to be distinctly different (8,9). While these differences could in part be explained by the non-exocrine components of WS, the appearance of multiple low molecular weight peptides in WS strongly points toward salivary protein fragmentation. Evidence for extensive proteolytic processing of salivary proteins in WS was also obtained in recent proteomic analyses. Specifically, peptides derived from acidic and basic PRPs, statherin and histatins were found to be major constituents of the whole saliva peptidome (10–12) indicating that these proteins in particular display a high level of susceptibility to whole saliva proteases.

While PRPs, statherin and histatins have been considered to be among the functionally most relevant salivary proteins, their structural instability in the oral environment has been poorly characterized or recognized with regard to their activities in the oral cavity. We hypothesize that the degradation of the parent glandular salivary proteins into smaller fragments by WS associated proteases will have important functional consequences. The aspect of salivary proteolysis is critical to make inroads into understanding the structure-function relationship of proteins derived from the oral exocrine system. Since WS is commonly expected to contain the elements crucial for the maintenance of oral health, the aim of the present study was to provide a quantitative comparison of histatins and acidic PRPs levels in WS and in PS, and to estimate their degradation rates in the oral environment.

Materials and Methods

Salivary proteins

Synthetic histatins 1, 3, and 5 were obtained from the American Peptide Company (Sunnyvale, CA) and from Quality Controlled Biochemicals (Hopkinton, MA). Acidic PRP1 and PRP3 were purified from parotid secretion (PS) by anion-exchange chromatography. Dialyzed and lyophilized PS proteins were dissolved in buffer A consisting of 50 mM Tris/HCl and 50 mM NaCl, (pH=8), applied to a MonoQ HR16/10 column (Amersham Biosciences, Uppsala, Sweden) and eluted at a flow rate of 2 ml/min with buffer B containing 50 mM Tris/HCl and 1 M NaCl (pH=8) using the following gradient steps: 0–38 min: 0–13% buffer B; 38–233 min: 13–22% buffer B; 233–250 min: 22–40% buffer B. All acidic PRP isoforms eluted in the shallow portion of the gradient. The purity of the synthetic histatins and isolated PRPs was verified by cationic, anionic PAGE, and reversed-phase analysis (13).

Subject selection

Twenty two orally healthy donors were recruited at Boston University Medical Center. All participating subjects consented to the collection of saliva in accordance with approved

protocols of the Institutional Review Board at Boston University. The donors ranged in age from 21 to 40 years of age and presented with good oral health conditions.

Collection of whole saliva (WS) and parotid saliva (PS)

All saliva samples were collected in graduated cylindrical tubes placed on ice. To collect unstimulated WS, the donors were asked to expectorate the fluid that had naturally accumulated in their oral cavity (~1 ml). To collect 5 ml of masticatory stimulated WS, subjects were provided with 1.5 g of parafilm for chewing. For histatin and acidic PRP quantitation studies, PMSF and EDTA (1 mM and 2.5 mM final concentrations, respectively) were added to WS immediately upon collection. Whole saliva supernatant (WSS) was obtained by centrifugation of the samples at $14,000 \times g$ for 10 min at 4°C. All WS derived samples were boiled and kept on ice before further processing. Parotid secretion (PS) was collected under conditions of gustatory stimulation using hard sour candies and a Carlson-Crittenden device (Lashley cup) positioned over the Stensen's duct (14). A 25 µl aliquot of PS was plated on blood agar (Hardy Diagnostics) to verify the sterility of the collected PS secretion (absence of WS contamination).

Histatin and PRP stability studies

For studying the stability of histatins and acidic PRPs in PS and in WS, saliva specimens were collected in the absence of protease inhibitors and not boiled. The stability of endogenous histatin and PRP levels in PS was determined by incubating a freshly collected PS sample in a 37°C water bath. After 0, 2, 4, 6, 8, 24, 48, 72 and 96 hours of incubation, 100 µl aliquots were removed, boiled and subjected to anionic or cationic PAGE. For stability studies in WS, synthetic histatin 1, 3 and 5 (40 µg/ml each) or pure isolated PRP1 (400 µg/ml) were added to freshly collected WS from seven individual subjects and incubated in a 37°C water bath for 0, 5, 10, 30, 60, 120, 180, and 240 min. Sample aliquots of 100 µl were taken at each of these time points, boiled and analyzed by cationic or anionic PAGE.

Cationic and anionic PAGE

To all boiled 100 µl sample aliquots, EDTA was added to a final concentration of 2.5 mM to chelate calcium and other divalent cations, thereby improving protein resolution during electrophoresis. All samples were dried in a Vacufuge speedvac (Eppendorf, Hamburg, Germany) and resuspended in sample buffers suitable for cationic or anionic PAGE. Cationic PAGE (15% w/v) was performed essentially as described (15,16). Gel polymerization was accelerated by exposure of the gel to a light source (60 Watts). After electrophoresis of the samples was completed, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid, and subsequently destained. Anionic PAGE was conducted as reported previously (17,18), using a separating gel of 7.5% (w/v). Gel staining was achieved with 2.5% (w/v) amido black in 7% (v/v) glacial acetic acid, followed by destaining in the same solution without the dye.

Densitometric analysis

Gels were scanned with the Versadoc Imager (BioRad, Hercules, CA), and the pixel intensities of the protein bands of interest were quantitated using Quantity One software (Biorad). Measured intensities were corrected for pixel intensities of a similar volume of gel not containing protein (background staining). The background-corrected pixel densities of the experimental protein bands were compared to standard curves generated for each of the major histatin and PRP proteins to yield quantitative measures for each of the proteins.

Statistical Analysis

Linear regression and Pearsons correlation coefficients were applied to estimate the associations among histatins, aPRP, and total protein concentrations. Wilcoxon non-parametric

analyses for 2 related samples were carried out to investigate differences in histatin and PRP levels in WS and PS samples. All statistical analyses were performed using SPSS for windows 15.0 (SPSS Inc, Chicago, IL).

Results

Range in histatin and acidic PRP concentrations in WS from 22 subjects

Proteins in WS samples were electrophoretically separated by cationic PAGE or anionic PAGE. In contrast to in SDS PAGE, histatins and PRPs can be separated from all other abundant salivary proteins by cationic and anionic PAGE, respectively, and display unique electrophoretic mobilities on each gel type (15,19). Histatin and PRP levels were quantitated by densitometric analysis. Standard curves were generated for each protein and differential staining efficiencies emphasized the need to compare each protein to be quantitated in the saliva sample to its respective standard. Two protein standards were present on each experimental gel to correct for potential gel to gel variations (not shown). Visual inspection of the cationic PAGE gels (Figures 2A, C and E) reveals a rather large inter-individual variation in histatin concentrations. Average total histatin levels (histatin 1+3+5) in WS were found to range from 15.7 to 64.9 µg/ml (Table 1). The median concentration of histatin 1 was highest (median=17.0 µg/ml), followed by histatin 5 (median=6.9 µg/ml) and histatin 3 (median=5.6 µg/ml). The acidic PRPs in WS from the same subjects were separated by anionic PAGE (Figure 2B, D, F) which allows partial resolution of the various acidic PRP isoforms. In this gel system PRP1 and PIF-s displayed similar electrophoretic mobilities as do PRP3 and PIF-f. PRP2 and PRP4 can be distinguished by their somewhat higher electrophoretic mobility compared to PRP1/PIF-s and PRP3/PIF-f, respectively. Since among the PRP genes, the PIF and PRP1 genes show the highest frequency of occurrence in the population (2,20), we used the intensities of the PRP1/PIF-s band and the PRP3/PIF-f band for PRP quantitation. For simplicity these bands are hereafter referred to as PRP1 and PRP3. The PRP1+PRP3 concentrations in WS from the 22 donors ranged from 267.2 to 720.6 µg/ml (Table 1). PRP1 and PRP3 showed virtually identical median values in WS of the 22 samples analyzed, namely 210.3 and 205.3 µg/ml, respectively.

Associations of histatin, PRP and total protein levels in WS

In Figure 3, WS total protein levels in the 22 samples are plotted against the respective WS total histatin levels (A), and total PRP levels (B). Total histatin levels were also plotted against total PRP levels (C). Visually, all three graphs suggested a positive correlation between the plotted parameters, which was confirmed by statistical analysis ($P<0.01$, $P<0.05$, and $P<0.01$, respectively). The findings indicate that subjects with overall higher salivary protein contents tended to have higher histatin levels as well as higher PRP levels.

Comparison of histatin and PRP levels in WS, WSS and PS

A subset of the saliva donors provided stimulated as well as unstimulated WS, stimulated and unstimulated WS supernatant, and stimulated PS. Histatin and acidic PRP concentrations were determined in all five salivary specimens (Table 2). In all WS samples, average histatin levels were histatin 1>histatin 5>histatin 3. Total histatin levels in stimulated and unstimulated WSS were lower than in stimulated and unstimulated WS ($p<0.05$), suggesting that a fraction of the histatins are cell-bound. The average PRP1 levels were similar to the average PRP3 levels in all WS samples analyzed. In contrast to histatins, total average PRP levels in WS and WSS showed no differences. Total average histatin levels in stimulated PS were substantially higher than in stimulated WS, showing a ratio of approximately 6 to 1 ($p<0.05$). The concentration differences of the acidic PRPs in stimulated PS and stimulated WS were not as dramatic as those observed for histatins, showing a ratio of PS to WS of 1.5 to 1 (NS).

Stability of endogenous histatins and acidic PRPs in PS

Further studies focused on the stability of histatins and PRPs in PS (Figure 4) and in WS (Figure 5). The histatin degradation patterns in a PS sample from 1 subject are shown in Figure 4, and similar studies were carried out with PS from 6 additional subjects. The stability of endogenous histatin 3 in PS as a function of time was determined by densitometric analysis of the histatin 3 band, producing a degradation curve as shown in Figure 4B. The “half life” values ($t_{1/2}$) of histatin 3 in PS were established from such plots, and averaged for the seven subjects (Table 3A). The $t_{1/2}$ values of histatin 3 in PS showed rather large inter-individual differences, ranging from 1.3 to 17.7 hours with an average of 7.2 ± 5.5 hr. The stability of acidic PRPs in PS was assessed by anionic PAGE (Figure 4C) and the intensity of the PRP1 band was quantitated and plotted (Figure 4D). The obtained $t_{1/2}$ values ranged for the seven subjects from 17.4 to 96.0 hours (Table 3A) demonstrating once more the rather pronounced inter-individual differences in PS proteolytic activity. On average, the half life values for PRP1 in PS were 50.3 ± 24.8 hrs.

Stability of histatin 3 and PRP1 added to WS

Since the natural histatin 3 levels in WS were low (Table 2 and Figure 5A far right lane), the degradation rates of histatin 3 in WS were determined by spiking WS from 7 individual subjects with a physiological concentration of pure synthetic histatin 3 (40 $\mu\text{g/ml}$; Figure 5A far left lane). The histatin 3 concentration selected for spiking was within the range of naturally occurring concentrations of this protein in PS. Plotted in Figure 5B is the pixel intensity of the added histatin 3 protein band as a function of incubation time. The resulting degradation curve clearly illustrates the rapid proteolysis of histatin 3 in WS as evidenced from the low $t_{1/2}$ values (Table 3B). For some subjects, the added histatin 3 protein disappeared in less than 1 minute (the first sampling time point). On average, $t_{1/2}$ values of 1.7 ± 1.6 min were observed. WS from the same subjects was also spiked with a physiological concentration of pure PRP1 (400 $\mu\text{g/ml}$). Added and endogenous PRP1 levels in WS are shown in the flanking gel lanes in Figure 5C. The total intensity of the PRP1 band as a function of time was plotted in Figure 5D. As for histatins, acidic PRP undergo rapid proteolytic degradation at an average $t_{1/2}$ value of 29.3 ± 15.3 min (Table 3).

Discussion

Knowing the availability and structural integrity of histatins and acidic PRPs in salivary fluids is of major importance with regard to their presumed roles in the maintenance of health in the oral cavity. The aim of this study was to gain insight into how much of the intact protein can be found in WS, the fluid representing the oral environment surrounding important structures such as teeth and the gingival tissues. The results showed that histatins and acidic PRPs are detectable in WS but, especially in the case of histatins, at substantially lower levels than in PS.

There are several factors that may contribute to the observed concentration differences. It has been well documented that in the oral cavity, proteins undergo complex formation with other proteins (21–23) and adsorb to tooth and buccal epithelial surfaces. For instance, intact histatins and other salivary proteins have been detected in the acquired enamel pellicle (24–26), and the oral mucosal pellicle (27). Adsorption of proteins to oral structures may in part contribute to the reduced concentration of these proteins in expectorated WS, although the amount of protein in the various pellicles (totaling μg levels) could not easily account for the concentration differences between PS and WS. WS proteolysis is another important factor to be considered. Oral bacteria and their products are presumably to a large extent responsible for the degradation of proteins in the oral environment (28). We aimed to estimate the degradation rates by determination of the half life ($t_{1/2}$) values of individual salivary proteins in the WS environment. From the obtained mean $t_{1/2}$ for histatin 3 in PS (7.2 hr, equaling 432 min) and in WS (1.7 min)

it can be calculated that the rate of disappearance of histatin 3 in WS is about 250x times faster than in PS. The mean $t_{1/2}$ of PRP1 in PS (50.3 hr, equaling 3018 min) and in WS (29.3 min) show that this protein is about 100x faster proteolytically cleaved in WS than in PS. There are several conclusions to be drawn from these observations. First, the rate of proteolysis of histatin 3 and PRP1 in PS is slow ($\mu\text{g}/\text{hour}$). It is questionable whether the low proteolytic activities in PS are of physiological relevance considering that the residence time of proteins in the ductal system, at least during flow conditions, is a matter of minutes. Second, the observed extremely fast degradation rates of proteins in the WS environment, in particular of histatin 3, provides strong evidence that proteolysis is a major determinant for protein disappearance in WS and the most plausible explanation for the concentration differences of histatin 3 and PRP1 in PS and WS.

The concentrations of histatin 3 and PRP1 selected for the WS spiking experiment differed by a factor 10 (40 $\mu\text{g}/\text{ml}$ and 400 $\mu\text{g}/\text{ml}$, respectively) and were chosen to approximate natural concentrations of these proteins in glandular secretions. The rate disappearance of the substrates (S), histatin 3 or PRP1, from WS, $\Delta S/\Delta t$, appeared constant up to $t_{1/2}$ as evidenced from the semi-linear rate of substrate degradation over time. Such linear relationships would indicate that the WS proteolytic enzymes involved in histatin and PRP1 proteolysis were saturated and operated at their V_{max} during this time interval. From the mean $t_{1/2}$ values it can be calculated that histatin 3 disappeared from WS at an apparent rate of $20 \mu\text{g}/1.7 \text{ min} = 11.8 \mu\text{g}/\text{min}$ whereas PRP1 was degraded at an estimated rate of $200 \mu\text{g}/29.3 = 6.8 \mu\text{g}/\text{min}$. On a molar bases, these values are 2.9 nmol/min for histatin 3 and 0.44 nmol/min for PRP1. The data indicate that on a gram and on a mole basis PRPs are slower degraded than histatins in the oral environment.

An important observation made in these studies is the apparent inter-subject variation, not only in the concentrations of PRPs and histatins, but also in their rates of degradation. Within subjects though, the results obtained were very reproducible. For instance, some individuals consistently showed high levels of histatins in PS whereas others were consistently low (not shown). Large inter-subject differences in the concentrations of bioactive salivary proteins without apparent clinical consequences is of high interest and could be explained by the redundancy principle. In this model, several different salivary proteins exhibit overlapping activities and therefore render saliva redundant with respect to function. For instance, besides histatins, also lactoferrin, lactoperoxidase, lysozyme and defensins display antimicrobial features in the oral environment. Similarly, remineralization properties ascribed to acidic PRPs are exerted by statherins as well. In view of this it is speculative to what extent overall saliva function is affected by WS proteolysis of certain susceptible proteins. The wide range in “normal” levels and the redundancy phenomenon are also important factors to be considered from a diagnostic point of view. It is now well recognized that multi-analyte approaches as opposed to single parameter comparisons in oral fluid diagnostics hold great promise for finding meaningful correlations between saliva composition and (oral) disease (29).

The aspect of salivary proteolysis is critical to make inroads into understanding the structure-function relationship of proteins derived from the oral exocrine system and relate the fate of these proteins to their role in maintaining oral health. The 6:1 ratio of PS:WS for histatins would imply that histatins in WS are for the most part present in a fragmented state. In this respect it is of interest to note that prior structure function studies involving histatins and acidic PRPs have demonstrated that both families of proteins contain functional domains constituting a part of the actual secreted salivary protein (30). For instance, antifungal domains of histatin 3 have been localized in the middle region of the molecule (31) as well as in an N-terminal 12 residue peptide designated as P-113 (32). In acidic PRPs, the phosphorylated N-terminus contains a segment responsible for the inhibition of calcium phosphate precipitation (33). It can thus be hypothesized that fragmentation of these proteins in the oral cavity would not

necessarily lead to a loss of function. Indeed, our lab recently demonstrated this to be the case for histatins by monitoring the anti-candidal activity of a histatin 5/WSS degradation mixture over time (13).

While proteolysis may be conceived as destroying salivary proteins, it can also be argued that oral fluid proteolysis could serve to liberate smaller bioactive peptides from larger, less or inactive, precursor proteins (34). In support of this, the group of Bennick has demonstrated that, on a weight basis, in vivo generated peptides derived from acidic PRPs were more effective in inhibiting the formation of hydroxyapatite crystals than intact acidic PRPs. (35,36). A full assessment on how salivary enzymes modulate salivary protein structure and function is likely to make a major contribution to the elucidation of the principal mechanisms allowing saliva to be important in host protection.

Acknowledgements

The authors thank Adel S. Alagl for conducting preliminary studies for this project and Nerline Grand-Pierre for technical assistance. This study was supported by NIH/NIDCR grants DE05672, DE07652, DE16699 and DE18132.

References cited

1. Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhorst EJ. Salivary proteome and its genetic polymorphisms. *Ann N Y Acad Sci* 2007;1098:22–50. [PubMed: 17303824]
2. Hay DI, Ahern JM, Schluckebier SK, Schlesinger DH. Human salivary acidic proline-rich protein polymorphisms and biosynthesis studied by high-performance liquid chromatography. *J Dent Res* 1994;73(11):1717–1726. [PubMed: 7983258]
3. Hay DI, Schluckebier SK, Moreno EC. Saturation of human salivary secretions with respect to calcite and inhibition of calcium carbonate precipitation by salivary constituents. *Calcif Tissue Int* 1986;39(3):151–160. [PubMed: 3093026]
4. Moreno EC, Varughese K, Hay DI. Effect of human salivary proteins on the precipitation kinetics of calcium phosphate. *Calcif Tissue Int* 1979;28(1):7–16. [PubMed: 115554]
5. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, et al. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. *J Biol Chem* 1988;263(16):7472–7477. [PubMed: 3286634]
6. Pollock JJ, Denepitiya L, MacKay BJ, Iacono VJ. Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptides on *Candida albicans*. *Infect Immun* 1984;44(3):702–707. [PubMed: 6373615]
7. Oudhoff MJ, Bolscher JG, Nazmi K, Kalay H, van 't Hof W, Nieuw Amerongen AV, et al. Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. *Faseb J*. 2008
8. Walz A, Stuhler K, Wattenberg A, Hawranke E, Meyer HE, Schmalz G, et al. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. *Proteomics* 2006;6(5):1631–1639. [PubMed: 16402355]
9. Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. *J Biol Chem* 2003;278(7):5300–5308. [PubMed: 12444093]
10. Helmerhorst EJ, Sun X, Salih E, Oppenheim FG. Identification of Lys-Pro-Gln as a novel cleavage site specificity of saliva-associated proteases. *J Biol Chem* 2008;283(29):19957–19966. [PubMed: 18463091]
11. Messana I, Cabras T, Pisano E, Sanna MT, Olianias A, Manconi B, et al. Trafficking and postsecretory events responsible for the formation of secreted human salivary peptides: a proteomics approach. *Mol Cell Proteomics* 2008;7(5):911–926. [PubMed: 18187409]

12. Vitorino R, Lobo MJ, Duarte JA, Ferrer-Correia AJ, Domingues PM, Amado FM. Analysis of salivary peptides using HPLC-electrospray mass spectrometry. *Biomed Chromatogr* 2004;18(8):570–575. [PubMed: 15386514]
13. Helmerhorst EJ, Alagl AS, Siqueira WL, Oppenheim FG. Oral fluid proteolytic effects on histatin 5 structure and function. *Arch Oral Biol* 2006;51(12):1061–1070. [PubMed: 16901460]
14. Jensen JL, Xu T, Lamkin MS, Brodin P, Aars H, Berg T, et al. Physiological regulation of the secretion of histatins and statherins in human parotid saliva. *J Dent Res* 1994;73(12):1811–1817. [PubMed: 7814752]
15. Baum BJ, Bird JL, Longton RW. Polyacrylamide gel electrophoresis of human salivary histidine-rich-polypeptides. *J Dent Res* 1977;56(9):1115–1118. [PubMed: 270513]
16. Flora B, Gusman H, Helmerhorst EJ, Troxler RF, Oppenheim FG. A new method for the isolation of histatins 1, 3, and 5 from parotid secretion using zinc precipitation. *Protein Expr Purif* 2001;23(1):198–206. [PubMed: 11570863]
17. Davis BJ. Disc Electrophoresis. Ii. Method And Application To Human Serum Proteins. *Ann N Y Acad Sci* 1964;121:404–427. [PubMed: 14240539]
18. Ornstein L. Disc Electrophoresis. I. Background And Theory. *Ann N Y Acad Sci* 1964;121:321–349. [PubMed: 14240533]
19. Oppenheim FG, Hay DI, Franzblau C. Proline-rich proteins from human parotid saliva. I. Isolation and partial characterization. *Biochemistry* 1971;10(23):4233–4238. [PubMed: 4331333]
20. Azen EA, Maeda N. Molecular genetics of human salivary proteins and their polymorphisms. *Adv Hum Genet* 1988;17:141–199. [PubMed: 3055850]
21. Bruno LS, Li X, Wang L, Soares RV, Siqueira CC, Oppenheim FG, et al. Two-hybrid analysis of human salivary mucin MUC7 interactions. *Biochim Biophys Acta* 2005;1746(1):65–72. [PubMed: 16203048]
22. Iontcheva I, Oppenheim FG, Troxler RF. Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. *J Dent Res* 1997;76(3):734–743. [PubMed: 9109822]
23. Young A, Rykke M, Rolla G. Quantitative and qualitative analyses of human salivary micelle-like globules. *Acta odontologica Scandinavica* 1999;57(2):105–110. [PubMed: 10445364]
24. Al-Hashimi I, Levine MJ. Characterization of in vivo salivary-derived enamel pellicle. *Arch Oral Biol* 1989;34(4):289–295. [PubMed: 2480770]
25. Vitorino R, Calheiros-Lobo MJ, Duarte JA, Domingues PM, Amado FM. Peptide profile of human acquired enamel pellicle using MALDI tandem MS. *J Sep Sci* 2008;31(3):523–537. [PubMed: 18266264]
26. Vitorino R, Calheiros-Lobo MJ, Williams J, Ferrer-Correia AJ, Tomer KB, Duarte JA, et al. Peptidomic analysis of human acquired enamel pellicle. *Biomed Chromatogr* 2007;21(11):1107–1117. [PubMed: 17516463]
27. Bradway SD, Bergey EJ, Jones PC, Levine MJ. Oral mucosal pellicle. Adsorption and transpeptidation of salivary components to buccal epithelial cells. *Biochem J* 1989;261(3):887–896. [PubMed: 2572218]
28. Makinen KK. Studies on oral enzymes. II. Fractionation and characterization of aminopeptidases in human dental plaque. *Acta odontologica Scandinavica* 1966;24(5):605–617. [PubMed: 5227457]
29. Wong DT. Salivary diagnostics powered by nanotechnologies, proteomics and genomics. *J Am Dent Assoc* 2006;137(3):313–321. [PubMed: 16570464]
30. Lamkin MS, Oppenheim FG. Structural features of salivary function. *Crit Rev Oral Biol Med* 1993;4(3–4):251–259. [PubMed: 8373982]
31. Raj PA, Edgerton M, Levine MJ. Salivary histatin 5: dependence of sequence, chain length, and helical conformation for candidacidal activity. *J Biol Chem* 1990;265(7):3898–3905. [PubMed: 2406266]
32. Rothstein DM, Spacciapoli P, Tran LT, Xu T, Roberts FD, Dalla Serra M, et al. Anticandida activity is retained in P-113, a 12-amino-acid fragment of histatin 5. *Antimicrob Agents Chemother* 2001;45(5):1367–1373. [PubMed: 11302797]

33. Hay DI, Carlson ER, Schluckebier SK, Moreno EC, Schlesinger DH. Inhibition of calcium phosphate precipitation by human salivary acidic proline-rich proteins: structure-activity relationships. *Calcif Tissue Int* 1987;40(3):126–132. [PubMed: 3105842]
34. Helmerhorst EJ, Oppenheim FG. Saliva: a dynamic proteome. *J Dent Res* 2007;86(8):680–693. [PubMed: 17652194]
35. Madapallimattam G, Bennick A. Phosphopeptides derived from human salivary acidic proline-rich proteins. Biological activities and concentration in saliva. *Biochem J* 1990;270(2):297–304. [PubMed: 2169237]
36. Minaguchi K, Madapallimattam G, Bennick A. The presence and origin of phosphopeptides in human saliva. *Biochem J* 1988;250(1):171–177. [PubMed: 3281658]

Figure 1.

Histatin and PRP standard curves. A, cationic PAGE of various amounts of synthetic histatin 1, 3, and 5. Loaded amounts for histatin 1, 3 and 5 in lanes 1 to 10: 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7, 3.0, 3.3, and 3.6 μg each, respectively; B, standard curves generated by densitometric analysis of the pixel intensity in the protein bands in A; C, anionic PAGE of pure PRP1 and PRP3. Loaded amounts for PRP1 and 3 in lanes 1 to 7: 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 μg each, respectively; D, densitometric analysis of the PRP1 and PRP3 bands.

Figure 2.

Cationic and anionic PAGE of WS samples from 22 subjects. In all lanes, 100 μ l sample was loaded. A, C, E, cationic PAGE results. Indicated are the positions of the histatin 1, 3, and 5 band; B, D, F, anionic PAGE results. Indicated are the positions of the PRP1/PIF-s band, the PRP2 band, the PRP3/PIF-f band, and the PRP4 band. Note the presence of lower molecular weight species indicative of WS proteolytic fragmentation.

Figure 3.

Correlation studies of WS salivary proteins. A, Total protein versus total histatin; B, total protein versus total acidic PRPs; C, total histatin versus total acidic PRPs. Each datapoint represents a single measurement of the indicated parameter. Linear regression yielded a line of which the mathematical characteristics are plotted in each graph.

Figure 4.

Proteolysis of endogenous histatins and acidic PRPs in PS. PS was collected from one subject and incubated for the indicated time intervals in a 37°C waterbath. A, cationic PAGE of the samples. Far right lane: standard containing 3 μg of synthetic histatins 1, 3 and 5 each; B, densitometric analysis of the histatin 3 band as a function of time; C, anionic PAGE of the samples. Far right lane: standard containing 21 μg of PRP1 and PRP3 each; D, densitometric analysis of the PRP1 band.

Figure 5.

Proteolysis of pure histatin 3 and PRP 1 added to WS. WS was collected from one subject, spiked with synthetic histatin 3 (40 µg/ml) or pure PRP1 (400 µg/ml) and incubated for the indicated time intervals in a 37°C waterbath. A, cationic PAGE of 100 µl samples. Left flanking lane: standard containing 4 µg histatin 3, right flanking lane: 100 µl WS before addition of histatin 3; B, densitometric analysis of the disappearance of the added histatin 3 as a function of time; C, anionic PAGE of 100 µl samples. Left flanking lane: standard containing 40 µg of PRP1, right flanking lane: 100 µl of WS before addition of PRP1; D, densitometric analysis of the disappearance of the PRP1 band.

Table 1
Concentration range of histatins and acidic PRPs in stimulated WS collected from 22 subjects.

	Concentration (µg/ml)		
	Mean ± SD	Range	Median
Histatin 1	19.7 ± 8.4	10.9 to 44.3	17
Histatin 3	5.7 ± 3.5	1.7 to 11.8	5.6
Histatin 5	8.0 ± 5.8	2.1 to 16.9	6.9
Total histatins	33.3 ± 16.7	15.7 to 64.9	28.8
PRP1	226.8 ± 66.8	149.3 to 399.0	210.3
PRP3	201.0 ± 62.5	79.8 to 323.8	205.3
PRP1 + PRP3	427.9 ± 123.3	267.2 to 720.6	412.8

Table 2
Concentrations of histatin and acidic PRPs in WS, WSS, and PS samples collected from 7 subjects.

Mean concentration (µg/ml) ± SD					
	WS		WSS		PS
	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
Histatin 1	15.5 ± 3.3	12.9 ± 3.7	11.9 ± 2.5	9.4 ± 3.7	36.8 ± 19.7
Histatin 3	4.6 ± 2.2	2.7 ± 0.1	1.9 ± 0.6	0.9 ± 0.6	33.6 ± 17.2
Histatin 5	7.0 ± 2.6	7.2 ± 1.7	4.9 ± 1.8	2.1 ± 0.3	42.5 ± 23.7
Total histatins	26.8 ± 7.3	22.8 ± 5.6	18.8 ± 4.5	12.4 ± 3.5	155.4 ± 58.4
PRP 1	303.6 ± 31.7	190.4 ± 18.5	270.3 ± 36.5	190.7 ± 39.9	478.2 ± 217.6
PRP 3	295.6 ± 37.1	207.7 ± 33.1	226.9 ± 32.5	158.7 ± 40.0	347.0 ± 240.8
PRP 1+3	599.2 ± 49.0	398.0 ± 48.5	497.1 ± 35.1	349.4 ± 75.6	825.1 ± 450.8

Table 3

Table 3A. "Half life" (t½) of endogenous histatin 3 and PRP1 in PS from 7 subjects			
Protein	t½ (hr)		
	Mean	Range	Median
Histatin 3 (endogenous)	7.2 ± 5.5	1.3 to 17.7	6.0
PRP1 (endogenous)	50.3 ± 24.8	17.4 to 96.0	42.5

Table 3B. "Half life" (t½) of histatin 3 (40 µg/ml) and PRP1 (400 µg/ml) added to WS from 7 subjects			
Protein	t½ (min)		
	Mean	Range	Median
Histatin 3 (added)	1.7 ± 1.6	<1 to 3.8	1.2
PRP1 (added)	29.3 ± 15.3	10.0 to 50.0	25.3