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# Oral fluid proteolytic effects on histatin 5 structure and function

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**Summary** Histatins are human salivary antifungal proteins that are prone to extensive enzymatic degradation upon their release into the oral cavity. Histatin proteolysis, leading to the disappearance of the intact protein can be expected to have functional consequences. Histatin 5, comprising 24 residues, is the smallest of the major salivary histatins and the most active in terms of its antifungal properties. The rate and mode of histatin 5 degradation were determined by incubating the protein in whole saliva supernatant for various time intervals. Fragmentation products were collected by reversed-phase high performance liquid chromatography (RP-HPLC), characterised structurally by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry and functionally in a fungal growth inhibition assay. Of the 19 fragments identified, 16 were derived from single proteolytic cleavage events in histatin 5. A remarkable finding was the inter-subject consistency in the histatin 5 degradation pattern. Added histatin 5 disappeared from whole saliva supernatant at an average rate of $105 \pm 22$ μg/ml/h, which in part could explain the virtual absence of histatin 5 in whole saliva. Despite the rapid proteolysis of histatin 5, the early degradation mixture was as active in antifungal assays as intact histatin 5. These data demonstrate that the oral-fluid mediated proteolysis of histatin 5 represents an intrinsic biological property of whole saliva. The data also reveal that the early proteolysis phase of histatin 5 does not abolish the antifungal properties associated with this protein.
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## Introduction

The parotid and submandibular/sublingual glands are the main contributors to oral fluid, more commonly called whole saliva. These glands secrete a wide variety of proteins, ranging in size from 3 kD to over 100 kD. Their functions are of critical importance in

*Abbreviations:* CHCA, alpha-cyano-4-hydroxy-cinnamic acid; kD, kilo Daltons; MALDI-TOF, matrix-assisted laser desorption/ionisation-time of flight; OD, optical density; RP-HPLC, reversed-phase high performance liquid chromatography; SDB, Sabouraud dextrose broth; WSS, whole saliva supernatant

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the maintenance of oral health since conditions of hyposalivation have serious clinical consequences such as caries, mucositis and gingivitis.[1–4] A major unresolved question in oral biochemistry is the discrepancy between the presence of salivary proteins in glandular secretions and the virtual absence of some of these proteins in their native form in freshly collected whole saliva.[5–8] This discrepancy is of functional importance since it calls for exploring how and when this apparent loss of protein occurs and what effect this loss has on the in vivo maintenance of oral hard and soft tissues. Proteolysis has been the obvious and major focus of investigations aimed at explaining the differences between proteins/peptides found in glandular secretions and those present in whole saliva. Studies on the degradation of salivary secretory products has so far shown that proteolysis occurs at least two different levels. First, the biosynthesis of salivary proteins comprises posttranslational proteolytic processing which occurs within the gland.[9–14] The enzymes responsible and their sub-cellular localisation have not yet been identified. While minor proteolytic activity has been reported in pure glandular secretions,[15,16] this proteolysis requires very long incubation times (hours) to be detectable and appears to be physiologically not relevant in view of the short residence time (minutes) of glandular saliva in the ductal system. The second level of proteolysis occurs in the oral environment triggered by bacterial and host-derived enzymes. The magnitude of this whole saliva associated proteolysis is very pronounced and the speed at which it occurs is extremely rapid.[17,18] This type of proteolysis seems to be in part responsible for the inability to detect, electrophoretically, chromatographically or by immuno-quantitation assays, some of the native salivary proteins in whole saliva.[5–8]

One of the major protein families expressed by the serous cells of the parotid and submandibular glands that are present at negligible levels in whole saliva are called histatins.[7,17,19,20] Total histatin concentrations in pure parotid and submandibular/sublingual secretions range between 30 and150 μg/ml, depending on the gland type and extent of stimulation.[21–23] In contrast, their concentrations in whole saliva are only 2–4 μg/ml[17] (Helmerhorst et al., unpublished observations). These proteins have gained considerable interest due to their antimicrobial properties.[19,24,25] The histatin family, as it is present in the pure glandular secretions, consists mainly of histatins 1, 3 and 5 each contributing about 20–30% to the total histatin pool.[23] Histatin 1 and histatin 3 are the expression products of the *HIS*1 and *HIS*2 genes, respectively, while histatin 5 is a posttranslational proteolysis product of histatin 3.[11] Processing of histatin 3 into histatin 5 most likely occurs within the acinar cell since the ratio of histatin 3 to histatin 5 in both glandular secretions is approximately 1:1 and this ratio is largely unaffected by the salivary flow rate.[21] While all levels of histatin processing are of interest, the proteolysis mechanisms associated with whole saliva are of major relevance considering its functional implications in the oral cavity.

Previous studies have shown that the addition of whole saliva to parotid salivary secretion promotes the degradation of histatins into smaller peptides.[17,18] Indeed, a multitude of very small histatin fragments, representing end-stage histatin degradation products, have been identified in whole saliva by chromatographic and proteomics approaches.[26–28] The high content of lysine (K) and arginine (R) residues in histatins makes these proteins extremely susceptible to tryptic-like digestion. This digestion is so rapid and efficient that it renders the mere analysis of whole saliva inadequate to determine the early sequence of events in the proteolytic degradation cascade in an oral environment.

Insight into the amino acid sequence of proteolytic fragments generated in whole saliva and the time-course of their generation is of importance in view of the fact that their functional capacity in the oral cavity is likely dependent on the extent of degradation. This is true not only for histatins but also for other functionally important salivary proteins. For this study, histatin 5 was selected as the model macromolecule, since it represents a major constituent of parotid saliva and the biological properties of this protein have been well defined. To capture the early proteolytic events in the histatin 5 proteolysis cascade the pure protein was added to whole saliva supernatant which was used as the enzyme source. It was investigated whether the nature of histatin 5 proteolysis is an inherent biological property of whole saliva or reflects random, subject-dependent proteolytic events. Furthermore, we investigated to what extent proteolysis affected the antifungal properties of this protein.

## Experimental procedures

### Saliva collection

Whole saliva samples were collected at least 1 h after the last meal from five orally healthy donors ranging in age from 25 to 38 years. Informed consent was received according to approved protocols of the Institutional Review Board at Boston University Medicial Center. Whole salivary secretion was stimulated by mastication using 1.4 g of paraffin wax

(Parafilm, American National Can™, Chicago, IL). Two to four millilitre of total volume was collected on ice. Immediately after collection, whole saliva was cleared from bacteria, cells and other debris by centrifugation for 10 min at 16,000 × *g* at 4 °C. The supernatant is subsequently referred to as whole saliva supernatant (WSS).

## Degradation of histatin 5 in WSS

To characterise the histatin 5 degradation products formed in the presence of whole salivary proteins, experiments were carried out with individual WSS samples from five donors, or with a pool of WSS from the same donors. Experiments were conducted either with undiluted or with 1:10 diluted WSS. Synthetic histatin 5 (American Peptide Company, Sunnyvale, CA) was added to undiluted or 1:10 WSS to a final concentration of 400 μg/ml. The control sample contained histatin 5 in water. Immediately after the addition of histatin 5 ($t = 0$), and after different incubation times in a water bath at 37 °C, 2 × 100 μl aliquots were removed and boiled to abolish proteolytic activity. To the first series of collected samples, 1 ml of 0.1% trifluoroacetic acid (buffer A) was added followed by sample filtration through a 0.22 μm filter (Pall Cooperation, Ann Arbor, MI) and RP-HPLC analysis. The duplicate series of the same samples (100 μl) were serially diluted and evaluated in a fungal growth inhibition assay.

## Reversed-phase high performance liquid chromatography (RP-HPLC)

RP-HPLC analysis was carried out on a HPLC Model 715 (Gilson, Middleton, WI) using a C-18 column (TSK-GEL 5 μm, ODS-120T, 4.6 mm × 250 mm, TOSOHaas, Montgomeryville, PA). Histatins were eluted using a linear gradient from 0 to 55% buffer B containing 80% acetonitrile and 0.1% TFA over a 74 min time interval at a flow rate of 1.0 ml/min. The eluate was monitored at 230 nm and 1 ml aliquots were lyophilised using a Speed Vac (Savant, Holbrook, NY) and subjected to cationic gel electrophoresis and mass spectrometric analysis. Protein peak areas in the RP-HPLC chromatograms were determined using Unipoint software (version 3.30, Gilson) with a default baseline setting and a sensitivity threshold of 5%.

## Cationic PAGE

Native, cationic polyacrylamide gel electrophoresis was performed as described previously.[19,29] Lyophilised samples were dissolved in sample loading buffer containing 20% sucrose and 0.04% methylgreen and proteins were separated in a 15% polyacrylamide gel at a constant voltage of 120 V. Gels were stained for 16 h with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 7% (v/v) acetic acid and 8% (v/v) methanol. Gels were destained with 40% (v/v) methanol and 10% (v/v) acetic acid.

## Mass spectrometric analysis

Aliquots of peptides obtained by RP-HPLC separation were lyophilised, dissolved in water and subjected to MALDI-TOF mass spectrometry. Data were collected using a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) in reflector mode, 25 kV accelerating voltage, delayed extraction, position ion mode and close external calibration. The matrix used consisted of alpha-cyano-4-hydroxy-cinnamic acid (CHCA).

## Fungal growth inhibition assay

Procedures for this assay have been described previously.[30] Histatin 5 (400 μg/ml) that had been incubated in 1:10 diluted pooled WSS for various time intervals was serially diluted in 10% Sabouraud dextrose broth (SDB) in a round-bottom microtiter plate to a final volume of 50 μl/well. In a control experiment, histatin 5 alone (400 μg/ml) or pooled 1:10 WSS alone were subjected to the same serial dilution. *C. albicans* cells (ATCC 10231) were grown for 24 h at 30 °C in 10% SDB and diluted to an OD at 620 nm of 0.002 which was pre-determined to contain $1 \times 10^4$ colony forming units/ml. A 50 μl aliquot was added to each dilution of the WSS-treated histatin/peptide samples such that the highest final concentration of histatin (intact histatin 5 and/or its fragments) was 200 μg/ml. After incubation for 48 h at 30 °C, cell growth was assessed by measurement of the optical density (OD) at 620 nm. Values were corrected for the absorbance of 10% SDB and $IC_{50}$ values were determined from the growth inhibition curves.

# Results

## Identification of the primary degradation fragments of histatin 5 added to WSS

To determine the mode of enzymatic processing of histatin 5 by whole salivary enzymes, synthetic histatin 5 (400 μg/ml) was incubated with 1:10 diluted WSS. The selected histatin 5 concentration and WSS dilution were chosen to retard proteolytic processes, and to minimise interference from endogenous salivary proteins in RP-HPLC and MS/MS

analyses of the generated histatin degradation fragments. After various incubation times of histatin 5 in 1:10 WSS, aliquots were boiled, and subjected to RP-HPLC (Fig. 1). The scales of the *Y*-axes were optimised to visualise the histatin proteolytic fragments. Degradation products started to appear after 4 h of incubation and eluted prior to histatin 5 which eluted after 40 min. After 4—14 h of incubation, at least 8 histatin fragmentation peaks were clearly discernable. In the 24 h sample, the intensity of the histatin 5 peak had decreased significantly concomitant with a further increase in the intensity of the eight histatin peaks. No additional histatin fragments were generated within this time frame. Longer incubation times, up to 48 h, ultimately resulted in the formation of smaller fragments eluting between 10 and 25 min, and in the complete disappearance of histatin 5.

## Reproducibility of the histatin 5 degradation pattern

To assess whether there were inter-individual differences in the efficiency and mode of histatin degradation, histatin 5 was added to 1:10 diluted WSS of five subjects individually. After various incubation times, the amount of histatin 5 in treated samples was determined relative to the peak area of the added amount of histatin 5 (Fig. 2A). Clear inter-individual differences were observed in the efficiency of histatin degradation. Among the five subjects, two showed relatively low histatin 5 degra-

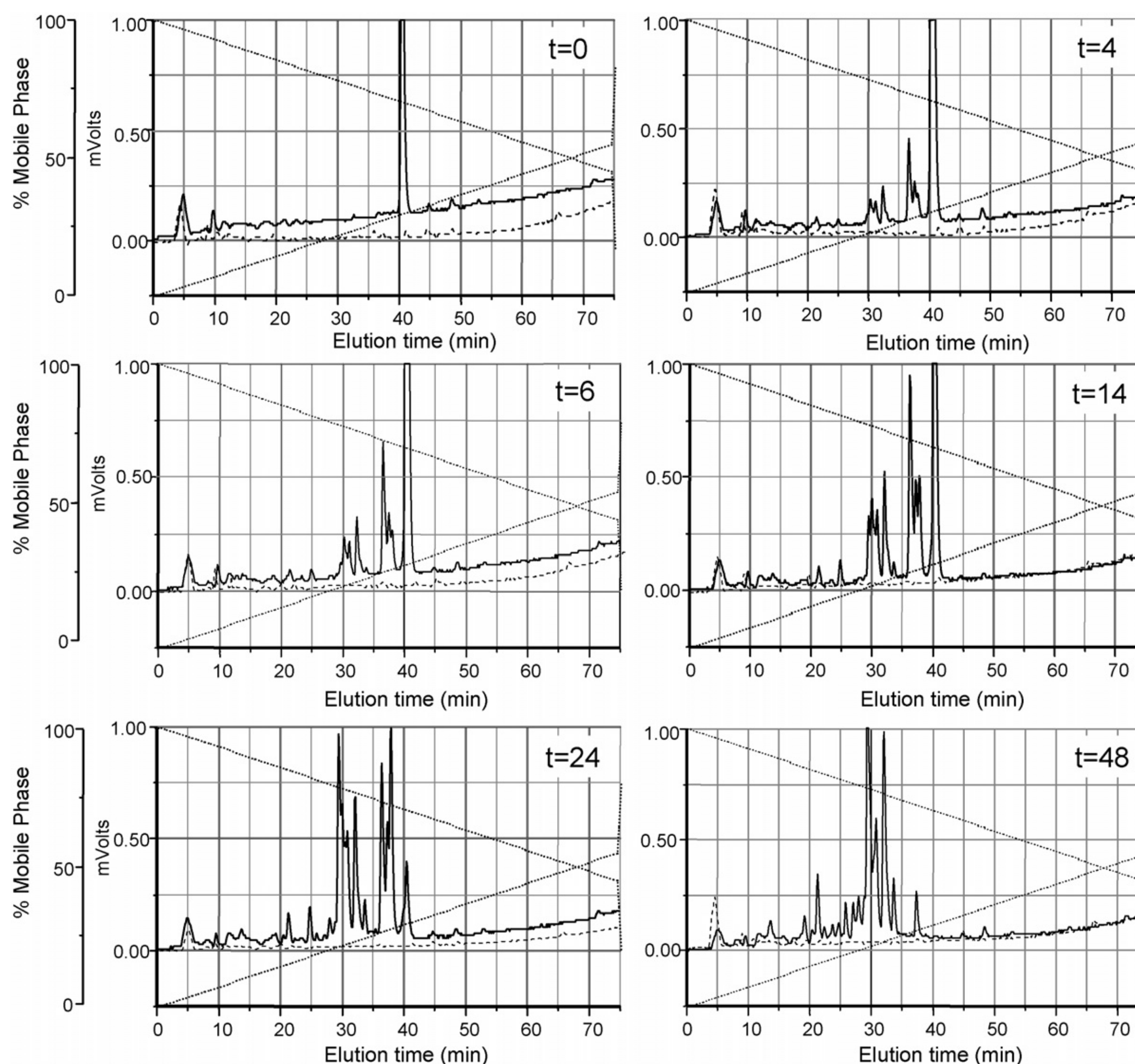


**Figure 1** RP-HPLC chromatograms showing the time-course of histatin 5 degradation in a whole salivary environment. Histatin 5 (400 μg/ml) was added to 1:10 diluted pooled WSS collected from five subjects. After incubation for 0, 4, 6, 14, 24 and 48 h at 37 °C, samples were boiled and analysed by RP-HPLC. The dashed lines represent proteins/peptides naturally present in 1:10 diluted WSS alone. The gradient used for elution (ratio of buffer A and buffer B) are indicated by dotted lines where buffer B equals 0% and buffer A equals 100% at $t = 0$.

dation rates ($\Delta$ = 3.3 and 3.8 $\mu$g/ml/h), one subject exhibited an intermediate rate ($\Delta$ = 10.5 $\mu$g/ml/h) and two subjects displayed relatively high histatin 5 proteolytic activities ($\Delta$ = 21.6 and 23.2 $\mu$g/ml/h). The observed differences were consistent with reported inter-subject variation in salivary proteolytic activities.[16] Despite these differences in histatin degradation efficiency, a high consistency in the histatin degradation pattern was observed (Fig. 2B). The data suggest that the mode of histatin degradation and the enzymes involved in this process is constant among subjects. The uniformity of these degradation patterns prompted us to characterise the primary cleavage products generated.

## Identification of the early histatin 5 degradation fragments

It was established that the fragmentation patterns generated after 24 h in 1:10 diluted pooled WSS was identical to that formed after 3 h in undiluted pooled WSS (Fig. 3A). This indicated that dilution of saliva modified the histatin 5 degradation efficiency but not the degradation pattern. To identify the early histatin 5 degradation fragments formed in 1:10 WSS, eight 1-ml fractions containing proteins eluting prior to histatin 5 were collected. The individual fractions were lyophilised and analysed by cationic PAGE (Fig. 3B). Fractions of 1:10 diluted WSS alone did not contain detectable levels of cationic proteins (data not shown). In most fractions containing the histatin 5 degradation fragments multiple peptides were present in the same fraction, due to incomplete resolution of peptides with the gradient employed. Peptides present in fraction 1 were not detectable in the gel. Peptides present in fractions 2—5 migrated faster than either histatin 5 or the fragments present in fractions 6—8. As expected, fraction 9 contained a protein with the same electrophoretic mobility as histatin 5 in the histatin standard.

## Mass spectrometric analysis of histatin degradation fragments

Fractions 1—9 were subjected to MALDI-TOF to obtain *m/z* values. Diluted WSS samples to which no histatin 5 was added were also fractionated and analysed to obtain a "background" *m/z* peak pattern. Differential analysis of the MALDI-TOF spectra of a particular 1:10 WSS fraction and the matching 1:10 WSS with added histatin 5 fraction clearly revealed which fragments were derived from the added histatin 5. The N- and C-terminal fragments were identified by comparing the obtained *m/z* values (mono-isotopic masses) with theoretical values of all possible fragments of histatin 5 using the FindPept tool on the ExPASy web server (http://us.expasy.org/tools/findpept.html). The sequences of a total of 19 histatin fragments were identified in the 8 collected fractions (Table 1). Peaks 1—5 contained mostly smaller histatin 5 fragments whereas peaks 6—8 contained larger fragments, consistent with the slower electrophoretic mobility of the latter peptides observed in cationic PAGE. Individual peptides identified were labelled $his_5$ *x/y*, where *x* refers to the N-terminal residue and *y* referes to the C-terminal residue in a particular fragment

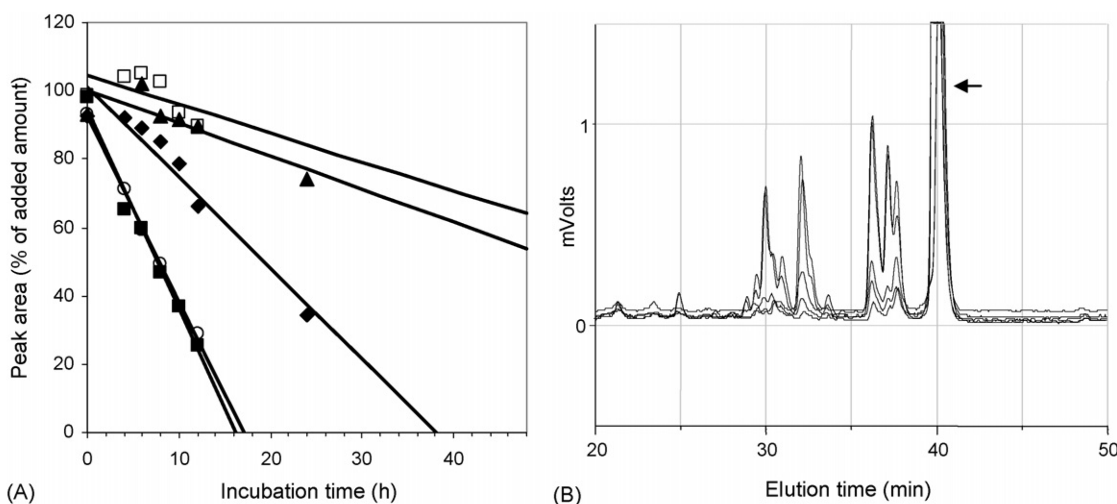


**Figure 2** Rate and pattern of histatin 5 degradation. (A) Rate of histatin 5 degradation at 37 °C in 1:10 diluted WSS of five subjects. Hundred percent (100%) equals to the added amount of histatin 5 (400 $\mu$g/ml). Subject 1 (◆); subject 2 (□); subject 3 (▲); subject 4 (○); subject 5 (■). (B) Elution profile of histatin 5 (indicated with an arrow) and its degradation products formed in diluted WSS from five subjects after 12 h of incubation. Note the overlap of the fragmentation pattern in all five subjects.

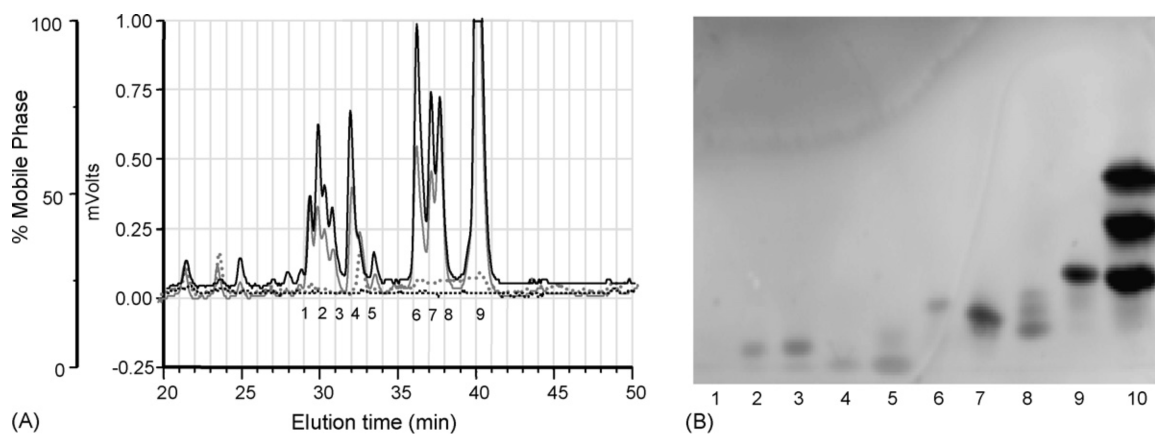


**Figure 3** Comparison of HPLC chromatograms of histatin degradation products generated in 1:10 diluted and undiluted pooled WSS (A) and cationic PAGE analysis of individual fractions containing the degradation products (B). Histatin 5 (400 μg/ml) was incubated for 3 h with undiluted pooled WSS (solid grey line) or for 24 h with 1:10 diluted pooled WSS (solid black line). The absorbance of pooled WSS and 1:10 WSS alone in the elution diagrams are indicated by the grey and black dotted lines, respectively. Eight fractions eluting prior to histatin 5 were collected, lyophilised and subjected to cationic PAGE. The lane numbers in the gel correspond to the fractions labelled in the chromatogram. Lane 10 contains 8 μg each of purified histatin 1, 3 and 5.

(nomenclature adapted from reference).[26] Peptides $his_5$ 1/10, 1/11, 1/12, 1/13, 1/15, 1/16, 1/17, 1/18, represented N-terminal histatin 5 fragments and in all cases, their C-terminal counterparts, $his_5$ 11/24, 12/24, 13/24, 14/24, 16/24, 17/24, 18/24 and 19/24, respectively, were identified. This indicates that various single proteolytic processing steps of histatin 5 had occurred. Only three fragments ($his_5$ 5/12, 6/13 and 14/22) had arisen from two proteolytic cleavages. Ten of the total 19 fragments formed were generated through tryptic-like enzymatic activity, targeting the peptide bonds associated with lysine and arginine residues. In these initial phases of histatin proteolysis, the R(6)—H(7) bond was the only trypsin site that remained intact, and indeed this peptide bond represents the least favourable trypsin cleavage site in the histatin 5 sequence.[31] Two of the 19 fragments were generated through chymotryptic-like activity at the Y(10)—K(11) bond, while the F(14)—H(15) bond, the less preferred cleavage site of chymotrypsin in histatin 5 was not cleaved.[32] We could not confirm previous observations that the R(22)—G(23) and the K(17)—H(18) bond would be resistant to proteolysis.[27] The remainder of the identified fragments were formed through various peptidase activities.

## The consequence of histatin 5 degradation on antifungal activity

Fungistatic properties of the histatin 5 degradation mixtures were assessed using a growth inhibition assay for cationic proteins and employing the most common fungus in the oral cavity, *C. albicans*, as the test organism. Histatin 5 was incubated with 1:10 diluted WSS from subjects 4 and 5 that showed high proteolytic activities (Fig. 2). The antifungal properties of the generated peptide mixtures were evaluated in a growth inhibition assay. Histatin 5 that had been incubated for up to 12 h with 1:10 diluted WSS (Fig. 4A) was equally effective in terms of its fungistatic activity as intact histatin 5 that had been incubated for the same time period in water (Fig. 4B). Upon extended incubation times in 1:10 WSS, some loss in antifungal activity was observed, but considerable activity was retained in samples incubated for 24, 48 and 72 h, despite the fact that histatin 5 in these samples had been completely degraded. The observed activity of the diluted WSS/his 5 mixture was not present in diluted WSS itself, which was completely devoid of fungistatic activity (Fig. 4B).

## Comparison of antifungal activity and levels of intact histatin 5

In order to make quantitative comparisons between antifungal activity and histatin 5 levels upon exposure to WSS-associated proteases for various time intervals, the $IC_{50}$ values and histatin 5 concentrations were expressed relative to the activities and amounts of histatin 5 in the control sample. The time delay between the loss of histatin 5 and the loss of antifungal activity was significant, 50% of intact histatin 5 was degraded after 8 h whereas 50% of the antifungal activity was lost only after 24 h (Table 2). The biological relevance of our observations points to the fact that histatin degradation fragments

**Table 1** Amino acid sequences and properties of 19 histatin fragments generated upon incubation of histatin 5 with diluted WSS

| Peak | Name | Observed (M+H)+ | Theoretical (M+H)+ | Sequence | Enzyme | PI | Activity[a] |
|---|---|---|---|---|---|---|---|
| 1 | his$_5$ 5/12 | 1081.701 | 1081.613 | K R H H G Y K R | Alanine-lysine endopeptidase/ trypsin-like | 11.1 | no[b] |
| 1 | his$_5$ 6/13 | 1081.701 | 1081.613 | R H H G Y K R K | Trypsin-like | 11.1 | no |
| 2 | his$_5$ 1/11 | 1335.762 | 1335.666 | D S H A K R H H G Y K | Trypsin-like | 9.7 | no |
| 2/3 | his$_5$ 1/13 | 1619.955 | 1619.863 | D S H A K R H H G Y K R K | Trypsin-like | 10.5 | no[b] |
| 3/4 | his$_5$ 1/12 | 1491.845 | 1491.768 | D S H A K R H H G Y K R | Trypsin-like | 10.3 | no |
| 4 | his$_5$ 14/22 | 1214.645 | 1214.593 | F H E K H H S H R | Trypsin-like | 8.8 | unknown |
| 4 | his$_5$ 19/24 | 756.380 | 756.354 | H S H R G Y | Histidine peptidase-like | 8.8 | no[b] |
| 4/5 | his$_5$ 18/24 | 893.450 | 893.412 | H H S H R G Y | Trypsin-like | 8.8 | no[b] |
| 5 | his$_5$ 1/10 | 1207.577 | 1207.571 | D S H A K R H H G Y | Chymotrypsin-like | 8.6 | no[b] |
| 5 | his$_5$ 16/24 | 1150.556 | 1150.550 | E K H H S H R G Y | Histidine peptidase-like | 8.7 | no[b] |
| 5 | his$_5$ 17/24 | 1021.507 | 1021.507 | K H H S H R G Y | Glutamyl endopeptidase-like | 10 | no[b] |
| 6/7/8 | his$_5$ 1/17 | 2161.102 | 2161.127 | D S H A K R H H G Y K R K F H E K | Trypsin-like | 10.2 | yes[c] |
| 6 | his$_5$ 1/18 | 2298.162 | 2298.186 | D S H A K R H H G Y K R K F H E K H | Histidine peptidase-like | 10.2 | yes[c] |
| 7 | his$_5$ 1/15 | 1903.990 | 1903.990 | D S H A K R H H G Y K R K F H | Histidine peptidase-like | 10.5 | unknown |
| 7/8 | his$_5$ 14/24 | 1434.688 | 1434.677 | F H E K H H S H R G Y | Trypsin-like | 8.6 | unknown |
| 8 | his$_5$ 1/16 | 2033.088 | 2033.032 | D S H A K R H H G Y K R K F H E | Glutamyl endopeptidase-like | 10 | yes |
| 8 | his$_5$ 11/24 | 1847.002 | 1846.968 | K R K F H E K H H S H R G Y | Chymotrypsin-like | 10.5 | yes |
| 8 | his$_5$ 12/24 | 1718.907 | 1718.873 | R K F H E K H H S H R G Y | Trypsin-like | 10.3 | yes |
| 8 | his$_5$ 13/24 | 1562.797 | 1562.772 | K F H E K H H S H R G Y | Trypsin-like | 9.7 | yes |
| 9 | histatin 5 | 3035.563 | 3035.522 | D S H A K R H H G Y K R K F H E K H H S H R G Y | | 10.3 | yes |

[a]Antifungal activity, described in references.[15,19,33–35]
[b]Anticipated activity, fragment represents a portion of a peptide with no activity.
[c]Anticipated activity, fragment is one or two residues longer and more cationic than a fragment with established antifungal activity.

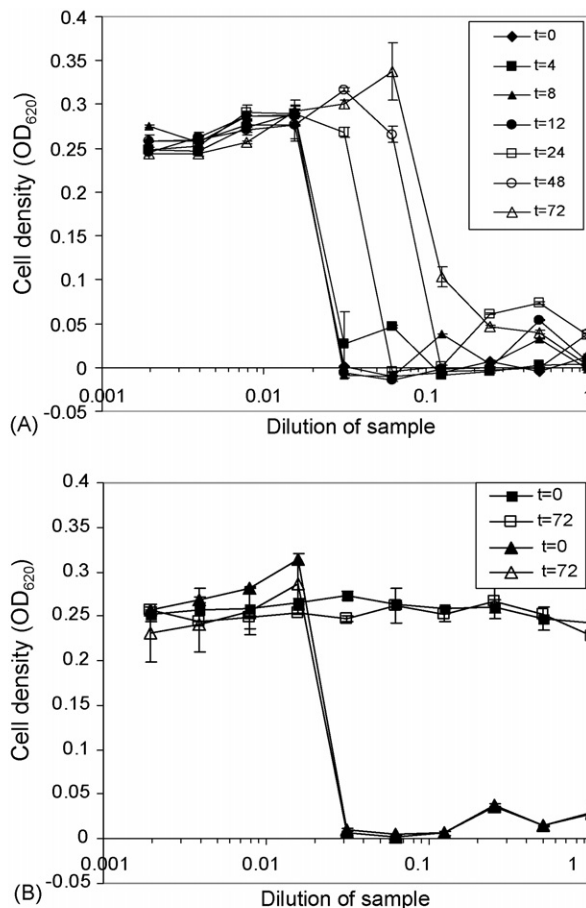


**Figure 4** Fungistatic activity of histatin 5 degradation mixtures. Histatin 5 (400 μg/ml) was incubated in 1:10 WSS of subjects 4 or 5. Samples were collected at the indicated time intervals. The fungistatic activities of these samples containing histatin 5 at various stages of its degradation (A) was compared to the activity of intact histatin 5 alone and WSS alone (B). Plotted is the dilution of the sample, with 1 representing a histatin protein concentration (intact or degraded) of 200 μg/ml. (A) The average and variation in activities in samples from the two subjects are plotted. (B) The average and variation among their 1:10 WSS samples are plotted (squares), along with the activity of histatin 5 alone (triangles).

**Table 2** Comparison of histatin 5 levels with antifungal activity

| Incubation time (h)[a] | Histatin 5 (%)[b] | Antifungal activity (%)[c] |
|---|---|---|
| 0 | $95.6 \pm 2.4$ | $100 \pm 0$ |
| 4 | $68.0 \pm 3.0$ | $95.6 \pm 4.3$ |
| 8 | $48.0 \pm 1.2$ | $100 \pm 0$ |
| 12 | $27.1 \pm 1.6$ | $100 \pm 0$ |
| 24 | $0 \pm 0$ | $48.6 \pm 0.8$ |
| 48 | $0 \pm 0$ | $23.6 \pm 0.3$ |
| 72 | $0 \pm 0$ | $19.9 \pm 1.6$ |

[a] Incubation time of histatin 5 in 1:10 diluted WSS from subjects 4 or 5.
[b] (Histatin 5 peak area of the sample incubated with 1:10 WSS/histatin 5 peak area of the sample incubated in water) × 100%.
[c] ($IC_{50}$ value of the histatin 5 control sample/$IC_{50}$ value of histatin 5 incubated in 1:10 WSS) × 100%.

exhibit considerable activity, and contribute as much to the observed fungistatic activity as the intact histatin 5 protein.

## Discussion

In this study we have identified the major initial degradation fragments of histatin 5 that are formed by free proteases present in whole saliva. Most fragmentations were trypsin-like in nature and arise mainly through the cleavage of a single peptide bond. A remarkable consistency was observed between subjects with regard to proteolytic specificity, but not proteolytic capacity. Furthermore, the data show that extensive histatin 5 degradation in an oral environment can occur without major loss in antifungal activity.

It can be expected that histatin degradation is initiated in the oral cavity as soon as the parent histatin molecules are released from the glandular excretory ducts. The in vivo time frame for this degradation is not known but the larger fragments which, as shown here, retain functional activity, appear first followed by smaller fragments representing ultimately the final degradation of histatins. In previous studies, various synthetic histatin fragments have been synthesised, and their activity toward *C. albicans* in antifungal assays have been analysed. Among these in vitro tested peptides, 7 were identical to the 19 fragments now identified to be generated in WSS. Of biological interest is the fact that fragment $his_5$ 1/16 comprising the N-terminal 16 residues of histatin 5 and fragments $his_5$ 14/24, 13/24 and 12/24 comprising the C-terminal 11, 12 and 13 residues of histatin 5 reportedly exhibit strong antifungal activities.[15,33–35] The antifungal activity of two other fragments ($his_5$ 1/17 and 1/18) can be anticipated based on the fact that they are larger and more cationic than the fungicidal peptide $his_5$ 1/16.[33] The various smaller N- and C-terminal fragments contained in fractions 1–5 are either inactive or expected to be inactive.[15,34] The observed antifungal activity of the early histatin 5 degradation mixture can therefore likely be ascribed to at least 6 of the 19 histatin fragments generated in WSS.

While the diluted WSS-promoted enzymatic activities described in this study with histatin 5 occur in hours of incubation, prolonged incubation with diluted parotid secretion for several days has

also shown to generate fragments of histatin 5. Similarly to our WSS-triggered fragmentation Xu et al.[15] determined that peptide $his_5$ 1/13 (designated HRP-5d at the time), $his_5$ 14/22 (HRP-5e) and $his_5$ 1/17 (HRP-5b) are formed by tryptic-like cleavages of histatin 5 in parotid secretion. In addition, the authors found three other histatin-5 fragments, $his_5$ 6/24 (HRP-5a), $his_5$ 15/24 (HRP-5c) and $his_5$ 6/12 (HRP-5f) which were not detected in our study with WSS. In another study, Hardt et al. focused on the inherent histatin fragments present in parotid secretion.[14] Their MS data indicated the presence of a large repertoire of possible cleavage products of histatin 5. The preponderant histatins (histatins 1, 3 and 5) in parotid secretion however are intact while they are virtually undetectable in whole saliva.[17] Thus, while some of the enzymatic specificities in parotid secretion and whole saliva may overlap, their enzymatic efficiencies do not.

In principle, the most likely sources of proteolytic enzymes in whole saliva are derived from oral microbiota, gingival fluid and epithelial cells. Bacteria, in particular those associated with periodontal disease, such as *P. gingivalis*, *B. forsythus*, *Capnocytophaga* species and the spirochete *Treponema denticola* possess trypsin-like activities.[36,37] Hydrolysis of a trypsin substrate analog has also been observed in whole saliva from edentulous subjects, arguing against gingival fluid or periodontal pathogens as the source of salivary proteases, but a positive correlation was observed with the number of epithelial cells in the cell pellet.[16] Despite the wide variety of enzymes in whole saliva that could lead to histatin 5 degradation, the observed early fragmentation pattern shows considerable specificity and consistency among subjects. This suggests the participation of one or a limited set of enzymes with similar specificities in this process.

The antifungal activity expressed in $IC_{50}$ of intact or mildly degraded histatin 5 is approximately 6–10 $\mu$g/ml (this study and reference[30]). The natural concentration of histatins in whole saliva is lower, probably explaining why WSS and 1:10 diluted WSS show no activity in fungal growth inhibition assays. To this end, the assessment of the biological activity of whole saliva itself, containing the very end-stage degradation fragments, is unsuitable for functional analysis. It is likely that there is a relative steady state between the proteolytic breakdown of histatins and the salivary flow-driven replenishment with intact glandular proteins. This continuous supply of native protein leads to a continuous generation of potentially functional peptides. While the half-life of these peptides is short, they can be expected to be highly significant for the beneficial role of saliva function.

We established that the average rate with which histatin 5 disappears from 1:10 diluted pooled WSS was $17.8 \pm 8.9$ $\mu$g/ml/h (average of five experiments). As expected, the rate of histatin 5 degradation was significantly higher in undiluted WSS ($105 \pm 22$ $\mu$g/ml/h, average of four experiments). Assuming a resting salivary flow rate of 12 ml/h, and a constant whole saliva volume in the oral cavity of 1 ml,[4] it can be calculated that the average time a given saliva sample stays in the mouth is 5 min. Assuming that parotid and submandibular/sublingual secretions would be the only contributors to whole saliva, and that the average histatin concentration in these secretions is 50 $\mu$g/ml, it can be calculated that at the established WSS degradation rate of approximately 2 $\mu$g/min, 10 $\mu$g of histatin 5 would disappear in 5 min. Thus, it appears that the proteolytic activity in WSS alone cannot fully account for the low histatin levels in whole saliva. It should be pointed out that the enzymatic rate in WSS is likely an underestimation of the in vivo rate of histatin 5 disappearance from whole saliva since additional cell-associated proteolytic activities[38] as well as protein–protein complex formation[39,40], binding of intact histatins to microbial cells[41,42] and to oral soft and hard tissues[8,43] may occur.

The evidence presented here for histatin, that proteolysis associated with whole salivary enzymes does not instantly abolish all functional activity, represents a novel concept in oral host defence mechanisms. This concept is likely to be of far reaching importance considering its possible applicability to other salivary proteins playing a role in a variety of oral homeostatic processes.

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