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Proteomic characterization of the acid-insoluble fraction of whole saliva from preterm human newborns

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ABSTRACT

The acid-insoluble salivary proteome obtained by addition of TFA to whole human saliva from adults, preterm and at-term newborns has been analysed by 2-DE in order to evidence differences among the three groups, and integrate data previously obtained on the acid-soluble fraction. 2-DE spots differentially expressed among the three groups were submitted to *in-gel* tryptic digestion and the peptide mixtures analysed by high resolution HPLC–ESI–MS/MS. By this strategy, we identified 3 over-expressed proteins in at-term newborns with respect to preterm newborns and adults (BPI fold-containing family A member 1, annexin A1, and keratin type 1 cytoskeletal 13), and several over-expressed proteins in adults (fatty acid-binding protein, S100 A6, S100 A7, S100 A9, prolactin-inducible protein, Ig kappa chain, cystatin SN, cystatin S/SA and α-amylase 1). Four spots, already detected but not characterized by other authors in human saliva 2-DE, were attributed to different protein species of S100 A9 (long-type and long-type monophosphorylated, short-type and short-type monophosphorylated) by MS/MS analysis of tryptic peptides and sequential staining of 2-DE gels with Pro-Q Diamond, for specific detection of phosphoproteins, and total protein SYPRO Ruby stain.

Significance: Differential protein expression analysis of the acid insoluble fraction of saliva from preterm, at-term newborns and adults has been performed in this study by coupling 2-DE analysis and high-resolution tandem mass spectrometry in order to complete the information previously obtained by top-down LC–MS only on the acid-soluble proteome. Several proteins identified in the acid insoluble fraction of both preterm newborn and adult saliva are not of glandular origin, being only prolactin-inducible protein, salivary cystatins, α-amylase and polymeric immunoglobulin receptor exclusive of salivary glands.

Three proteins resulted increased in at-term newborns with respect to preterm newborns and adults: BPI fold-containing family A member 1, two proteoforms of annexin A1 and keratin type 1 cytoskeletal 13, while several proteins were significantly increased in adults.

1. Introduction

Saliva is a bodily fluid that can be collected by non-invasive methods, and several studies have pointed out its relevance in evidencing significant variations in the concentration of specific proteins and peptides in subjects affected by oral and systemic diseases [1]. Even if saliva is easily collected from subjects of any age, and thus also from children, very few studies have been performed on infant saliva. Some of them were focused on determining the variation in concentration of one or few proteins as a function of age; for example, it has been shown that immunoglobulin (sIgA, IgD) can be determined in saliva even in the first day of life [2], and α-amylase [3], and serum albumin [4] concentrations increase significantly from birth up to one year of age. Very few studies were focused on determining the proteome composition of infant saliva, i.e. Manconi et al. examined the age range 0–48 months by a top-down platform based on RP–HPLC–ESI–MS [5], Morzel et al. the age range 3–6 months by SDS-PAGE coupled to MALDI–TOF/TOF MS [6], and in both cases major changes in the salivary protein composition in early childhood were evidenced.

A study by Inzitari and coll. [7] analysed concentration variation of several isoforms of acidic proline-rich proteins in human preterm and at-term newborns during one year follow-up. It appeared evident that saliva sampling may be performed immediately after birth both in preterm and in at-term newborns without causing stress or pain.

A comprehensive study that explored the peptide and protein composition of the acid-soluble fraction of whole saliva from preterm

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human newborns recently provided a deep proteomic characterization of the peptide/protein composition as a function of post-conceptional age (PCA). >40 protein masses often undetectable in adult saliva have been evidenced and their PTMs have been characterized [8]. A very recent paper has analysed, by means of HPLC-low- and high-resolution ESI-MS platforms, the time dimension variability of the salivary proteome in a very high number of samples from preterm newborns with a PCA at birth varying from 178 to 217 days, children between 0 and 17 years and adults [9]. The paper evidenced that proteins peculiar of adults appeared in saliva with different time courses during human development, for example histatin 1, statherin and P-B peptide appeared at 7 months of PCA while basic proline-rich proteins appeared roughly at 4 years of age. It should be outlined that in both studies only acid-soluble proteins were analysed.

Aim of the present study was the characterization of the acid-insoluble fraction of whole saliva from preterm human newborns by 2-DE and high-resolution tandem mass spectrometry, thus extending the results of previous studies [8,9]. This approach allowed the identification of several proteins, the majority, but not all, not detected in the acid-soluble fraction.

2. Material and methods

2.1. Subjects enrolled, sample collection and treatment

The study protocol and written consent form were approved by the Ethical Committees of the Catholic University of Rome and by the Ethical Committee of the Neonatal Intensive Care Unit of the Institute of Clinical Pediatrics of the Catholic University of Rome and, therefore, it has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All rules were observed and written consent forms were signed by one parent of each child or by the adult donor. For ethical reasons, saliva was only collected when sample collection caused no stress. We collected whole saliva samples from:

a) 7 preterm newborns (4 males, 3 females) with a PCA at birth of 169–197 days (24–28 weeks). Newborns with major congenital malformations or prenatal infections were excluded from the study. Sample collection was performed on the same preterm newborn during several weeks after birth at fixed time intervals (1 or 2 weeks). When possible, it was also performed after discharge from the neonatal unit, during the periodical check visits within about 1 year follow-up. In this way, 41 saliva specimens were analysed covering a period from 169 to 249 days of PCA.
b) 6 at-term newborns (5 females, 1 male) with a PCA comprised between 273 and 294 days (39–42 weeks)
c) 6 adults (2 males, 4 females) with an age comprised between 18 and 56 years.

Collection time was established between 10 and 12 a.m. and donors, except preterm newborns, did not eat or drink 2 h before the collection. The donors were in healthy clinical conditions. Whole saliva was collected with a soft plastic aspirator as it flowed into the anterior floor of the mouth.

After collection, each salivary sample was immediately diluted 1:1 (v/v) with 0.2% TFA in an ice bath. The solution was then centrifuged at 8000 ×*g* for 5 min (4 °C) and the pellet was separated from the acidic supernatant and either immediately analysed by 2-DE or stored at −80 °C until the analysis was performed.

2.2. Acid-insoluble fraction pre-treatment

Pellets obtained after TFA treatment of whole saliva from preterm newborns were used in 2-DE experiments without any other pre-treatment or after removal of salts or after TCA/acetone treatment. Ionic strength was reduced by washing the pellets four times with 500 μL of H_2O on Amicon Ultra-0.5 centrifugal filters with a molecular weight cut off of 3000 Da (Millipore, Billerica, MA, USA). Saliva pellets were dispersed and emulsified in 1.5 mL of 10% TCA, 20 mM DTT in acetone by a pestle in a 1.5 mL microtube. This procedure was performed to improve the resolution of the 2-DE separation in experiments to be used for sequential staining protocol. Samples were then incubated overnight at −20 °C to precipitate the proteins. After centrifugation at 20,000 ×*g* for 30 min at 4 °C, TCA pellets were washed 3 times with 3 mL of cold 20 mM DTT in acetone, centrifuging each time at 20,000 ×*g* for 30 min. Pellets were dried in air for approximately 3 h, and stored at −80 °C until analysed.

Following the treatment chosen, pellets were neutralised by adding 5 μL of 0.1 M NaOH under 2 min vortexing and then re-suspended in rehydration solution (DeStreak, GE Healthcare). After approximately 3 h of incubation under agitation, insoluble materials were removed by centrifugation at 20,000 ×*g* for 10 min at 4 °C and supernatants were used for IPG strip overnight rehydration after determination of total protein concentration.

2.3. Determination of total protein concentration

2D-Quant Protein Kit (GE Healthcare, Uppsala, Sweden) was used to quantify the acid-insoluble fractions from whole saliva after re-solubilisation in DeStreak Rehydratation solution. 15 μL of each sample was used for quantification, performed in duplicate, following the manufacturer's instructions.

2.4. First-dimension 2-DE

All the IPG strips utilised in this study contain a 3–10 nonlinear immobilized pH gradient (BioRad, Hercules, CA, USA). For analytical gel analysis, 20–90 μg of total protein were loaded on each strip for overnight rehydration, while 150 μg were used for preparative analysis.

A first set of experiments was performed on 7.0 cm IPG strip after re-solubilisation of each sample with 125 μL of DeStreak. The electrical running conditions applied consisted of four steps, for a total time of about 3.5 h, setting the maximum current applied to 50 μA. For the first step, voltage was set at 300 V for 60 min or until reaching 200 V/h. The second and the third steps were carried out in gradient mode from 300 to 1000 V in 30 min and from 1000 to 5000 V in 90 min, respectively. For the fourth step, voltage was set at 5000 V for 30 min or until reaching a total of 8000 V/h.

The second and the third sets of experiments were performed on 11.0 cm IPG strips after re-solubilisation of each sample with 250 μL of DeStreak. The electrical running conditions applied consisted of four steps, for a total time of about 5 h, setting the maximum current applied to 50 μA. For the first step, voltage was set at 500 V for 90 min or until reaching a total of 300 V/h. The second and the third steps were carried out in gradient mode from 500 V to 1000 V in 1 h and from 1000 V to 6000 V in 2 h, respectively. For the fourth step, voltage was set at 6000 V for 30 min or until reaching a total of 11,000 V/h.

2.5. Equilibration of IPG strips and second-dimension of 2-DE

Before starting the second dimension, both 7.0 and 11.0 cm strips were equilibrated in 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl pH 8.8, 0.01% bromophenol blue and 1% DTT for 15 min. Afterwards, a second equilibration step with the same buffer but in the presence of 2.5% iodoacetamide (IAM) instead of DTT was performed for additional 15 min.

Second dimension SDS-PAGE was performed for 7.0 cm strips using home-made Tris-HCl polyacrylamide gels (T = 12.5%) on a Mini-PROTEAN Tetra System (Bio-Rad), and for 11.0 cm strips using Tris-HCl precast Criterion gels (T = 10–20%) on a Criterion Cell (Bio-Rad), in both