ARCHIVES OF ORAL BIOLOGY 56 (2011) 634–642

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# Saliva electrophoretic protein profiles in infants: Changes with age and impact of teeth eruption and diet transition

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ARTICLE INFO

*Article history:*
Accepted 30 December 2010

*Keywords:*
Saliva
Infants
Proteins
Proteome
Teeth eruption
Diet
Introduction of solid foods
Amylase
Cystatins

ABSTRACT

*Objective:* The objective of this study was to describe the changes in salivary protein profiles in infants between the ages of 3 and 6 months, and to evaluate the impact of teeth eruption and introduction of solid foods on such profiles.

*Design:* 73 infants were followed longitudinally at 3 and 6 months of age. Their whole saliva proteins were separated by SDS–PAGE electrophoresis and semi-quantified by image analysis. Amylase activity was also measured on a sub-sample of the population ($n$ = 42 infants). Bands which abundance was significantly different between the two ages according to paired comparisons were identified by mass spectrometry techniques.

*Results:* Out of 21 bands, 13 were significantly different between 3 and 6 months of age. Two short variants of amylase increased in abundance with age, as did amylase activity. Other changes possibly translated developmental physiological events, for example maturation of the adaptive immune system. The balance between S-type cystatins and cystatins A and B was modified, in favour of S-type cystatins at 6 months of age. Teeth eruption resulted in an increase in albumin abundance, whilst introduction of solid foods was associated with higher levels of β-2 microglobulin and S-type cystatins.

*Conclusions:* Salivary profiles were modified substantially between the ages of 3 and 6 months. Both teeth eruption and diet had an impact on abundance changes for some proteins, revealing dynamic interactions between saliva proteome, oral physiology and diet.



## 1. Introduction

Being a biological fluid which can be collected by non-invasive methods, saliva has gained tremendous interest as a source of biomarkers of oral or systemic diseases.[1,2] The vast majority of potential markers are proteins and peptides, which explains that description of saliva proteome has been the object of many recent publications.[3–5] However, the saliva protein composition of infants is rather poorly documented. The literature on the matter has mainly focused on immunoglobulins.[6–8] Other abundant saliva proteins or their

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*Abbreviations:* ACN, acetonitrile; CHAPS, cholamidopropyl dimethylammonio-propanesulfonate; CHCA, cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; LC-IT ESI MS/MS, liquid chromatography–ion trap electrospray ionisation tandem mass spectrometry; MALDI-TOF, matrix-assisted laser dissociation ionisation-time of flight; PRPs, proline-rich proteins; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl) phosphine; TFA, trifluoroacetic acid.

0003–9969/$ – see front matter 
doi:10.1016/j.archoralbio.2010.12.015

activities have been measured in infant's saliva, in particular amylase,[9] mucins and albumin[10] or acidic PRPs.[11]

The first months of an infant's life are marked by major changes affecting the oral cavity, in particular teeth eruption and dietary transition. Common sense would dictate that such events may affect the saliva protein composition. For example, emergence of teeth creates the gingival crevices, allowing crevicular fluid to become an integral part of whole saliva. Teeth eruption is associated with a shift in oral microbial ecology[12] which could theoretically result in environmental regulation of antibacterial salivary proteins. Similarly, intake of solid foods introduces a wider variety of food-borne antigens to the oral cavity and gut with possible consequences on salivary components of the adaptive immune system. New dietary constituents may also modify the expression of specific proteins. For example, induction of amylase has been demonstrated in mice exposed to a tannin-rich diet[13]. When it comes to human infants' saliva, nonetheless, data on the subject are extremely scarce. Teeth eruption has been reported to modify the levels of immunoglobulins[7] and of albumin but without affecting substantially electrophoretic profiles[10]. As to introduction of solid foods, it increased the levels of IgA and IgG.[7]

In order to further document the saliva protein composition in infancy, we followed longitudinally a group of infants at 3 and 6 months of age and monitored their saliva electrophoretic SDS–PAGE profiles. We also evaluated whether profile modification was dependent on teeth eruption or diet.

## 2. Materials and methods

### 2.1. Participants

Participants were recruited in the frame of OPALINE (Observatory of food preferences in infants and children), a prospective longitudinal study on the development of food preferences in the first 2 years of life. Here, we report results concerning 73 infants (41 male and 32 female) from whom a minimum of 300 μL of saliva was collected at 3 and 6 months of age. Information concerning the infants' teeth and diet (more specifically the introduction of solid foods or ''weaning'') was collected from parents using feeding diaries filled out one week per month over the course of the first year and prospective questionnaires, and cross-checked during interviews. Since some infants were offered occasionally some foods as early as the second month, we used an operational definition of ''weaning''. It was thus defined as the regular introduction of any food other than milk, i.e. two successive feeds should be separated by three days or less. A weaning starting date could therefore be identified for each infant, and the duration of exposure to solid foods at 6 months was the number of days between weaning and the age of 6 months.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki. The general OPALINE procedure and saliva sampling procedures were approved by the local ethical committee (Comité de Protection de Personnes Est I Bourgogne). Information about the study was communicated and written informed consent was obtained from the infants' parents.

### 2.2. Saliva sampling

Whole saliva was collected using a Salivette® (Sarstedt, Nümbrecht, Germany). Practically, this was performed by allowing the infant to suck on the swab firmly held by the parent's hand covered by a surgical glove until no longer accepted or for a maximum of 2 min. Salivette tubes were stored frozen at −30 °C for a maximum of 5 days until further treated. Salivettes were thawed at room temperature for 15 min and recovery of saliva from the swabs was performed by centrifugation at 2000 × *g* for 5 min at 4 °C. Aliquots of 300 μL of saliva were subsequently applied to vivaspin 500 ultrafiltration device (Vivascience, Hannover, Germany) with a molecular weight cut-off of 5000 Da, and centrifuged at 15,000 × *g* for 30 min at 10 °C. Concentrates issued from the same samples were pooled and frozen at −80 °C until analysed. Protein concentration was measured in the concentrates following Bradford's method.

### 2.3. Measurement of amylase activity

Amylase activity was determined by measuring the rate of maltose release during incubation at 30 °C of starch with saliva extracts, using the 3,5-dinitrosalicylic acid (DNS) assay.[14] Briefly, saliva extracts were added to a solution containing 0.5% Extra Pure Starch (Merck) in phosphate buffer 0.02 M, pH 6.9. An aliquot was added to a 1% DNS solution in Na–K tartarate at 2, 4, 6, 8 and 10 min and absorbance was measured at 540 nm. The standard curve was established using a maltose solution. The activity was finally expressed in μM maltose/mg of protein/min.

### 2.4. SDS–PAGE gel electrophoresis

SDS–PAGE was performed in a PROTEAN II Multi Cell (Bio-rad, Richmond, USA) using 12% polyacrylamide gels, 20 cm long and 1 mm thick. Protein samples were denatured in a Laemmli's buffer containing 100 mM DTT and the protein load was adjusted to 20 μg per lane. Electrophoresis was carried out at 15 mA per gel, at 40 V for 30 min and 300 V for 8 h. Gels were subsequently stained using the so-called Blue silver protocol.[15]

### 2.5. Image analysis

Densitometric scans of SDS–PAGE gels were acquired using a flatbed scanner (GS-800, Bio-Rad). Intensities of the protein bands were analysed by the software Quantity-One (Bio-Rad). Peak area values of bands were normalized by calculating the ratio of each band's quantity to the total quantity of all matched bands in a lane. Data were expressed in percentage and finally transformed into centered log ratio.

Molecular weights were estimated according to the molecular weight standards (PrecisionPlus, Bio-Rad) run with protein samples.

### 2.6. Identification of bands of interest by MALDI-TOF/TOF mass spectrometry

Electrophoretically separated protein bands were excised from gels using surgical blades. Gel pieces were washed twice