*Indian Journal of Clinical Biochemistry, 1998, 13(1), 46-51*

# SALIVARY PROTEOLYTIC ACTIVITIES IN PERIODONTITIS, GINGIVITIS AND DIABETES MELLITUS

**Praveen Kumar Shetty and T.N. Pattabiraman**

Department of Biochemistry, S.D.M. College of Dental Sciences, Sattur, Dharwad-580 009.

## ABSTRACT

**Elastase activity was found to be significantly increased in periodontitis (0.872 ± 0.4270 absorbance units/mg protein, mean ± S.D., 1.05 ± 0.61 units/ml saliva), gingivitis (0.772 ± 0.416 units/mg protein, 1.515 ± 0.952 units/ml) and diabetes (0.549 ± 0.286 units/mg protein, 1.20 ± 0.769 units/ml) compared to normals (0.255 ± 0.089) units/mg protein, 0.264 ± 0.079 units/ml). Chymotryptic activity was not found to be increased in these disease conditions over the normal level (0.284 ± 0.096 units/mg protein). Aminopeptidase activity was elevated only in periodontitis (0.670 + 0.140 units/mg protein) compared to normals (0.349 ± 0.100 units/mg protein). Trypsin-like activity was also found to be significantly raised in periodontitis compared to normals when Pro-Phe-Arg-p-nitroanilide (0.666±0.204 units/mg protein), Ile-Pro-Arg-p-nitroanilide (1.59 ± 0.260 units/mg protein) and Pyroglu-Pro-Arg-p-nitroanilide (1.82 ± 0.380 units/mg protein) were used as substrates. The normal values with these three substrates were respectively, 0.454±0.110, 1.04±0.231 and 1.15 ± 0.312 units/mg protein. Total protein level in saliva was found to be significantly elevated in gingivitis (209± 66.8 mg/dl) and diabetes (204 ± 68.0) compared to normal values (107 ± 20.7). Increase in periodontitis was marginal (127 ± 28.3 mg/dl). Expression of proteolytic activities normalized to protein level was useful in differential diagnosis. Increase in elastase level in saliva is not a specific marker for periodontal diseases.**

KEY WORDS : Elastase, Proteases, Saliva, Periodontitis, Gingivitis, Diabetes mellitus

## INTRODUCTION

Human saliva contains trace quantities of proteolytic enzymes, both of host and microbial sources (1). While kallikrein is of submaxillary origin, collagenase and elastase are considered to arise from leukocytes (2-4). The latter two enzymes enter the oral cavity through gingival sulcus. In addition, tryptase, cathepsins B and L and a dipeptidyl aminopeptidase have been identified in gingival crevicular fluid (5). Presence of a serine proteinase has been demonstrated in the secretion of human submandibular gland (6). A trypsin-like activitiy of microbial origin has also been identified in sublingual plaque (7).

Proteolytic activities, especially that of elastase in crevicular fluid, are considered to be useful markers for periodontitis (5,8,9). Elastase activity in whole saliva and water-rinse samples of oral cavity was shown to be significantly increased in periodontitis and the levels correlated well with the severity of the disease (10). Elastase activity was not found to be a good index of gingivitis (10). Darany et. al. observed that elastase activity in gingival fluid when normalized to protein content, was significantly higher in periodontal diseases (11). These workers also found a significant correlation between specific activity of elastase and gingival flow rate. No correlation studies between protease activities and protein level in saliva are available in normal and disease conditions.

Total protein content in gingiva and crevicular fluid is known to be increased in periodontitis (12). Total protein level is also shown to be elevated in saliva in periodontitis and to a lesser extent in gingivitis (13). Salivary protein is increased in

**Author for correspondence**

Dr. Praveen Kumar Shetty, at above address.

diabetes and Kertesz et. al. (14) attributed this increase to enhanced basement membrane permeability. It can be expected that proteolytic activity may be increased in saliva in diabetes and this would minimize the diagnostic significance of assessing proteolytic activities in saliva and crevicular fluid in oral diseases. In this communication, we report the levels of trypsin-like, chymotrypsin, elastase and aminopeptidase activities in saliva in normals, gingivitis, periodontitis and diabetes. The alterations in terms of protein level are evaluated as diagnostic indices.

## MATERIALS AND METHODS

Four groups of subjects were chosen for the study. The control group consisted of student and staff volunteers who had normal gingiva and absence of loss of attachment as determined by probing. They were healthy with no systemic diseases and their blood sugar levels were in the normal range. The second group consisted of 36 patients who attended the clinics of Department of Oral diagnosis, S.D.M. College of Dental Sciences, Dharwad. They presented symptoms of gingivitis (severe inflammation, marked redness, edema, ulceration, tendency to spontaneous bleeding and loss of attachment) as determined by the clinicians. The third group consisted of 30 patients who were referred to the Department of Periodontology for treatment. The subjects were selected based on radiographic evidence of alveolar bone loss and having at least 6 to 8 sites with a probing pocket depth of $\geq$5mm. The fourth group consisted of 23 subjects with high post-prandial blood glucose values. They had normal gingiva and had no symptoms of periodontal diseases. They were confirmed diabetics.

Unstimulated whole saliva samples were collected around 9-10 A.M. and centrifuged at 800 g for 10 minutes. The clear supernatants were assayed immediately for proteolytic activities and protein concentraiton. A total of 35 samples from healthy subjects (20 males, 15 females) and 89 samples from patients (50 males, 39 females) were analysed. The age of the subjects varied from 24 to 60 years. All the diabetic subjects had post-prandial blood glucose values in the range, 210-340 mg/dl. There was no correlation between blood glucose level and salivary protein concentration in this group (n=23).

Total salivary protein was determined by the bicinchoninic acid method using bovine serum albumin as standard (15). Proteolytic activities using the dye-bound protein substrates, azocollagen, congocollagen, azocasein, and orcein-albumin (products of Calbiochem, San Dieogo, U.S.A.) were estimated as described by Rinderknecht et. al. (16). Ten mg of the substrate was incubated with 0.2 ml of saliva in 3.0 ml (0.18 M Tris-HCl buffer pH 7.6) at 37°C for 4 hours. After centrifugation at 800 g for 15 minutes, absorbance in the clear supernatants were measured. Absorbance was quantitated at 490 nm for congocollagen, 520 nm for azocollagen, 410nm for azocasein and orcein-albumin.

The following peptide linked p-nitroanilides (obtained from Kabi Vitrum Diagnostika, Molndal, Sweeden) were also used as substrates, Pro-Phe-Arg-pNA (S-2302, a substrae for kallikrein), Val-Leu-Arg-pNA (S-2266, a substrate for glandular kallikrein), Pyroglu-Pro-Arg-pNA (S-2366, a substrate for protein C), Ile-Pro-Arg-pNA (S-2288, a substrate for plasminogen activator), Val-Leu-Lys-pNA (S-2251, a substrate for plasmin) and Phe-Pip-Arg-pNA (S-2238, a substrate for thrombin). More than one serine proteinase can hydrolyze these compounds (17). Specific substrates for elastase (MeSuc-Ala-Ala-Pro-Val-pNA, MAPV), chymotrypsin (Suc-Ala-Ala-Pro-Phe-pNA, SAPP) and aminopeptidase (Leu-pNA, LP) were purchased from Sigma Chemical Company, St. Louis, U.S.A. p-Nitroaniline liberated from these substrates by the action of salivary enzymes was quantitated by measuring absorbance at 405 nm (18). The assay system consisted of one mg of the substrate, aliquots of saliva (0.2 ml), 100 µmoles of phosphate buffer pH 7.6 in a volume of 2.0 ml. After incubation for definite time intervals (15-60 minutes) at 37°C, the reaction was arrested by the addition of one ml of 30% acetic acid and the absorbance was measured with the clear supernatants obtained by centrifugation at 800g for 10 minutes. Proteolytic activities are expressed as absorbance units per 30 minutes.

Phenylmethylsulfonyl fluoride (PMSF), Lima bean trypsin inhibitor (LBTI) and soyabean trypsin inhibitor (SBTI) were obtained from Sigma Chemical Company, St. Louis, U.S.A. The inhibitory effects

of these compounds on salivary-protease activities were assessed as follows. An aliquot of saliva (0.2 ml) was incubated with 0.9 mg of PMSF, 0.2 mg of LBTI or 0.2 mg of SBTI for 15 minutes in phosphate buffer at 37°C. The substrate was added and the residual proteolytic activity was measured as described above. Controls without the inhibitors were run simultaneously.

## RESULTS

Normal salivary samples showed very low proteolytic activities when the dye-bound proteins, azocollagen, congocollagen, azocasein and orcein-albumin were used as substrates. Absorbance units per hour per ml saliva were found to be 0.055-0.063 for congocollagen, 0.007-0.013 for azocollagen, 0.010-0.030 for azocasein and 0.03-0.080 for orcein-albumin (data for 4 samples each). However, significant activities were noticed with synthetic peptide p-nitroanilide substrates. Preliminary studies showed fairly high activities with S-2366 (1.39 absorbance units per ml per 30 minutes, mean of three samples), S-2288 (1.05) and S-2266 (1.05). Moderate activities were seen with S-2302 (0.465), S-2238 (0.51) and S-2251 (0.42). All these compounds are substituted arginyl or lysyl p-nitroanilides and are substrates for trypsin-like enzymes. The hydrolytic activities with MAPV (elastase subtrate), SAPP (chymotrypsin substrate), and LP (aminopeptidase substrate) were lower in saliva. The mean values for three samples were respectively, 0.260, 0.290 and 0.350 absorbance units.

The salivary proteolytic activities were fairly heat stable. Exposure to 60°C for 60 minutes destroyed 30% of elastase activity, 46% of chymotrypsin activity and 60% of trypsin-like activity (with S-2302). Heating at 80°C for 10 minutes destroyed all the three activities. Activities were progressive with time of incubation when S-2302, S-2266 and S-2288 were used as substrates upto one hour. With elastase and chymotrypsin substrates, activities reached a plateau at 30 minutes. The enzyme activities were also susceptible to different extent to inhibitors. PMSF inhibited activities with S-2266, S-2302, MAPV and SAPP to the extent to 30.4%, 47.4%, 59.0% and 68.9% respectively. On the other hand, LBTI had neglible action on glandular kallikrein and elastase activities. Percent inhibition with S-2266, MAPV, S-2302 and SAPP were found to be respectively, 5.1, 6.4, 56.6 and 55.6. SBTI had feeble action on elastase, but powerfully inhibited kallikrein and chymotrypsin activities. Magnitudes of inhibition with SAPP, S-2302, MAPV and S-2266 were 6.4%, 57.8%, 64.5% and 26.0%. pH optima of the proteolytic activities were also found to be slightly different. Elastase (MAPV) activity was maximal over the broad pH range of 7.5-10.0. Optimal chymotryptic (SAPP) activity was observed in the pH range, 8.0-8.5. S-2266 and S-2288 activities showed sharp pH optimum of 9.0. S-2302 activity alone was maximal at pH 10.0.

A total of 35 salivary samples from healthy subjects were assayed for S-2302 hydrolytic activity and protein. Absorbance units with S-2302 per ml saliva and per mg of protein, varied in the range, 0.280-0.605 and 0.219.-0.625, respectively. Protein levels ranged from 72.8 mg/dl to 147 mg/dl. The age of the subjects varied from 24 to 45 years. No correlation between age and S-2302 activity or protein concentration was noticed. No significant difference with respect to proteolytic activity or protein in regard to sex was observed (males 20, females 15). The patterns were similar with other substrates in the control group. In table 1, hydrolytic activity with S-2302 in saliva in health and diseases is represented. In gingivitis, periodontitis and diabetes, proteolytic activities were significantly elevated when expressed as values per unit volume. In terms of protein concentration, the increase was more significant in periodontitis (t=4.41) than in gingivitis (t=2.19). The values in diabetic samples were not statistically different from normal values. In gingivitis and diabetes, salivary protein was significantly elevated. In periodontitis, the increase was marginal.

In Table 2, hydrolytic activities with S-2288 and S-2366 as substrates are shown. Units per ml of saliva were found to be significantly increased in gingivitis (t=6.41 and 5.66, respectively), periodontitis (t=7.69 and 6.22) and diabetes (t=5.55 and 5.35). However, when activities were expressed as functions of protein concentration, only in periodontitis, S-2288 (t=5.40) and S-2366 (t=4.73) activities were elevated.

**Table 1. Hydrolytic activities in saliva on S-2302**

| Group | units/ml | Units/mg protein | Protein, mg/dl |
|---|---|---|---|
| Normals (n=26) | 0.464 ± 0.081 | 0.454 ± 0.110 | 107 ± 20.7 |
| Gingivitis (n=26) | 1.08 ± 0.332[a] | 0.534 ± 0.146[b] | 209 ± 66.8[a] |
| Periodontitis (n=20) | 0.802 ± 0.169[a] | 0.666 ± 0.204[a] | 127 ± 28.3[b] |
| Diabetes (n=23) | 1.05 ± 0.466[a] | 0.548 ± 0.228[c] | 204 ± 68.0[a] |

Values are mean ± S.D. Activities are in absorbance units. a: P<0.01 compared to normals. b: P<0.05, c:P>0.05.

**Table 2. Hydrolytic activities with S-2288 and S-2366**

| Group | S-2288 | | S-2366 | |
|---|---|---|---|---|
| | units/ml | Units/mg protein | Units/ml | Units/mg protein |
| Normals (n=16) | 1.07 ± 0.160 | 1.04 ± 0.231 | 1.19 ± 0.209 | 1.15 ± 0.312 |
| Gingivitis (n=10) | 2.01 ± 0.540[a] | 1.08 ± 0.244 | 2.35 ± 0.760[a] | 1.24 ± 0.310[b] |
| Periodontitis (n=10) | 1.73 ± 0.260[a] | 1.59 ± 0.260[a] | 1.99 ± 0.420[a] | 1.82 ± 0.380[a] |
| Diabetes (n=15) | 2.27 ± 0.820[a] | 1.31 ± 0.610[b] | 2.24 ± 0.730[a] | 1.30 ± 0.590[b] |

Values are mean ± S.D. Activities are in absorbance units. a: P<0.01 compared to normals b: P>0.05.

**Table 3. Hydrolytic activities on MAVP, SAPP and LP**

| Group | MAVP | SAPP | LP |
|---|---|---|---|
| | Units/mg protein | Units/mg protein | Units/mg protein |
| Normals (n=16) | 0.255 ± 0.089 | 0.284 ± 0.096 | 0.349 ± 0.10 |
| Gingivitis (n=16) | 0.772 ± 0.416[a] | 0.280 ± 0.150[b] | 0.480 ± 0.210[b] |
| Periodontitis (n=10) | 0.872 ± 0.427[a] | 0.400 ± 0.210[b] | 0.670 ± 0.140[a] |
| Diabetes (n=15) | 0.549 ± 0.286[a] | 0.340 ± 0.200[b] | 0.490 + 0.170[b] |

Values are mean ± S.D. Activities are in absorbance units. a: P<0.01 compared to normals b: P>0.05.

In Table 3, hydrolytic activities in saliva on MAPV, SAPP and LP are indicated. Elastase activity (MAPV hydrolysis), was found to be significantly increased in periodontitis (t=5.20), gingivitis (t=4.68) and diabetes (t=3.76). The values were also quite high, in terms of saliva volume in disease conditions (absorbance units per ml: normal 0.264 ± 0.079, mean ± S.D.; gingivitis 1.515 ± 0.952, diabetes 1.20 ± 0.767; periodontitis 1.05 ± 0.611). On the other hand, chymotryptic activity (SAPP hydrolysis) in disease conditions did not differ statistically from normal levels, when expressed as units/mg protein. Absorbance units per ml saliva were 0.290 ± 0.111 (normals), 0.490 ± 0.230 (gingivitis), 0.440 ± 0.230 (periodontitis) and 0.670 ± 0.610 (diabetes). Chymotryptic activity varied over a wide range, especially in diabetic samples. With reference to aminopeptidase (LP) activity, only in periodontitis, the specific activity was found to be significantly elevated (t=6.53). As absorbance units per ml saliva, aminopeptidase activities were 0.370 ± 0.108 (mean ± S.D.) in normals, 0.870 ± 0.330 in gingivitis, 0.740 ± 0.190 in periodontitis and 930 ± 0.500 in diabetes. Increase in aminopeptidase activity per unit volume of saliva was found to be statistically significant in all the three disease conditions.

## DISCUSSION

Quantitative measurement of elastase and other proteases in terms of specific activity in crevicular fluid for diagnostic purposes, is not possible as a routine investigation (11). Measurement of proteolytic activity in saliva in terms of volume is not of much significance in identifying and differentiating oral diseases. The present studies indicate that a host of activities including elastase, aminopeptidase, chymotrypsin, kallikrein and trypsin-like enzymes were found to be increased in saliva in periodontitis, gingivitis and diabetes. When normalised to protein level, elastase activity was found to be increased in all the three disease conditions, indicating that salivary elastase measurement is not of much significance in differential diagnosis. On the other hand, normalised activities of trypsin-like enzymes (with the substrates S-2288 and S-2366) and aminopeptidase activity were found to be selectively elevated in periodontitis. While S-2288 is maximally acted upon by trypsin followed by thrombin, S-2366 is the preferred substrate for protein C and thrombin.

Protein concentration in saliva was found to be elevated to a lesser degree in periodontitis than in gingivitis or diabetes. This contradicts the observation of Henskens et. al. who reported higher protein levels in periodontitis than in gingivitis (13). Salivary protein level varied over a wide range in diabetes (82.4-357 mg/dl) and gingivitis (118-433 mg/dl) than in periodontitis (80.0-161mg/dl). The nonspecific increase of proteolytic activities in the former two conditions can be attributed to changes in basement membrane permeability or due to leukocyte infiltration.

## ACKNOWLEDGEMENT

The help of Dr. N.T. Desai, Professor and Head, Department of Oral diagnosis and Dr. Srinath L.Thakur, Professor and Head, Department of Periodontology, S.D.M. College of Dental Sciences, Dharwad, in the diagnosis and grouping of the subjects is acknowledged. The help of S.B. Javali for statistical analysis of the data is acknowledged.

## REFERNECES

1. Nakamura, M. and Slots, J. (1983) Salivary enzymes: Origin and relationship to periodontal disease. J. Periodont. Res. 18, 559-569.

2. Matsuda, Y., Fujimoto, Y., Watanabe, Y., Obara, T. and Akhihama, S. (1989) Human salivary kallikrein and submaxillary gland kallikreins. Adv. Expt. Med. Biol. 247/B, 169-175.

3. Lijima, K., Ando, M., Nakashizuka, J. and Hayakawa, T. (1983) Collagenase activity in human saliva J.Dent. Res. 62, 709-712.

4. Gustaffson, A., Asman, B., Bergstrom, K. and Soder, P.O. (1992) Granulocyte elastase in gingival crevicular fluid. A possible discriminator between gingivitis and periodontitis. J.Clin. Periodont. 19, 535-540.

5. Cox, S.W. Detection of cathepsin B and L, elastase, tryptase, trypsin and dipeptidyl peptidase IV like activities in crevicular fluid from gingivitis and periodontitis patients with peptidyl derivatives of 7-amino 4-trifluoromethyl coumarin. J. Periodont. Res. 24, 353-361.

6. Piotrowski, J., Czajkowski, A., Murty, V.L., Slomiany, A. and Slomiany, B.L. (1992) Identification of human salivary protease activity towards mucin. Differences with caries. Biochem. Int. 28, 939-947.

7. Loesche, W.J., Syed, S.A. and Stoll J. (1987) Trypsin-like activity in sublingual plaque. A diagnostic marker for spirochetes and periodontal disease. J.Periodontol. 58, 266-273.

8. Palconis, K.G., Larjava, I.K., Wells, B.R., Suggs, K.A., Landis, J.R., Chadwick, D.E. and Jeffcoat, M.K. (1992) Elastase as an indicator of peridontal disease progression J.Periodontol. 63, 237-242.

9. Armitage, G.C., Jeffcoat, M.K., Chadwick, D.E., Taggart, E.J. Jr., Numabe, Y., Landis, J.R., Weaver, S.L. and Sharp, T.J. (1994) Longitudinal evaluation of elastase as a marker for the progression of periodontitis. J. Periodontol. 65, 120-128.

10. Uitto, V.J., Nieminen, A., Coil, J., Hurttia, H. and Larja, H.(1996) Oral fluid elastase activity as an idicator of periodontal health. J. Clin. Periodontol. 23, 30-37.

11. Darany, D.G., Beck, F.M. and Walters, J.D. (1992) The relationship of gingival fluid leukocyte elastase activity to gingival fluid flow rate. J. Periodontol. 63, 743-747.

12. Akalin, F.A. Sengun, D., Eratalay, K., Renda, N. and Caglayan, G. (1993) Hydroxyproline and total protein levels in gingiva and gingival crevicular fluid in patients with juvenile, rapidly progressive and adult periodontitis. J. Periodontol. 64, 323-329.

13. Henskens, Y.M.C., Van der Velden, U., Veerman, E.C.I. and Amerogen, A.V.N. (1993) Protein, albumin and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. J. Periodont. Res. 28, 43-48.

14. Kertesz, P., Banoczy, J. and Koesis, L. (1994) Salivary proteins in healthy and diabetic subjects. J. Dent. Res. 73, 421-424.

15. Smith, K.P., Krohn, I.R., Hermanson, G.T., Mallia, K.A., Gartner, H.F., Provenzano, D.M., Fujc moto, K.E., Goekan, N.M., Olson, B.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76-82.

16. Rinderknecht, H., Geokas, M.C., Silverman, P. and Haverback, B.J. (1968) A new ultra sensitive method for the determination of proteolytic activity. Clin. Chim. Acta. 21(2), 197-203.

17. Kabivitrum bulletin on synthetic chromogenic substrates, page 6, 1983.

18. Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271-278.