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Top-down HPLC-ESI–MS proteomic analysis of saliva of edentulous subjects evidenced high levels of cystatin A, cystatin B and SPRR3

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ABSTRACT

Objective: This study aims to analyze the salivary peptidome/proteome of edentulous subject with respect to dentate control subjects.
Design: Unstimulated whole saliva, collected from 11 edentulous subjects (age 60–76 years) and 11 dentate age-matched control subjects, was immediately treated with 0.2% aqueous trifluoroacetic acid and the acidic soluble fraction analyzed by High Performace Liquid Chromatography-Mass Spectrometry. The relative abundance of the salivary peptides/proteins was determined by measuring the area of the High Performace Liquid Chromatography-Mass Spectrometry eXtracted Ion Current peaks which is linearly proportional to peptide/protein concentration under identical experimental conditions. Levels of salivary peptides/proteins in the two groups were compared by the nonparametric Mann-Whitney test to evidence statistically significant differences.
Results: Levels of cystatin A, S-glutathionylated, S-cystenylated, S-S dimer derivatives of cystatin B and S-glutathionylated derivative of SPRR3, were found significantly higher in edentulous subjects with respect to dentate controls. The major peptides and proteins typically deriving from salivary glands did not show any statistically significant differences.
Conclusions: Cystatin A, S-glutathionylated, S-cystenylated, S-S dimer derivatives of cystatin B and S-glutathionylated derivative of SPRR3, which are mainly of intracellular origin and represent the major constituents of the cornified cell envelope are a clue of inflammation of mucosal epithelia.

1. Introduction

Although complete tooth loss has declined over the last decade, edentulism remains a major disease worldwide, especially among older adults (Douglass, Shih, & Ostry, 2002). Several non-disease factors such as attitude, behavior, dental attendance, characteristics of health care system and socio-demographic factors play important roles in the aetiopathogenesis of edentulism (Emami et al., 2013), but the predominant cause of tooth loss in developed countries is periodontal disease (Famili, Cauley, Suzuki, & Weyant, 2005). Edentulism has a series of deleterious consequences for oral and general health (Kandelman, Petersen, & Ueda, 2008). Oral consequences vary from the well-known residual ridge resorption to an impaired masticatory function, an unhealthy diet, social disability, and poor oral health quality of life. Moreover, the long term related medications often encountered in edentulous subjects, with removable dentures, may be linked to denture stomatitis, ulcerations and oral candidiasis (Iosif, Preoteasa, Murariu-Măgureanu, & Preoteasa, 2016). Another frequent consequence of edentulism is the alteration of the normal salivary functions, an important factor for the maintenance of oral health (Preoteasa et al., 2014). The reduction of the salivary flow, which is relatively frequent in geriatric patients, leads to discomfort in wearing dentures (Turner et al., 2008) and may represent a risk factor for inflammation of soft tissues in response to the oral

Abbreviations: PRP, proline-rich proteins; SPRR3, small proline rich protein 3; XIC, eXtracted Ion Current.

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microbiota. The salivary peptidome/proteome of totally edentulous subjects has been previously studied with the aim of evidencing qualitative and quantitative variations related to the absence of teeth. The observed decreased level of α-defensins, with respect to dentate subjects, was most likely related to the absence of the gingival sulcus in edentulous, in agreement with the crevicular origin of this antimicrobial peptide. On the contrary, the level of histatins, antimicrobial peptides with the highest specific activity against *Candida albicans*, secreted from both major glands, did not change with respect to control subjects (Fanali et al., 2008). More recently, investigation of the salivary proteome of edentulous subjects with type 2 diabetes evidenced the presence of 52 salivary biomarkers that may be related to those present in serum; these included 14 salivary gland secreted proteins, 4 plasmic proteins, 6 immunoglobulins, and 32 additional proteins (Border et al., 2012).

The current development of new proteomic platforms offers powerful instruments for deciphering the variation of the salivary peptidome/proteome linked to physiological or pathological conditions (Messana et al., 2013). The proteomic platforms can be divided in two main categories, bottom-up and top-down, depending on the sample treatment (Bogdanov & Smith, 2005). In the bottom-up approach, proteins and peptides are identified by High-Performance Liquid Chromatography-Mass Spectrometry analysis of very complex proteolytic digestion mixtures typically obtained by using trypsin. Top-down proteomics differs from the bottom-up strategy, since it explores the intact proteins and peptides naturally occurring in the proteomes, minimizing, as much as possible, any sample alteration (Tipton et al., 2011). With an integrated top-down/bottom-up approach, more than 250 different salivary peptides/proteins have been characterized in the last 10 years (Cabras et al., 2014; Castagnola et al., 2012; Wu et al., 2014). At the light of the latest discoveries in the salivary proteome field, the present study aims to assess whether the level of other proteins present in saliva of edentulous subjects could vary qualitatively and quantitatively with respect to an age-matched control group.

2. Methods and materials

2.1. Sample collection

Resting whole saliva (from 0.2 to 1 mL) was collected with a soft plastic aspirator at the basis of the tongue from 3 to 5 p.m., when salivary secretion is at a maximum (Dawes, 1972). Samples were collected at least 30 min after any food or beverage had been consumed and teeth had been cleaned. After collection salivary samples were immediately mixed with an equal volume of 0.2% aqueous 2,2,2-trifluoroacetic acid (v/v; TFA) in an ice bath. The acidic solution was centrifuged at 9000*g* for 3 min to remove the precipitate and the acidic clear solution was either immediately analyzed by RP-HPLC-ESI–MS (100 μL, corresponding to 50 μL of saliva) or stored at −80 °C until analysis.

2.2. Participants and ethics statements

11 edentulous patients aged between 60 and 76 years in healthy condition and 11 control subjects, aged between 57 and 74 years, having a minimum of 25 teeth and with clinically normal gingival mucosa, were enrolled for the comparative study. The study protocol and written consent forms were approved by the Medical Ethics Committee of the Faculty of Medicine of the Catholic University of Rome.

2.3. Reagents and apparatus

Chemicals and reagents, all of LC–MS grade, were purchased from J.T.Baker (Deventer the Netherlands), Merck (Darmstadt, Germany) and Sigma Aldrich (St. Louis, MO, USA). RP-HPLC-ESI–MS apparatus was a Thermo Finningan (San Jose CA, USA). Surveyor HPLC system connected by a T splitter to a PDA diode-array detector and to an LCQ Deca XP Plus mass spectrometer. The mass spectrometer was equipped with an ESI source. The chromatographic column was a Vydac (Hesperia, CA, USA) C8 middle-bore column, with 5 μm particle diameter (column dimensions 150 × 2.1 mm).

2.4. RP-HPLC–low resolution ESI–MS analysis

The following solutions were utilized for the chromatographic separation: (eluent A) 0.056% aqueous TFA and (eluent B) 0.050% TFA in acetonitrile/water 80/20 (v/v). The gradient applied was linear from 0 to 55% in 40 min, at a flow rate of 0.30 mL/min. The T splitter addressed a flow-rate of about 0.20 mL/min towards the diode array detector and 0.10 mL/min towards the ESI source. During the first 5 min of separation the eluate was not addressed to the mass spectrometer to avoid instrument damage due to the high salt concentration. The diode array detector was set at wavelengths of 214 and 276 nm. The ion trap apparatus operated in positive

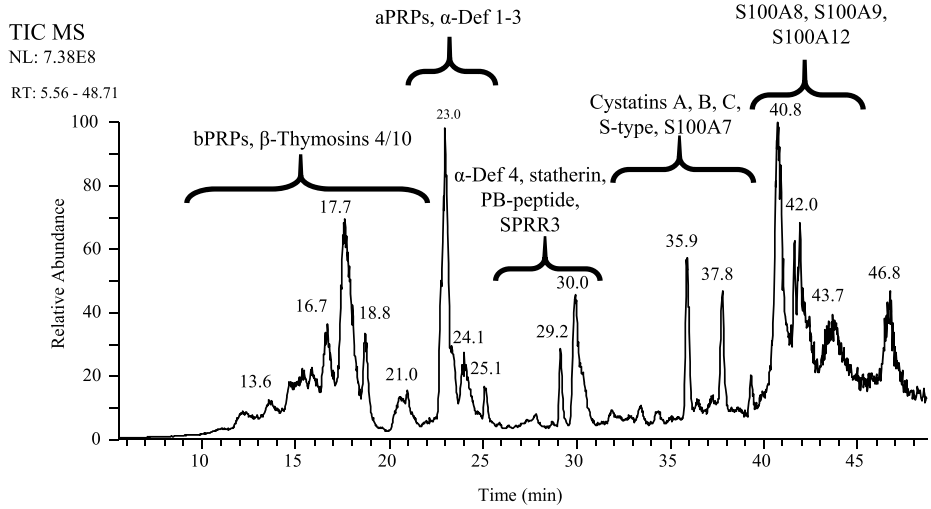

Fig. 1. Typical HPLC low resolution ESI–MS profile of the acidic soluble fraction of saliva from an edentulous subject with the elution range of the principal salivary peptides/proteins.