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Inter-individual variability of protein patterns in saliva of healthy adults

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ABSTRACT

In order to document inter-individual variability in salivary protein patterns, unstimulated whole saliva was obtained from 12 subjects at 10 am and 3 pm of the same day. Saliva proteins were separated using two-dimensional gel electrophoresis, and semi-quantified using image analysis. One-way ANOVA was used to test the effects "time of sampling" and "subject". Data were further explored by multivariate analyses (PCA, hierarchical clustering). Spots of interest were identified by mass spectrometry (MALDI-TOF MS/MS and nanoLC ESI-IT MS/MS). A dataset of 509 spots matched in all gels was obtained. There was no diurnal statistical effect on salivary patterns while inter-individual variability was high with 47 spots differentially expressed between subjects ($p<1\%$). Clustering of these spots revealed that subjects could be discriminated first based on several proteins participating to the non-specific immune response (cystatins, lipocalin 1, parotid-secretory protein and prolactin-induced protein). Independently, subjects were also differentiated by their level of proteins originating from serum and involved in the immune system (complement C3, transferrin, IgG2), as well as the relative abundance of enzymes involved in carbohydrates metabolism (amylase and glycolytic enzymes). Inter-individual variability should be accounted for when searching for salivary biomarkers or when studying in-mouth biochemical mechanisms.

1. Introduction

Studies on characterisation of human saliva proteome have recently flourished with the advent of efficient methods, combining electrophoretic or chromatographic separation of protein mixtures with mass spectrometry-based protein identification [1–5]. 2-D electrophoresis allows the comprehensive characterisation of relatively abundant proteins, while LC-MS may in some cases detect proteins present in extremely low quantities. Such highly resolutive and complementary techniques have revealed the complexity of saliva. For example, a recent publication of the "Human Salivary Proteome Consortium" reports the identification of 1166 proteins from parotid and submandibular/sublingual salivas [6]. Even more proteins are expected in whole saliva, since it also contains proteins from other glands and from the gingival crevicular fluid, a transudate of blood plasma.

Besides being complex, saliva is also a dynamic fluid and its protein composition for a given subject may qualitatively vary. Thus, taste stimulation induces changes in proteome patterns [7]. Differences in the proportions of specific low molecular weight peptides have also been detected in parotid saliva at

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different times of the day [8]. Other studies, although they did not strictly compare the same subjects, suggest that saliva protein composition may vary with age in infants. For example, the proportions of the various isoforms of Proline-Rich Proteins (PRPs) varied with post-conception age in newborns [9]. Finally, proteome pattern modifications were evidenced between groups of subjects either healthy or suffering from oral or systemic pathologies [10,11]. In addition to this plasticity in response to age, condition or environmental factors, one should also consider the variability from one subject to another due to genetic polymorphism. The highly polymorphic character of several salivary proteins, in particular PRPs, has long been recognised (for a review, see [12]).

In this context, any study based on proteome analysis of saliva, be it for diagnostics purposes [13] or comprehension of in-mouth mechanisms, needs to take into account variability in protein profiles within a group of subjects. However, very few studies provide data on this topic. Morales-Bozo et al. [14] used 1-D electrophoretic separation to characterise polypeptide salivary profiles in a healthy population, and found overall no age or sex effect but observed inter-individual variations. Variability was also examined using intact protein LC/ESI-TOF mass spectrometry [15], which technically restricts the observations to low molecular proteins (<15 kDa). The authors demonstrated that day-to-day intra-subject and even more so inter-subject salivary protein variation is significant. The objective of the present work was therefore to formally document inter-subjects variability in 2-D electrophoretic salivary patterns.

2. Materials and methods

2.1. Saliva collection and processing

Twelve volunteers (6 women, 6 men; 26–47 years old; non smokers; salivary flow in resting conditions >0.5 ml min^{-1}) were recruited for the study. Written informed consent was obtained from all participants. Two sampling sessions took place at 10:00 am and 3:00 pm of the same day. Subjects were instructed to refrain from eating or drinking at least one hour before the sessions. They spat out saliva accumulating freely in their mouth as frequently as desired over a duration of 2 min. Samples were immediately centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was submitted to ultrafiltration at 15,000 g for 30 min at 10 °C using spin columns with a 5 kDa cut-off (Vivaspin 500, Sartorius AG, Germany). Aliquots of the resulting protein extracts ($n=24$) were stored at −80 °C until analysis.

2.2. 2-DE analysis

The samples were analysed in series of 6 gels. Samples were randomly assigned to the series. Protein concentration was measured in the extracts following the Bradford's method. The first dimension was performed using 17 cm 3–10NL IPG strips (Bio-Rad) on a PROTEAN (Bio-Rad) IEF cell. Protein extracts were suspended in a buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.1% w/v DTT, 1% v/v 3–10 carrier ampholytes and 0.3% v/v protease inhibitors (SIGMA). Strips were loaded with 150 μg of protein for analytical gels and 800 μg for preparative gels, and rehydrated at 20 °C for 13 h at 0 V and 8 h at 50 V. Isoelectric focusing was carried out at a final voltage of 8000 V for a total of 60 kVh. Thereafter, strips were equilibrated for 15 min and 20 min in two consecutive solutions of 6 M Urea, 50 mM Tris–HCl pH 8.8, 30% v/v glycerol, 2% w/v SDS, to which was added DTT at 1% w/v or iodoacetamide at 2.5% w/v. Strips were then placed on 12% SDS-polyacrylamide gels and electrophoresis was performed at 50 mA per gel on a PROTEAN II Multi Cell (Bio-Rad). Analytical gels were silver stained [16] and preparative gels were stained using the so-called Blue silver protocol [17].

2.3. Image analysis and statistical treatment

Gel digital images were acquired with the Image Scanner (Amersham Biosciences) and analysed using SameSpots software v.3.0. Quantities of matched spots were normalised by calculating the ratio of each spot's quantity to the total quantity of valid spots in a gel. Data were expressed in ppm. Finally, in order to make the variance independent of the mean, data were transformed into log before statistical treatment. The dataset was analysed using the Statistica software (StatSoft, Tulsa, OK, USA). It was first submitted to a Principal Component Analysis using all 509 spots as variables. Data were then submitted to one-way ANOVA testing three factors: time of sampling (morning/afternoon), gender, and subject. A spot was considered significant when it was associated to a p-value<1%, and a fold-change above 2 for the two-level factors "time of sampling" and "gender". Specifically for the description of intra-individual variability, significant spots were selected and they were subjected to a Principal Component Analysis. Data for these 47 spots and 12 subjects were visualised by heatmap and ascendant hierarchical cluster analysis (method of Ward based on Euclidian distances) using the software PermutMatrix [18].

2.4. Mass spectrometry-based protein identification

Spots of interest were excised manually, washed in NH_4HCO_3 0.1 M for 10 min and dehydrated in ACN for 10 min. Dry spots were incubated successively in 10 mM TCEP/0.1 M NH_4HCO_3 for 30 min at 37 °C, in 55 mM iodoacetamide/0.1 M NH_4HCO_3 for 20 min, in 0.1 M NH_4HCO_3 for 5 min and in ACN for 5 min. Digestion was performed in two steps: spots were first pre-incubated for 30 min at 4 °C in 20 μL of a 40 mM of NH_4HCO_3/10% ACN solution containing 10 ng/μl of trypsin (V5280, Promega, USA). 15 μl of this solution was subsequently removed and 10 μL of 40 mM NH_4HCO_3/10% ACN was added, prior to incubation at 37 °C for 2 h. Peptides were collected in the two successive supernatants obtained after addition of 0.5% TFA and ACN (1 μl and 10 μl, respectively) and sonication for 10 min.

For MALDI-TOF analysis, peptides were further concentrated on C18 beads (Invitrogen) following the manufacturer's instructions. 0.5 μl of the concentrate was loaded onto a Ground Steel target, mixed with 1 μl of matrix solution (3.5 mg/ml CHCA in ACN 50%, TFA 0.25%) and allowed to dry. The target was introduced in a mass spectrometer MALDI-TOF (Ultraflex, Bruker Daltonics, Bremen, Germany). Ionisation was performed in MS and MS/MS (PSD-LIFT technology) by irradiation of a nitrogen laser (337 nm) operating at 50 Hz. Data were acquired at