Journal of Oral Science, Vol. 45, No. 2, 85-91, 2003

Original

Hyaluronan (hyaluronic acid) and its regulation in human saliva by hyaluronidase and its inhibitors

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(Received 20 November 2002 and accepted 19 May 2003)

Abstract: The presence of hyaluronan (HA), hyaluronidase and hyaluronidase inhibitors has been assayed in pure resting and stimulated parotid saliva and also in resting and stimulated mixed saliva utilizing an ELISA-type assay and its modifications. Results confirmed the presence of hyaluronan in all saliva specimens which generally decreased upon stimulation. Hyaluronan in parotid saliva was of high molecular weight (> 200,000 kDa) whilst that in whole saliva in the floor of the mouth had a molecular weight between 20,000 kDa and 200,000 kDa, presumably because of cleavage by bacterial hyaluronidases. Hyaluronidase detection was variable in saliva, being present in some specimens of unstimulated parotid saliva, but in fewer specimens of stimulated saliva. Hyaluronidase was detected in parotid and whole saliva, both in the resting and stimulated state, at pH 3.7. Unstimulated whole saliva also showed hyaluronidase activity at pH 6.8, suggesting a different origin for this hyaluronidase. Hyaluronidase inhibitors were identified in both parotid and mixed whole saliva. There was an inverse relationship between the presence of hyaluranidase and the presence of hyluronidase inhibitors, suggesting a feedback mechanism. The possible significance, interactions and function of hyaluronan, hyaluronidase and its regulation by hyaluronidase inhibitors in saliva is discussed, particularly in relation to intra-oral wound healing and periodontal disease. (J. Oral Sci. 45, 85-91, 2003)

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Key words: hyaluronan; human; saliva; hyaluronidase; inhibitor.

Introduction

Hyaluronan, one of the most important components of the ground substance of the subcutaneous tissues, also has important roles in growth, development and repair of tissues. It is known that hyaluronan levels are elevated during embryological development (1), wound healing (2) and tumorigenesis (3). In the early stage of morphogenesis, when cell growth and proliferation predominate, hyaluronan levels characteristically rise (4,5).

Levels of hyaluronan result from a balance between biosynthesis by hyaluronate synthases (6,7) and enzymatic degradation. Levels decrease with cell differentiation, permitting compaction of tissue and allowing increased cell to cell communication. The presence of hyaluronan appears to inhibit differentiation (8). The key event in the onset of differentiation appears to be the degradation of hyaluronan by the concerted activities of an endoglycosidase, hyaluronidase and two exoglycosidases, ß-glucoluronidase and ß-*N*-acetylglucosamidase (9). Of these, the hyaluronidase appears to be the most important. In processes such as wound healing, the balance between hyaluronan deposition and hyaluronidase activity appears to be critical in modulating the various stages (10).

As noted, hyaluronan levels are high during cell morphogenesis (5) but decrease during cell differentiation, and at this time there is a corresponding rise in hyaluronidase levels. The control mechanism for hyaluronidase has not been as well elucidated, but by extrapolation from other mammalian enzyme systems (11), it seems likely that the presence, or action, of

hyaluronidase may be controlled by hyaluronidase inhibitors whose removal permits hyaluronidase activity to become manifest (12).

Saliva is important in the preservation and maintenance of oral health (13). Self and communal licking and grooming among animals are instinctive behaviors which become more pronounced following injury (14). The healing properties of saliva have been recognized, but little study has been devoted to the presence and possible role of hyaluronan and its metabolism in saliva. Hyaluronan may be a carrier of growth factors which can play a role in maintaining the health of the oral tissues. Hyaluronan has been shown to be an important factor in fetal scarless wound healing (14). It has also been noted that intraoral wounds heal rapidly with little or no scarring, and thus have some similarities to fetal wound healing (15). The interaction of saliva with the oral mucosa may be responsible for these intriguing properties, with similarities between intraoral wound healing and fetal wound healing. This article describes the presence and interactions of hyaluronan, hyaluronidase and hyluronidase inhibitors in human saliva.

Materials and Methods

Ten healthy adult volunteers (8 males, 2 females) between the ages of 19 and 34 years participated in this study. They were non-smokers, taking no medications, nor receiving medical care for systemic disease, and had no evidence of salivary dysfunction or local oral disease. Unstimulated and stimulated pure parotid saliva and mixed whole saliva specimens were collected under standardized conditions utilized in salivary flow rates studies (16,17). Participants were seen at 1:00 p.m. by one clinician (MAL). All subjects had taken nothing by mouth for a minimum of two hours prior to the study. This included gum chewing. Stenson's duct was cannulated and the saliva secreted during the initial two minutes of outflow were discarded since it is known that during this period, salivary components can vary significantly (18). Approximately 2 ml of parotid saliva was then collected in a 15 ml polystyrene clinical tube and stored on ice, to prevent degradation of salivary proteins by digestive enzymes. After the collection of parotid saliva, mixed saliva was aspirated from the floor of the mouth utilizing a 10 ml monojet syringe. Following the collection of unstimulated secretions, stimulated parotid and mixed saliva was obtained using 2% citric acid as the gustatory stimulus for salivation. The citric acid was applied uniformly over the tip of the dorsum of the tongue with a cotton swab for 5 seconds at 30 second intervals until between 2 and 5 ml of saliva had been collected.

Preparation of Specimens

Following collection, each saliva sample was treated with 0.1% ethylene diamine tetraacetic acid (EDTA) to prevent the formation of calcium proteinates which may affect protein composition (19). The samples were clarified by centrifugation at 12,400 rpm for 20 minutes at 4°C to remove any insoluble material. The supernatant was stored at -20°C until analysis. After thawing at 4°C, all samples were kept on ice while being prepared for analysis.

Protein Assay

The protein content of each saliva sample was determined by the Bio-Rad Protein Microassay (Bio-Rad Laboratories, Richmond, CA). One hundred sixty microliters of a 30-fold dilution of saliva in calcium- and magnesium-free phosphate-buffered saline (PBS-CMF) was added to 40 microliters of concentrated dye reagent (Coomassie Brilliant Blue G-250, phosphoric acid, methanol) in a 96-well microtiter plate and mixed thoroughly. After 5-60 minutes, the absorbance of samples was measured at 595 nm in an automated plate reader (Lab Systems, Helsinki, Finland). PBS-CMF served as the reagent blank and bovine serum albumin (BSA) (Sigma, St. Louis, MO) was used to generate a standard curve. Reported values represented the mean protein content of saliva samples assayed in triplicate which were obtained from the standard curve.

ELISA-like Assay for Hyaluronic Acid (HA)

Quantitation of the HA content in saliva was determined using an ELISA-like assay as described by Fosang et al. (20), with a modification described by Stern et al. (21), in this laboratory. In brief, a fixed amount of HA was immobilized on the solid support, and a fixed quantity of a HA-specific indicator molecule was allowed to competitively bind to HA. HA (ICN Biochemicals, Costa Mesa, CA) prepared from human umbilical cords, was first applied to a microtiter plate. One hundred microliters of 0.1 mg/ml HA in 0.42 mM n-hydroxysulfosuccinimide (Pierce, Rockford, IL, USA) and 3.2 mM 1-ethyl-3-carbodiimide (Sigma, St. Louis, MO) were adsorbed to a 96-well microtiter plate (Covalink-NH) for 17 hours at 4°C. After the coupling of HA to the well, the plates were rinsed three times with a solution of 2 M sodium chloride and 41 mM magnesium sulfate in PBS (Buffer A). All subsequent incubations were followed by triplicate washes in PBS with the addition of 0.05% Tween-20 (Fisher Scientific, Fair Lawn, NJ).

A biotinylated HA-binding peptide (HABP) served as the indicator molecule, similar to a labeled antibody, to bind competitively to HA in saliva samples, and subsequently to decrease the HABP available for binding

to HA-coated wells. The binding protein has been shown, in this laboratory by the third author, to be highly specific for HA (22), and when incubated with other high-molecular-weight polysaccharides and glycosaminoglycans, binding to HA does not decrease. The specificity of the HA-HABP reaction appears to be strong with an affinity approaching that of the avidin-biotin reaction (23).

The HABP was prepared from bovine nasal cartilage and biotinylated in this laboratory (23) by the technique of Stern (21) which itself is a modification of the technique of Tengblad (24). This cartilage-derived peptide from proteoglycan core protein, specifically and irreversibly binds to HA. Hence, when coupled to an immuno-peroxidase-linked avidin-biotin reaction, this complex is easily detected. An equal volume of undiluted saliva sample and diluted biotinylated HABP were incubated at 37°C for one hour. HABP was diluted 100-fold in 25 mM sodium phosphate, 1.5 M sodium chloride, 0.3 M guanidine-HCl, 0.8% BSA and 0.02% sodium azide, pH 7.0. A standard curve for each ELISA plate was established using serial dilutions of Healon,® a highly purified high-molecular-weight HA (Pharmacia AB, Uppsala, Sweden) prepared in PBS. A negative control to serve as a reagent blank, and a positive control to determine the maximum binding of HABP to HA fixed to the plate, were also included.

Following the blocking of nonspecific binding with 300 microliters per well of 0.5% nonfat dry milk in PBS at 37°C for 30 minutes, 100 microliters of the sample preincubated with HABP was added to the plate in triplicate. The remaining biotinylated HABP in the sample was allowed to bind to the HA-coated wells for one hour at 37°C and was then detected utilizing the avidin-biotin peroxidase technique. The avidin-biotin complex (ABC) reagent (Vector, Burlingame, CA) was prepared in PBS containing 0.1% Tween-20. One hundred microliters per well of the reagent was incubated for 30 minutes at room temperature. After five thorough washes with buffer B, the substrate, 0.5 mg/ml o-phenylenediamine (Calbiochem, San Diego, CA) in 33 mM citrate, 67 mM dibasic sodium phosphate, pH 5.3, and 0.015% hydrogen peroxide was added to terminate the enzymatic reaction. Utilizing a Titertek Multiscan Plus MK 2 automated plate reader, absorbance values were obtained at 492 nm. Reported mean saliva HA concentrations represent the remaining percentage of biotinylated HABP bound to the plate as related to the standard curve for known concentrations of Healon® HA.

ELISA-like Assay for Hyaluronidase Activity

The ELISA-like assay for hyaluronidase is based on the hydrolysis of hyaluronan absorbed to microtiter plates (22). The assay has been described in detail (22) by the third author of this article and has been shown to be 1,000 times more sensitive than other assays such as those described by Reissig et al. (25). Based on the hydrolysis of hyaluronan absorbed to microtiter plates by hyaluronidase, the first step of the assay involves the equal addition of 0.4 mg/ml hyaluronan (ICN Biochemicals, Costa Mesa, CA) dissolved in water and 0.2 M carbonate buffer at pH 9.2. One hundred microliter aliquots of this coating solution are absorbed to 96-well microtiter plates (Co-Star, Cambridge, MA) for 17 hours at 4°C.

Relative hyaluronidase activity was calculated as the difference in absorbance between control wells with no hyaluronidase and wells containing enzyme. Units of activity were expressed in National Formulary Units (NFU), defined as the amount of enzymatic activity required to reduce turbidity by 50% in 30 mins, reflecting hydrolysis of 50% of the substrate.

Buffers

Several enzymatic buffering solutions were compared at different pH ranges to assess optimal salivary hyaluronidase activity. An acetate buffer, a citrate phosphate buffer, and a phosphate buffer were utilized with pH profiling from pH 3.3 to 7.3. Each buffer also contains 0.05 M NaCl and 0.02% bovine serum albumin (BSA). Dilutions of 1:10 saliva in buffer were prepared in all cases in order to achieve linearity. This is in accordance with Afify et al. (26) in that any biological extract must be diluted first to demonstrate this proportionality to hyaluronidase enzyme activity.

ELISA-like Assay for Hyaluronidase Inhibitor Activity

The hyaluronidase assay was adapted to measure hyaluronidase inhibitors by assaying the activity of commercially purified Streptomyces hyaluronidase of known activity after the addition of increasing quantities of the salivary samples. A 1:10 dilution of parotid and whole saliva was utilized. A decrease in activity of the Streptomyces hyaluronidase as measured by the ELISA-like assay indicated the presence of hyaluronidase inhibitors in saliva.

Separation of Hyaluronan

The hyaluran in the samples was separated on a Superose 6 column (0.75 × 93 cm) (Pharmacia, Uppsala, Sweden) eluted with 50 mM pyridine acetate, pH 5.0. Fractions of 1.5 ml were collected at a flow rate of 0.75 ml/min. The fractions containing hyaluronan were identified by using

hyaluronan-binding peptide prepared from bovine nasal cartilage and biotinylated, as described by Stern (21). This cartilage-derived peptide specifically binds to hyaluronan (27). When coupled to an immunoperoxidase-linked avidin-biotin reaction, the complex is easily detected. The apparent molecular weight of the hyaluronan fractions was estimated using hyaluronan molecular weight markers covering a range of 20-225 kDa.

Results

The hyaluronan content of stimulated and unstimulated parotid and whole saliva is summarized in Tables 1 and 2 in nanograms per milligram of saliva and nanograms per milligram of protein respectively. Results are the mean of 3 assays on each sample. The greatest concentrations were detected in unstimulated whole saliva while concentrations decreased in stimulated whole saliva. The levels in parotid saliva were lower than in whole saliva and were lowest in the stimulated parotid saliva. This latter result, however, was variable since some subjects showed similar or even higher levels of hyaluronan in stimulated parotid secretions.

As seen in Table 1, the decrease in hyaluronan on stimulation of whole saliva was statistically significant at $P = 0.0098$ from nonparametric Wilcoxon signed-rank test applied to logarithms of the ratios. However, the decrease in hyaluronan in stimulated parotid saliva was not statistically significant.

As seen in Table 2, the decrease of hyaluronan in whole saliva was again statistically significant ($P = 0.0039$) utilizing the same test as that in Fig. 1, and again the change in parotid hyaluronan secretion upon stimulation was not statistically significant.

Several buffers and a range of pH profiles were utilized to detect hyaluronidase in the saliva samples. The whole saliva samples showed two enzyme peaks, one at pH 3.7 in citrate buffer and another at pH 6.8 in phosphate buffer (Fig. 1). Unstimulated parotid saliva exhibited one activity peak at pH 3.7 in the presence of 0.05 M NaCl and 0.02% BSA. The parotid activity therefore required a greater salt concentration to demonstrate hyaluronidase activity. Since neutral enzyme is present only in mixed saliva, it can be assumed that it does not originate from the parotid gland.

Hyaluronidase activity was variable. In the parotid saliva samples, four subjects showed hyaluronidase activity at rest, of whom two also exhibited it in stimulated samples. Stimulated parotid saliva exhibited slightly increased total levels of hyaluronidase activity as compared to unstimulated samples. In whole saliva, 6 subjects demonstrated hyaluronidase activity at pH 3.7 when unstimulated, whereas only three subjects demonstrated activity at pH 6.8. On stimulation of whole saliva the same 6 subjects showed hyaluronidase activity at pH 3.7 but none showed hyaluronidase activity at pH 6.8. No results approached statistical significance due to the small numbers involved.

The modification of the ELISA-like assay to detect hyaluronidase inhibitors in saliva did show that the samples from 4 subjects showed inhibition of hyaluronidase activity by saliva, and Fig. 2 shows a correlation in that specimens of saliva showing higher levels of hyaluronidase activity showed low levels of inhibitor activity, and vice versa. However, with the small number involved, even assuming a normal distribution, the correlation was not statistically significant by unpaired *t*-test ($P = 0.078$).

Molecular weight determination of the hyaluronan showed that the hyaluronan in pure parotid saliva had a predominantly high molecular weight (> 200,000 kDa), whilst hyaluronan present in the mixed saliva of the mouth

Table 1 Calculations of hyluronan in salivary samples (ng/ml) - averaged triplicate assays

Subject	Unstimulated Parotid	Stimulated Parotid	Unstimulated Whole	Stimulated Whole
1	76	80	300	189
2	307	150	240	150
3	78	84	655	375
4	37	43	148	160
5	195	64	265	355
6	56	104	369	73
7	56	99	235	89
8	57	59	1270	140
9	46	40	710	94
10	75	104	400	132
Mean	98.30	82.70	459.20	175.70

Table 2 Calculations of hyluronan in saliva (ng/ml of protein) - averaged triplicate assays

Subject	Resting Parotid Saliva	Stimulated Parotid Saliva	Resting Whole Saliva	Stimulated Whole Saliva
1	110	163	441	233
2	376	283	293	188
3	848	95	590	274
4	43	59	231	157
5	229	85	265	341
6	51	128	351	52
7	33	55	143	49
8	26	32	1187	85
9	107	1333	866	159
10	40	63	408	62
Mean	186.3	229.6	477.5	160

floor had a molecular weight predominantly within the range 20,000-200,000 kDa, with only 3% above 200,000 kDa (Fig. 3).

Discussion

This study demonstrates that hyaluronan is present in saliva, showing higher levels in unstimulated whole saliva, and lower levels in stimulated saliva. Lower levels are detected in pure parotid saliva and again these tend to decrease upon stimulation, although less consistently.

The presence of hyaluronidase in saliva samples was more difficult to detect, but did show two pH peaks: one acidic and one neutral in whole saliva, with only an acidic peak in parotid saliva. Not all samples demonstrated this activity. Comparison of the levels of hyaluronidase and hyaluronidase inhibitor appeared to show a reciprocal relationship between them.

The exact role of this hyaluronan cascade remains to be elucidated. Although the hyaluronan levels in saliva are lower than in fluids such as synovial fluid, its mere presence in saliva may contribute to the functional qualities of saliva. The high glycoprotein content may help to provide the lubricating properties of saliva which serve to protect the oral mucosa. Hyaluronan in saliva may also act locally on the mucosa, providing elevated levels during the everyday microtrauma that occurs with mastication, in order to ensure that wounds heal with a minimum of scarring and fibrosis (28).

The origin and function of the hyaluronidase also remain speculative. Low levels at a single pH value were found in parotid samples, and levels were detected at both acidic

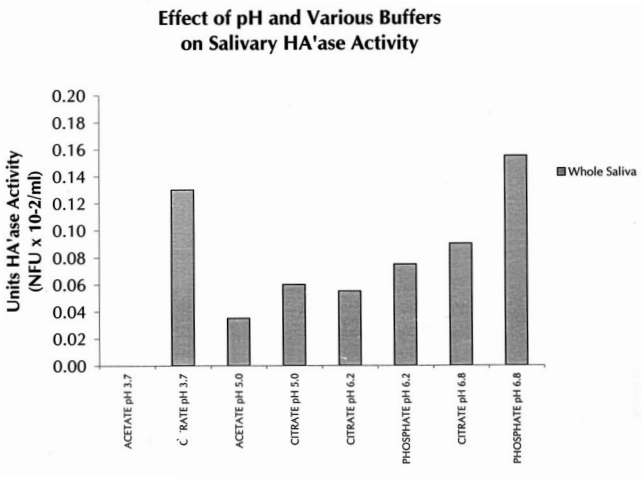

Fig.1 Salivary hyaluronidase activity in whole saliva buffered at several pH levels. Each buffer contained 0.05m NaCl and 0.02% BSA. Two peaks of activity are apparent, at pH 3.7 in citrate buffer, and pH 6.8 in phosphate buffer.

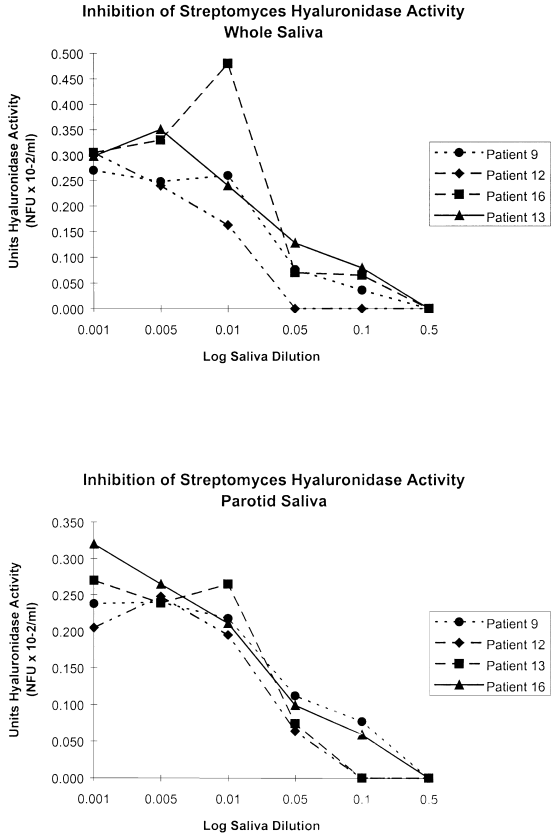

Fig. 2 Demonstration of hyaluronidase inhibitors in saliva. Increasing amounts of unstimulated whole saliva (A) and parotid saliva (B) samples were assayed for their ability to inhibit activity of commercially purified Streptomyces hyaluronidase. The incubation buffer consisted of 0.5 M acetate, 0.05 M NaCl, 0.02% BSA, pH 5.0. Samples from the same 4 subjects exhibiting either positive or negative hyaluronidase activity were analyzed. Inhibition of Streptomyces hyaluronidase activity was apparent in both whole and parotid saliva samples in which hyaluronidase activity had or had not been detected previously by the ELISA-like assay for hyaluronidase.

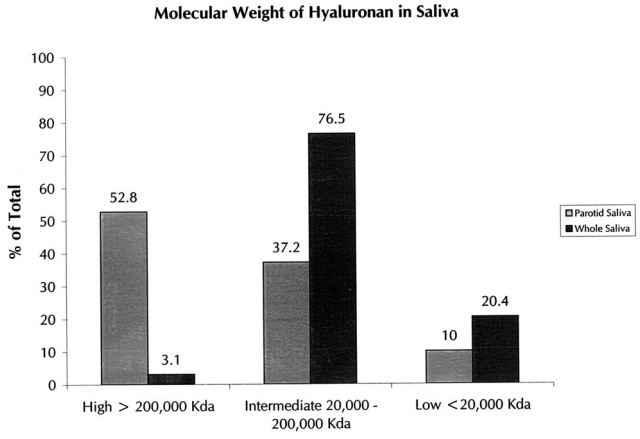

Fig. 3 Percentages of different molecular weight hyaluronan species in whole and parotid saliva.

and neutral pH in some whole saliva specimens, indicating an origin from two sources. It is possible that the acidic hyaluronidase in whole saliva originates from the parotid gland while the neutral hyaluronidase arises from the submandibular or other salivary glands, but it is equally likely that the neutral hyaluronidase arises from the gingival crevice and may be as a result of bacterial activity. Its relationship to physiological activity is unknown since if produced by bacteria, it may be related to destruction within the periodontal tissues. The fact that hyaluronan in parotid saliva is at predominantly one molecular weight only, whereas hyaluronan in mixed saliva shows two molecular weight bands may well be of physiological and clinical significance. It seems likely that the low-molecular-weight hyaluronan found in mixed saliva results from cleavage by the additional hyaluranidase which is found in specimens from the floor of the mouth. In turn it appears that this additional hyaluranidase may well be of bacterial origin, and would therefore be expected to show higher levels in patients with periodontal disease. Since one possible role of hyaluronan in the oral cavity is to act as a carrier for growth factors, the lower-molecular-weight hyaluronan is a less efficient carrier of growth factors. Thus one sees the potential for a feedback loop whereby bacteria present in the saliva of patients with periodontal disease would break down hyaluronan and therefore make it a less efficient carrier of growth factors to induce healing, therefore leading to progression of the periodontal disease.

It appears that hyaluronidase levels may be masked in some cases by the presence of hyaluronidase inhibitors which appear to be present in some specimens of both parotid and whole saliva. It must be assumed that these hyaluronidase inhibitors, which have not been detected previously in saliva, are responsible for controlling the hyaluronidase levels, which in turn control hyaluronan levels. The mechanism controlling the hyaluronidase inhibitors is unknown at this stage.

A recent study by the third author has shown that one species of hyaluronidase inhibitor is not present in serum and may be unique to saliva (29). This suggests that saliva is not merely a transudate of normal serum, but has specific properties and activities separate from serum.

The presence of such an inhibitor would be a possible defense mechanism against the oral bacteria that secrete hyaluronidases. Such activities are often virulence factors, and the hyaluronidase inhibitor activity may be a host countermeasure against bacterial invasion by the oral route. Since the mouth is often the first defense against ingested bacteria, the presence of salivary hyaluronidase inhibitors has obvious evolutionary and survival value.

The development of saliva-specific enzyme inhibitors could be viewed as an evolutionary adaptation survival mechanism to protect against bacterial invasion.

The presence of hyaluronan, its catabolic enzyme and enzyme inhibitors in samples of saliva from different areas in the mouth obviously requires considerably more investigation.

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