

## Secretory proteins in the saliva of children

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**Abstract:** The protein composition of oral fluid is modulated by environmental factors and physiological states, i.e. chemical, mechanical and pharmacologic stimuli, pathologic conditions, and psychological stress. Secretory protein concentrations in samples of whole saliva (WS) from children were measured and the results were subjected to statistical analysis. Protein expression was determined using electrophoresis and Western blotting. Protein profiles of children were significantly different from those of adults ( $n = 50$ ,  $P < 0.05$ ). All samples of saliva from children contained a group of high-molecular-weight (>90 kDa) proteins, whereas fewer than 5% of samples from adults had comparable bands. The ratio of the regulatory subunits (RII) of type II protein kinase A (an enzyme that regulates secretion) to total protein was stable in children's saliva, but variable in saliva from adults. Alpha amylase ( $\alpha$ -amylase), an enzyme that digests carbohydrates, was less degraded in WS of children than in that of adults. Gingival crevicular fluid of both children and adults did not contain  $\alpha$ -amylase or RII. No significant gender-based differences were found, but Caucasian children had higher salivary protein levels than children with an African background. Saliva collection is rapid, painless, non-invasive, economical, and yields findings that are reproducible. Objective, biochemical monitoring of secretory proteins in oral fluid of children may reveal responses to stressful

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### Introduction

Molecular changes in the composition of body fluids may serve as indices of cellular signal processing during acute or chronic stress. These changes, in the form of altered expression of secretory proteins, can be measured in oral fluids. The protein composition of saliva reflects cellular signal processing that results from day-to-day environmental influences as well as from acute or chronic stress (1,2). Individuals subjected to environmental or physiological stimuli (3-5) exhibit specific changes in the composition of oral fluid. Components of saliva, therefore, may serve as biomarkers because the composition of oral fluid is responsive to behavioral, mechanical, genetic or ontogenetic stimuli (6-11). The use of saliva to test for genetic markers and drugs has long been recognized (12-14). Analyses of proline-rich proteins (PRPs) and immunoglobulin A (IgA) have been reported (14,15). Other proteins with functions possibly correlated with physiologic reactions, such as alpha amylase ( $\alpha$ -amylase), mucins and cyclic AMP-receptor proteins, also have been identified in oral fluid of adults (16-21). More recently, a series of growth factors have been identified as secretory proteins whose function in saliva is not precisely known, but may reflect systemic conditions (22).

Salivary cortisol levels have been frequently used as an objective measure of stress in psychological studies (23-25). Differences in salivary  $\alpha$ -amylase have been used as a marker of the adrenergic component of the stress response (26). These differences reflect sympathetic nervous system (SNS) activation, based on the premise that salivary  $\alpha$ -

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amylase levels increase under stressful conditions known to increase plasma catecholamines, heart rate, systolic blood pressure, cardiac output, and a shortened pre-ejection period (1,27-29). Measurement of  $\alpha$ -amylase activity in children's saliva has been used as an indicator of the aggression response involving the SNS (1,26,30). Signal transduction of catecholamine hormones is mediated by increases in cyclic AMP, which regulates the activity of cyclic AMP-dependent protein kinase (PKA, E.C.2.7.1.37). Salivary exocytosis stimulated by catecholamine hormones and neurotransmitters is mediated via the cyclic AMP pathway. The type II regulatory subunit (RII) of PKA, previously thought to be solely a tissue protein, has been shown to be a secretory protein present in both animal and human saliva, and is sensitive to environmental stimuli (19,31,32). A zero gravity environment (space travel), mechanical manipulation (orthodontic treatment) and pharmacologic stimulation (injection of a  $\beta$ -adrenergic agonist) all elicit changes in salivary RII levels that are correlated with the duration and extent of the stimulus (3,4,6). Basal levels of salivary RII are stable in normal individuals under everyday conditions. However, during acute stress, sharp, transient increases occur, while chronic stress causes a significant decrease in RII concentration below basal levels (3,4).

The present study was conducted to examine the relationships between the specific proteins  $\alpha$ -amylase and RII, their ratios to total protein, and the characterization of individual protein profiles in the saliva of children. Standard biochemical methods described here reveal individual protein profiles and changes in the concentrations of specific proteins that can be quantified using public domain software, and which may serve as clinical markers. Ultimately, in order to provide a complete analysis of the salivary proteome, analytical methods using multi-dimensional separation and mass spectrometry will be employed for broad-spectrum peptide separation followed by bioinformatic analyses (33,34). A biochemical test for levels of specific secretory proteins may be useful when treating children (2).

## Materials and Methods

This study was authorized by the Institutional Review Board at the University of Connecticut Health Center and conducted in accordance with NIH Guidelines. Before any dental treatment, patients in good general health, and not taking medication, were invited to participate. Informed consent forms were signed by the subjects (and parent or guardian, in the case of minors) after all procedures had been explained, and the subjects were advised that participation could be terminated at any time and would

not affect their treatment or relationship with their dental professional in any way. All subjects had a healthy periodontal status, and understood and were compliant with the procedures. Samples were identified by a code to protect the subjects' privacy.

Samples of whole saliva (WS) were collected as described previously (5,6). Briefly, before collecting WS, the patients rinsed their mouths thoroughly with water. Afterward they were asked to drink about 25 ml of water and then to allow a small quantity, 1 to 2 ml, of saliva to passively flow into a sterile cup. The collected WS was combined with an equal volume of a buffer solution containing a protease inhibitory cocktail (1% sodium dodecyl sulfate [SDS], 0.2% beta-mercaptoethanol, 0.1% bromphenol blue and  $1 \times 10^{-6}$  M benzamidine and phenylmethyl sulfonyl fluoride) and centrifuged at  $10,000 \times g$  for 10 min. A 50- $\mu$ l aliquot was taken for microscopic analysis and the remainder of the sample was stored at  $-70^\circ\text{C}$  until the time of analysis. Samples with the same total protein concentrations were obtained from 50 juvenile (aged 6 to 15 yr) and 50 adult subjects (aged 18 to 65 yr) selected at random from a total of 100 subjects. Sample collection and the experimental steps were conducted under identical conditions. Of the 50 samples, 10 each were used for Western blotting with antibodies against  $\alpha$ -amylase, RI and RII.

Gingival crevicular fluid (GCF) was collected using flexible strips of filter paper (Periopaper, HARCO, Tustin, CA USA), causing no pain or tissue injury (35). After inserting 1-2 mm of a paper point into the gingival sulcus of the canine teeth, the paper point was withdrawn, and cut just above the point of 'wetting' to avoid sample diffusion. The cut tip was dropped into a tube containing 50  $\mu$ l buffer and protease inhibitor solution (as described above) and stored frozen at  $-70^\circ\text{C}$  until analysis. A measure of filter paper wetting comparable to the level of the GCF was 5-10  $\mu$ l, and therefore the dilution of GCF was approximately 1:10 compared to WS, which at this point was 1:2.

In general, the analytical procedures were conducted as described previously (6). Total proteins in each sample applied to nitrocellulose membranes were determined by staining with Ponceau S (Sigma Chemical Co., St Louis, MO USA) (36) and digital image analysis using NIH Image (public domain software). Concentrations were calculated based on values of bovine serum albumin (BSA, Sigma Chemical Co.) that showed linear gray values within the range 0.5-25  $\mu$ g (4-6). The proteins were separated by denaturing polyacrylamide gel electrophoresis (PAGE) using identical protein concentrations and a MiniGel System (E-C Apparatus Corp., St. Petersburg, FL

USA) by modification of standard methods (37). Protein size, in kDa, was determined by comparing the relative migration of each band with the banding pattern of standard proteins (Rainbow Markers, Amersham Co., Arlington Heights, IL) of known molecular size: 220, 97, 66, 30, 21 and 14.3 kDa. The separated proteins were then transferred from the PAGE gel to a nitrocellulose (NC) membrane by electroblotting (38), and the protein patterns were visualized by staining with Ponceau S dye (0.3% w/v in 10% acetic acid) (36,39). The protein profiles were developed by washing away excess dye with distilled water, then scanned and saved as digital image files. Peak heights of proteins of specific kDa size were measured according to their relative intensity and recorded in arbitrary density units (36).

For Western blotting the membranes were washed free of the dye and incubated with antibodies against  $\alpha$ -amylase and RI (Sigma Chemical Co.) raised in rabbit, and anti-RII (not cross-reactive with RI) prepared in this laboratory (licensed to Affinity BioReagents, Golden, CO USA). The secondary antibody was monoclonal anti-rabbit

immunoglobulin-horseradish peroxidase conjugate from mouse (Sigma Chemical Co.). The procedures were carried out according to previously described modifications of standard methods (4,6). Quantification and analyses were carried out by densitometry using NIH Image J (public domain) software. Data were subjected to statistical analysis of variance evaluation using the ANOVA program.

## Results

The collected saliva was a clear fluid that contained no cells, microorganisms or food particles when the centrifuged sample supernatant was viewed using a light microscope. Total protein concentrations in WS of children varied among individual subjects, with a mean of 0.88 mg/ml (Table 1). A comparison between WS and GCF is shown in Table 1; generally, GCF had a 2 to 5 times greater protein concentration than WS.

No significant differences in salivary protein concentration were found between children of the two age groups (Table 2). The results from children with mainly primary or mixed dentition, aged 6 to 9 yr, were similar to those

Table 1 Total Protein Concentration in Children's Saliva and GCF

Sample	Mean mg/ml	SD	<i>n</i>
GCF	6.36	3.37	25
Saliva	0.88	0.61	49

Protein concentration of WS was compared with that of gingival crevicular fluid (GCF). Protein determination was carried out as described in Methods and the concentrations calculated in mg/ml, taking into account sample dilution and based on standard curve values obtained using commercially available proteins of known concentration as standards. There is a statistically significant difference between the protein concentrations of GCF and WS ( $P < 0.05$ ). SD, standard deviation; *n*, sample number.

Table 2 Average Salivary Protein Concentration Based on Age, Sex and Ethnicity

Value	Age, years		Sex		Ethnic Background	
	6 – 9	10 – 15	female	male	A/H	EC
mean	0.76	1.00	0.79	1.02	0.62*	1.15*
SEM	0.27	0.35	0.33	0.28	0.15	0.32
<i>n</i>	29	20	27	22	26	23

Average protein concentration (mg/ml) calculated from densitometry data as mean and standard error; *n* = the number of subjects in each group. The division according to age correlated generally with primary and secondary dentition. Ethnicity was reported by parent or guardian as American or Hispanic with an African background (A/H) and Caucasian with a European background (EC). The only significant difference was between the average protein concentrations of A/H\* and EC\*,  $P < 0.05$ .

of children with largely permanent dentition, aged 10 to 15 yr. In this study, the type of dentition did not appear to influence the salivary secretory protein concentration.

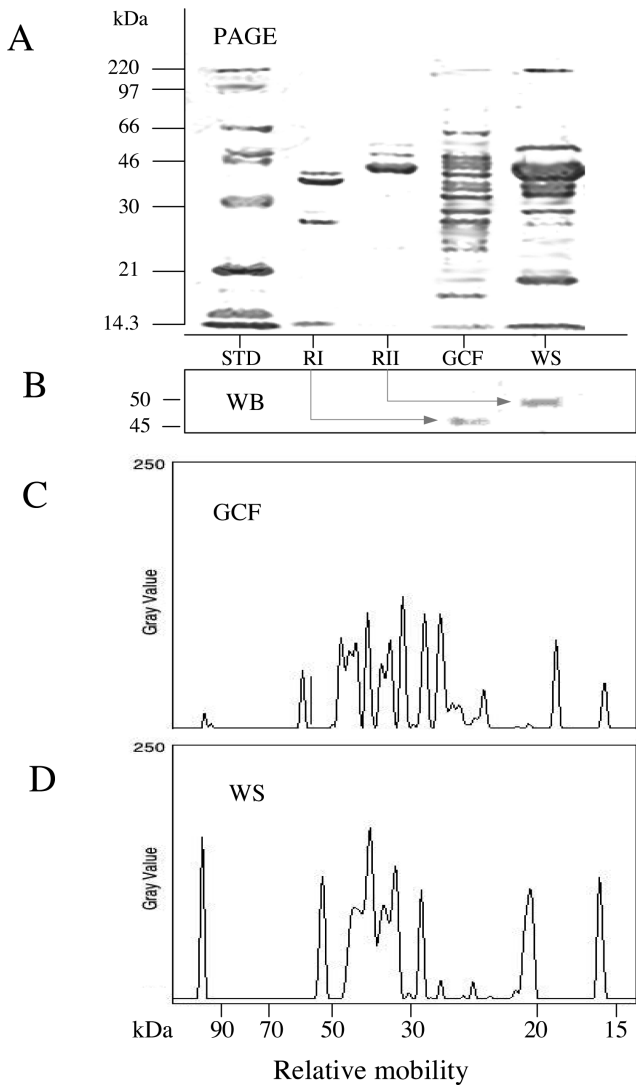


Fig. 1 Electrophoretic analysis of oral fluid. Panel A: Polyacrylamide gel electrophoresis. The leftmost lane shows colored standards (STD) in order of descending molecular size in kDa: myosin, 220; phosphorylase b, 97; bovine serum albumin, 66; ovalbumin, 46; carbonic anhydrase, 30; trypsin inhibitor, 21; lysozyme, 14.3. Lanes 2 and 3 are PKA RI and RII, respectively, lane 4 is gingival crevicular fluid (GCF), and lane 5 is whole saliva (WS). Panel B Western blotting. Reactivity of GCF is with RI while WS is with RII. Panel C: GCF and WS Panels C and D: Densitometric profiles of GCF and WS, respectively. The peak height is defined by arbitrary density units indicated as gray values on the ordinate. The abscissa shows molecular size in kDa calculated on the basis of relative mobility compared with the standard proteins of known molecular size.

Similarly, there were no significant differences in salivary protein concentration between boys and girls (Table 2). When protein concentrations were compared on the basis of ethnicity, some differences were observed. Notably, children of European Caucasian ethnic background had higher total protein concentrations than children with an African ethnic background (Table 2). These findings, although based on a sample of children (where  $n = 50$ ), indicate that there may be genetic or racial differences in salivary protein composition.

Notable differences were seen between the protein

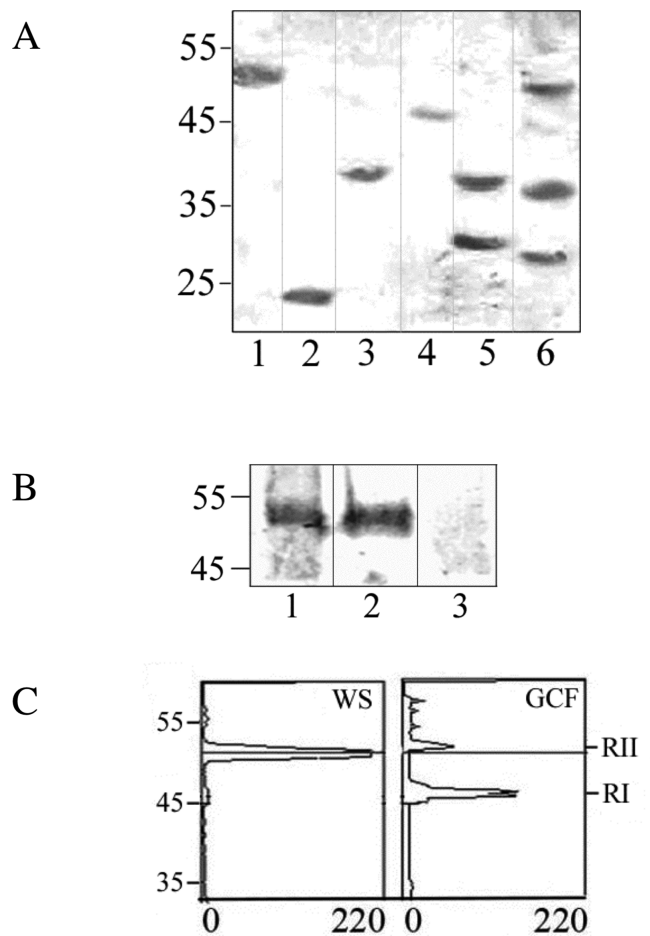


Fig. 2 Western blotting of amylase and RII. Panel A, amylase in samples of whole saliva from five (lanes 2 to 5) randomly chosen adults, ranging in age from 19 to 61 yr. Lane 1 is a commercial standard for human alpha amylase. Panel B, a representative sample of saliva from a child, and GCF Western blotted for amylase where lane 1 is an amylase standard, lane 2 whole saliva and lane 3 GCF. Panel C, densitometric analysis of saliva from children tested for RII where the abscissa is density measured in arbitrary units. The ordinate in all three panels is molecular size in kDa.

banding patterns of WS and GCF (PAGE and densitometry, Fig. 1). Western blotting (WB, Fig. 1, and Fig. 2) showed that the R subunits of PKA in GCF were different from those of WS (RI and RII, respectively). Type I and type II PKA are different gene products, indicating that WS and

GCF are separate compartments of oral fluid in individuals with healthy gingival tissues (Fig. 2, panels B and C.) In addition,  $\alpha$ -amylase was not found in children's GCF but was a prominent component of saliva (Fig. 2, panel B). These compartments are distinct in terms of protein composition.

The mean data for  $\alpha$ -amylase and RII in WS of children and adults are shown in Fig. 3. Panel A. Both  $\alpha$ -amylase and RII concentrations were significantly higher in children than in adults ( $P < 0.05$ ). Significant differences between adults and children were also observed for high molecular weight (HMW) and low molecular weight (LMW) proteins ( $P < 0.01$ ) (Fig. 3, Panel B). Secretory proteins with the lowest mobility, the HMW proteins, with a molecular weight of  $>90$  kDa, were present in all children's saliva tested, but generally absent in adults. Among the saliva samples tested, all of those from children, and 90% of those from adolescents, had a HMW ( $>90$  kDa) band while  $<10\%$  of adults showed comparable bands. The electrophoretically fastest moving group, LMW, containing the smallest-sized proteins, 30 kDa and smaller, was also more prominent in saliva from children than in that from adults.

Each sample of saliva from children contained a significant peak with the relative mobility and immunoreactivity of  $\alpha$ -amylase. Most of the samples of adult saliva had little or no reactivity in the region of mobility of intact  $\alpha$ -amylase, but generally showed numerous faster-moving immunoreactive components, presumably  $\alpha$ -amylase degradation products. A distinct difference between adults and children was also seen in the R subunit compartmental distribution (Fig. 2, Panel C); only RII was present in whole saliva, while only RI was present in GCF.

## Discussion

The present results showed similarities as well as differences in salivary protein expression between children and adults. In healthy children and adults, the protein composition of WS was distinctly different from that of GCF. The average protein concentration of WS was comparable to that reported previously for children's saliva (11). However, the protein concentration in GCF was lower in children than in adults (40). The expression or maintenance of the specific proteins  $\alpha$ -amylase and RII, tested in this study, differs between children and adults. In children  $\alpha$ -amylase is stable, whereas it is markedly degraded in most adults (Fig. 2, panel A). The differences in the stress protein RII, although significant, appear to be less extensive than those of  $\alpha$ -amylase. Children's saliva had a prominent HMW peak that was largely absent in

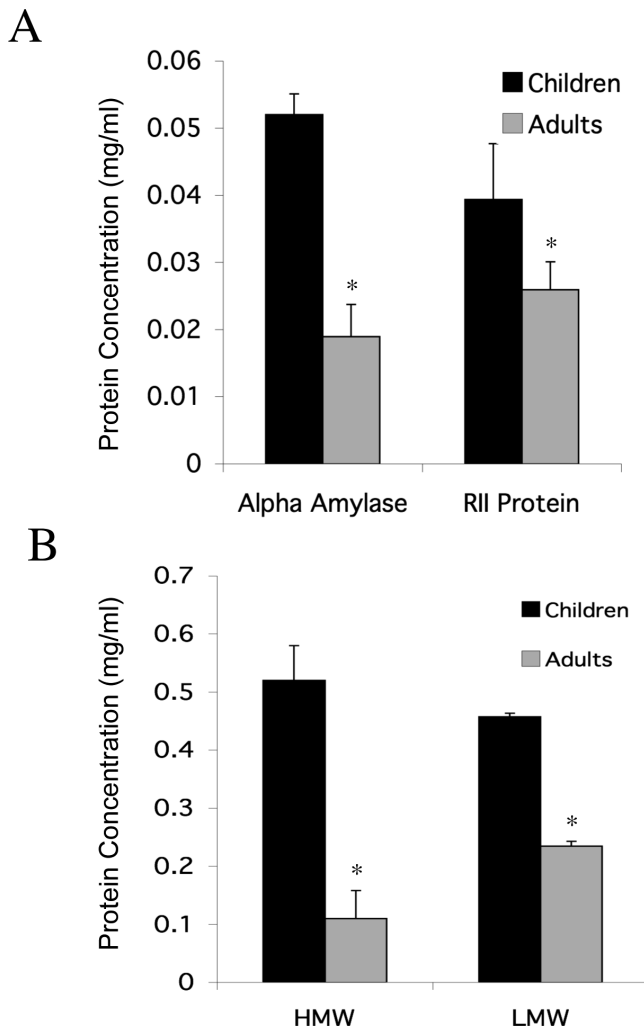


Fig. 3 Comparison of salivary proteins from children and adults. Sample collection and electrophoresis were carried out under comparable conditions. The adult data were obtained from 50 of a group of 100 randomly selected individuals. Panel A: Levels of  $\alpha$ -amylase and RII. Both proteins were significantly lower in adult saliva (indicated by \* on the bar graph,  $P < 0.05$ ) than in children's saliva. Panel B: Protein groups selected according to electrophoretic mobility: the high molecular weight (HMW) group of  $>90$  kDa proteins comprise a larger proportion of the total proteins in children's saliva than in adult saliva, as does the low molecular weight (LMW) group of  $<30$  kDa proteins. Note, the values (mg/ml) on the ordinate are different for each panel. The I-bars denote standard deviation.

adults. Few data are available regarding the prevalence of high molecular weight proteins in the saliva of children. A possible explanation of these findings may be that there are differences in either the expression or activation of salivary proteases. This is supported by the apparent stability of  $\alpha$ -amylase in saliva of children compared to that of adults.

Expression of cyclic AMP-receptor proteins (RII) reflects responses to a variety of stressors and has been demonstrated experimentally in salivary glands and other tissues (3-6,19,41,42). Cyclic AMP-receptor proteins may be among several non-immune proteins, such as the mucins, histatins, lysozyme,  $\alpha$ -amylase, proline-rich proteins and peroxidase, that are involved in or are indices of defense mechanisms and are measurable in oral fluid (16,21,43,44). It remains to be determined whether secretory responses to pharmacological or environmental stress in children are comparable to those of adults. The present results indicate that the synthesis and/or processing of secretory proteins is developmentally related and can serve to distinguish adult from juvenile saliva as well as an index of systemic changes in the signaling pathways of secretory proteins in oral fluid.

Information complementary to that obtained from analysis of saliva may be obtained from GCF, which has more serum-like components. Although the volume of fluid obtained is very small, a collection method was devised that yielded consistent results so that analysis of GCF components can yield key information about systemic conditions. Collection of GCF using filter paper strips that are thin and flexible causes no injury or anxiety to the patient. Recovery of GCF was easy when a paper probe was used (35). We have shown previously that GCF can be collected without any mixing with WS (6). We found that  $\alpha$ -amylase was abundant in WS, while none was found in GCF. Moreover, the PKA type I and type II R subunits are specific to each fluid.

Differences in salivary protein composition described in this report can be studied using standard biochemical tests that are low-cost, require only standard instrumentation, and have a high throughput (up to ten samples can be analyzed in an hour from collection to results). When complex procedures and sophisticated instrumentation are not available, the tests described in this study can be adapted for use in the field or for forensic applications.

An objective saliva-based test might be especially useful in young patients. A child coming to the dental or medical clinic might be anxious, and the ability to measure the stress level of a juvenile patient biochemically could aid health care providers in determining the best course of treatment. Our data suggest that RII and amylase in children can be

applicable as indices of individual stress levels. The development of standardized salivary tests for diagnosis of potential susceptibility to oral or systemic diseases, especially in children, may have potential value for adding the protein profiles of oral fluid to a comprehensive diagnostic array.

Since both WS and GCF can be collected quickly and non-invasively, they may prove to be useful protein profiling media for comparing responses of secretory protein expression to environmental stimuli.

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