CHAPTER V

SALIVARY ANTIPROTEASE ACTIVITIES
INTRODUCTION

In the previous two chapters, salivary proteolytic activities with chromogenic substrates and their diagnostic importance are described. This chapter deals with a study of proteolytic inhibitors in saliva.

The $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI) concentration in gingival crevicular fluid of healthy sites is reported to approximate serum level of this protein (about 2.8 mg/ml) presumably reflecting the serum transudate nature of gingival crevicular fluid (218).

The gingival crevicular fluid $\alpha_1$-PI concentration was found to be increased by 200% and 290% in gingivitis and adult periodontitis patients respectively (229), reflecting not only increased serum exudation into crevices/pockets due to the vascular component of the inflammatory response, but also presumably elevated production of this protein by monocytes/macrophages infiltrating the periodontal lesions. Circulating monocytes, after they extravasate into gingival connective tissue during inflammation and after they are transformed into macrophages, produce elevated levels of $\alpha_1$-PI (230).

An individual with a normal amount of $\alpha_1$-PI is known to cope up with the burst of developing infection by increasing the amount of $\alpha_1$-PI produced by liver. A person with a deficient phenotype, e.g., MZ is often unable to increase the production of $\alpha_1$-antitrypsin to the same level during inflammatory challenge. The proteases activated in the tissues and released from polymorphonuclear leucocytes thus remain unneutralized. Ohlson and co-workers have shown that some of the proteases released in inflammatory destructive periodontal disease remain free and active, due to the insufficiency of the local protease inhibitory capacity in the periodontal pocket (231). In combination with plasma-derived protease inhibitors, the production of additional protease inhibitors by oral bacteria would protect the host tissue from degradation (232).
There are a few reports in the literature on the serum levels of $\alpha_2$-MG in periodontitis. Saito et al reported elevated serum levels of $\alpha_2$M in three different types of periodontal disease: periodontitis, periodontosis and "gingival recession with inflammation" (145). $\alpha_2$MG in GCF is thought to be mainly plasma derived (226). However, in view of the high molecular weight of this globulin, it has been proposed that a proportion in healthy sulci might represent local synthesis rather than vascular leakage (233, 234).

The concentration of total $\alpha_2$MG was always found to be lower in GCF than in plasma from the same patient. At highly inflamed sites, the concentration of total $\alpha_2$-MG in GCF is significantly associated with the plasma concentration, thereby reflecting the increased vascular permeability at these sites. Elevated protease levels which may lead to or which may be caused by increased inflammation are associated with elevated levels of total $\alpha_2$-MG in GCF (235). It is likely that the GCF concentration of $\alpha_2$-MG is influenced by factors other than protease activity, such as local production by macrophages (236), fibroblasts (237) and the concentration of vasodilator agents.

Number of investigators had measured $\alpha_2$-MG level and a contradictory relationship between healthy and diseased periodontal sites has emerged. According to Skeleri and others (238) the concentration of total $\alpha_2$-MG (protease bound and free form) is lower in gingival fluid taken from sites with more inflammation. Its concentration decreases with an increase in the pocket depth and the alveolar bone loss, at the sites of fluid collection. The concentration of $\alpha_2$-MG bound form in gingival fluid is low or absent at sites with more pronounced bone loss (above 15%). These results support the hypothesis that unbalanced protease activity damages the periodontal tissue. This observation was confirmed by Gustafson and others, who have studied the relationship between elastase level to $\alpha_2$-MG. They came to the conclusion that periodontal tissue destruction is associated with increased levels of elastase activity and decreased levels of $\alpha_2$-MG (239).
Cystatins are cystein protease inhibitors present in a variety of tissues and body fluids including saliva. They protect the organism against the controlled action of endogenous and exogenous cysteine proteases (240). Originally, members of the cystatin superfamily were grouped into three families: family I or stefins, family II or cystatins (including salivary cystatins) and family III or kininogens (241). Aguirre and others estimated the levels of salivary cystatins in periodontally healthy and diseased individuals. They did not find any significant difference in the levels between healthy and diseased individuals. These findings suggest that comparing the levels of cystatins in glandular saliva may not be a suitable indicator of periodontal disease status (242).

Histatins are a family of salivary polypeptides characterized by their high content of histidine residue. For example, Histatin-5, comprises about 29% of histidine. In addition to its antibacterial and antifungal activities, it also acts as a protease inhibitor. It acts as a strong inhibitor of a protease form Bacteroides gingivalis, an oral bacterium suspected of being a pathogen of adult periodontal disease (243).

Impaired regulation of proteolysis is an important biochemical aspect in the aetiology of periodontal disease (244). Most of the recent studies have shown increased levels of proteolytic enzymes with increasing severity of inflammation and the varied levels of different protease inhibitors in crevicuar fluid and gingival homogenates. However, it has not been determined if levels of salivary protease inhibitors can be useful in assessing periodontal disease status. Such an association might serve as an indicator of disease activity. Accordingly, the goal of this study was to see if there is any correlation between the levels of salivary protease inhibitory activity and periodontal status. The present study reports levels of total antitryptic, antielastase and antichymotryptic activities in saliva in normal condition and in periodontitis and gingivitis.
MATERIALS AND METHODS

Saliva samples from healthy controls and patients were collected as mentioned in Chapter II.

Salivary total protein was estimated as mentioned in Chapter II.

Estimation of salivary antielastase activity

To 0.2 ml of saliva sample, 0.5 ml of sodium phosphate buffer (0.2Moles, pH 7.6) and 0.1 ml of appropriately diluted enzyme (4.3 µg porcine elastase) was added in a final volume of 1 ml with distilled water. After preincubation at 37°C for 15 minutes, the enzyme reaction was started by the addition of 1ml substrate solution (1mg STANA). Incubation was continued for a further period of 15 minutes after which the reaction stopped by the addition of 1 ml 30% acetic acid. A blank (without enzyme, without saliva), control (without saliva) and a second blank (without enzyme) were run simultaneously. The readings were taken at 410 nm. Uncorrected antielastase activity is the difference between the test and control systems. The second blank value is a measure of salivary elastase activity.

Estimation of salivary antitryptic activity

The assay system consist of 0.4 ml of 0.1M sodium phosphate buffer (40 µMoles, pH 7.6), 0.2 ml saliva and 0.2 ml of appropriately diluted enzyme (20 µg bovine trypsin) solution in a total volume of 1 ml. After preincubation at 37°C for 15 minutes, the enzyme reaction was started by the addition of 2ml substrate solution (BAPNA, 4µM). Incubation was continued for a further period of 15 minutes, after which the reaction was stopped by the addition of 1 ml of 30%, acetic acid. Antitryptic and tryptic activity were evaluated as indicated for antielastase activity.
Estimation of salivary antichymotryptic activity

The test consisted of 0.5 ml of 0.2Moles sodium phosphate buffer pH 7.6, 0.2 ml saliva and 0.15 ml of appropriately diluted enzyme (0.3 μg bovine chymotrypsin) in a final volume of 1 ml. After preincubation at 37°C for 15 minutes, the reaction was started by the addition of one ml substrate solution (S-2586, 1.53 μmoles). Incubation was continued for further period of 15 minutes after which the reaction was stopped by the addition of 1 ml 30% acetic acid. A blank (without enzyme, without saliva), control (without saliva) and a second blank (without enzyme) were run simultaneously. The readings were taken at 410 nm. Uncorrected antichymotryptic activity is the difference between the test and control systems. The second blank value is a measure of salivary chymotrypsin activity with S-2586 substrate.

RESULTS

Salivary antitryptic activity

Normal saliva samples showed low tryptic activity with BAPNA as substrate. However, it is fairly efficiently hydrolyzed by bovine trypsin used in these studies. For this reason, BAPNA was chosen to evaluate antitryptic activity in saliva. Normal salivary samples showed BAPNA hydrolytic activity to the tune of 0.1-0.265 absorbance units/ml under the assay conditions.

The measured antitryptic activity was found to be insignificant. In fact, in six out of 12 samples, test values (absorbance in presence of trypsin plus saliva) were higher than control values (absorbance in presence of trypsin alone). This is partially due to the endogenous BAPNA hydrolytic activities of saliva. After correction for this activity, which was measured simultaneously, the antitryptic activity was observed in nine out of 12 samples. The range of activities before and after correction were respectively, (+) 0.33 to 0.09 and (+) 0.22 to 0.355/ml saliva. Mean ± S.D. values (n=12) were (+) 0.044 ± 0.158 before correction and 0.128 ± 0.199 after correction. The (+) sign indicates activation rather than inhibition.
The patterns of antitryptic activities in periodontitis and gingivitis were grossly similar. Before correction for in situ salivary trypsic activity, only in one sample in periodontitis, antitryptic activity could be detected. The values ranged from (+) 0.555 to 0.385/ml. After correction, in four out of 10 samples antitryptic activity was noticed. The calculated corrected values ranged from (+) 0.345 to 0.485 absorbance units/ml saliva ((+) 0.038 ± 0.243 Mean ± S.D.). In gingivitis, in none of the ten samples, antitryptic activity could be noticed. The values (difference between control and test) ranged from (+) 0.290 to (+) 0.03. After correction for salivary trypsic activity in 8 out of 10 samples antitryptic activity was noticed. The values ranged from (+) 0.11 to 0.195 absorbance units/ml saliva (0.0675 ± 0.1061 Mean ± S.D.).

These data indicate that measured antitryptic activity in normal saliva is low. In gingivitis and periodontitis, the increase in trypsic activity, more than compensate for the increase in antitryptic activity, if any. Statistical analysis showed that there is no difference between the three groups.

In some salivary samples, there was an apparent activation of BAPNA hydrolytic activity on addition of saliva to bovine trypsin. In other words, absorbance measured when trypsin and saliva were present together was more than the sum of the values with trypsin and saliva individually. Typical data are shown in Table 5.1. Even though the activation was small, it was highly reproducible in individual samples.

Bovine trypsin was preincubated with saliva samples for different time intervals (0 to 30 minutes) before the addition of BAPNA and then the trypsic activity was measured. Time of preincubation did not alter the magnitude of activation (data not shown). This indicates that bovine trypsin does not activate latent trypsin-like activity in saliva, as function of time. The alternate possibility for activation observed, could be due to protection of bovine trypsin by salivary constituents against denaturation.
TABLE 5.1  
SALIVARY TRYPTIC (BAPNA HYDROLYSIS) ACTIVITY

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Trypsin</th>
<th>Trypsin + Saliva (0.2ml)</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (P)</td>
<td>0.461</td>
<td>0.531</td>
<td>+0.054</td>
</tr>
<tr>
<td>2 (N)</td>
<td>0.442</td>
<td>0.506</td>
<td>+0.044</td>
</tr>
<tr>
<td>3 (P)</td>
<td>0.460</td>
<td>0.507</td>
<td>+0.031</td>
</tr>
<tr>
<td>4 (N)</td>
<td>0.444</td>
<td>0.488</td>
<td>+0.024</td>
</tr>
<tr>
<td>5 (G)</td>
<td>0.411</td>
<td>0.450</td>
<td>+0.020</td>
</tr>
</tbody>
</table>

The assay conditions are described under methods
P: Periodontitis.
N: Normal
G: Gingivitis

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TABLE 5.2

SALIVARY ANTIELASTASE ACTIVITY

Absorbance units per ml saliva : Mean ± S.D.

<table>
<thead>
<tr>
<th>Group</th>
<th>Uncorrected</th>
<th>Corrected for salivary elastase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.0254 ± 0.110</td>
<td>0.181 ± 0.111</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>0.553 ± 0.279&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14 ± 0.505&lt;sup&gt;al&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>(+)0.135± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0185 ± 0.240&lt;sup&gt;bl&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>(+)</sup> = Activation rather than inhibition

- a  p<0.01  t = 5.51  {Periodontitis Vs Normal}
- a<sup>l</sup>  p<0.01  t = 5.86
- b  p<0.01  t = 6.01  {Periodontitis Vs Gingivitis}
- b<sup>l</sup>  p<0.01  t = 6.04
Salivary antielastase activity

The data on antielastase activities are summarized in Table 5.2. The observed (uncorrected) values in normal samples ranged from (+) 0.115 to 0.195. In 6 out of 12 samples antielastase activity could be measured. In others, absorbance in test samples was higher than in control samples due to endogenous salivary elastase activity. After correction for these activities, in individual samples, antielastase activity was noticed in all but one sample. The values ranged from 0 to 0.360 absorbance units/ml saliva. Thus, normal antielastase activity was found to be low in saliva, like the antitryptic activity.

In periodontitis, in all the ten samples, antielastase activity could be directly measured. The values ranged from 0.13 to 0.915. After correction for endogenous (salivary) elastase activity, the calculated values ranged from 0.38 to 2.07 absorbance units per ml saliva. Compared to normals, the increase in antielastase activity was statistically significant in periodontitis both for uncorrected (p<0.01, t=5.51) and corrected (p<0.01, t = 5.86) values. It has been indicated in Chapter IV that elastase activity is also increased in periodontitis. Under the present assay conditions (0.2 ml saliva, 15 minutes incubation) also, STANA hydrolytic activity by saliva was significantly increased in periodontitis (0.05 - 0.255 absorbance units) compared to normals (0.021 -0.06).

The uncorrected antielastase activity in salivary samples from gingivitis cases was low. The values ranged from (+) 0.465 to 0.095. Only in one out of 10 samples, antielastase activity could be detected. After correction for salivary elastase activity, the values ranged from (+) 0.245 to 0.36 absorbance units/ml saliva. In four samples there was no measurable antielastase activity. Statistical analysis of the data indicated that salivary antielastase activity (after correction) was significantly increased in periodontitis than in gingivitis (p<0.01, t=6.04). Since protein level in saliva is higher in gingivitis than in periodontitis, the difference is also significant when the values are normalized for protein levels (p<0.01, t=5.44).
Salivary antichymotryptic activity

In Table 5.3 the data on antichymotryptic activity are summarized. Unlike antitryptic and antielastase activities, significant antichymotryptic activity could be detected in all normal salivary samples even without correction for endogenous chymotryptic activity. The values ranged from 0.620 to 1.88 absorbance units per ml. After correction the values were in the range, 0.735 - 2.07 absorbance units/ml.

In periodontitis, the uncorrected values for antichymotryptic activity ranged from 0.33 to 2.26 and the corrected values were in the range 0.59-2.90 absorbance units/ml. They were well within the normal range. The antichymotryptic activities in gingivitis were in the range 0.41-2.37 (uncorrected) and 0.560-2.94 (corrected) respectively. The values were not different in three groups based on statistical analysis.

It is worthwhile to emphasize that even though antichymotryptic activity in salivary samples is higher than antielastase or antitryptic activities in terms of absorbance units, when expressed as quantity of enzyme(s) inhibited, the antichymotryptic activity is not high. This is because the amount of bovine chymotrypsin used in the assay system (0.3 µg) is much higher than the amount of bovine elastase (4.3 µg) or bovine trypsin (20 µg) used to get comparable absorbance values (0.45-0.50). The calculated maximal amount of enzyme inhibited by one ml of normal saliva was 1.22 µg chymotrypsin (absorbance unit 2.07), 3.1 µg elastase (absorbance unit 0.36) and 14.8 µg trypsin (absorbance unit 0.355). Similarly, maximal inhibition observed in periodontitis was 1.41 µg chymotrypsin, 17.8 µg elastase and 20.2 µg of trypsin per ml of saliva. These data also emphasize that salivary antielastase activity is more of diagnostic significance in differentiating normal group from periodontitis patients.
<table>
<thead>
<tr>
<th>Group</th>
<th>Uncorrected</th>
<th>Corrected for salivary chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.924 ± 0.420</td>
<td>1.09 ± 0.450</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>0.639 ± 0.792</td>
<td>1.820 ± 0.74</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>1.46 ± 0.770</td>
<td>1.72 ± 0.877</td>
</tr>
</tbody>
</table>
DISCUSSION

The reported values of antitryptic and antielastase activities in serum using methods similar to those described in this chapter are 70-120 and 140-230 absorbance units per ml in normal condition (245). Comparison of these values with the values in normal saliva indicates that antitryptic and antielastase activities are several hundred times lower in saliva. The gradient is much steeper for inhibitors than for proteases reported in Chapter IV. Ohlsson et al reported that in both healthy and inflammed crevices of gingival tissue, the protease inhibitory capacity of α-1 protease inhibitor was saturated whereas elastase and collagenase activities of polymorphonuclear leukocyte origin was 5-7 fold higher in inflamed gingival pockets. In the light of these data, it is not clear as to where the antiprotease activities in saliva originates.

From the diagnostic view point, the present studies show that measurement of antitryptic activity and antichymotryptic activity is not of much significance. These parameters in saliva are not significantly altered in gingivitis or periodontitis. This is in contrast to the generalized increase in trypsin-like activities in periodontitis (vide Chapters III and IV). However, antielastase activity was found to be significantly elevated in periodontitis but not in gingivitis. This is in contrast to the increased elastase activity in saliva in both periodontitis and gingivitis as reported in Chapter IV.

These data suggest that protease inhibitors and proteases in saliva, in gingivitis and periodontitis will have different origins.

The nature of the factors responsible for the observed protease inhibitory activities in saliva is not known. It is unlikely, α-2 macroglobulin can be a significant contributory factor. Because of its large size, it cannot be expected to enter form plasma into saliva through crevicular fluid to any significant extent. Further, α-2 macroglobulin bound proteases are fairly efficient in hydrolyzing the synthetic substrates used in these studies. Plasma α1-protease inhibitor acts
efficiently on elastase, trypsin and chymotrypsin in that order. The rate of inactivation, in terms of $K_{association}$ for this factor is $6.5 \times 10^7$ M$^{-1}$ Sec$^{-1}$ for elastase and $4.8 \times 10^4$ M$^{-1}$ Sec$^{-1}$ for trypsin. In practical terms, preincubation of the target protease with $\alpha_1$ – protease inhibitor for time upto 15 minutes is necessary to elicit maximal inhibitory activity. Since the molecular weight of $\alpha_1$ – protease inhibitor is much smaller than that of $\alpha_2$MG, it can be expected to find its origin from plasma to saliva relatively easily. $\alpha_1$.Antichymotrypsin a minor inhibitor in plasma is highly specific for chymotrypsin. It has a molecular mass comparable to that of $\alpha$-1 protease inhibitor. Fairly constant antichymotryptic activity in normal saliva compared to variable values for antitryptic (and antielastase) activity suggests that it can arise from plasma.

However wide variations in the levels of antitryptic, antielastase and antichymotryptic activities in saliva in periodontitis compared to normals suggests that there are sources other than plasma for salivary inhibitory factors. It is known that human mucous secretions contain low molecular weight factors that inactivate elastase, cathepsin-G and chymotrypsin like enzyme. The action of histatins on elastase and chymotrypsin are not studied. Further investigations are needed to identify the inhibitory factors present in saliva.