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RP-HPLC–ESI-MS evidenced that salivary cystatin B is detectable in adult human whole saliva mostly as S-modified derivatives: S-Glutathionyl, S-cysteinyl and S–S 2-mer

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ABSTRACT

An HPLC–ESI-MS analysis of adult human whole saliva evidenced three protein masses (M average 11,487±2, 11,301±2 and 22,362±3 Da) eluting in the 32.5–35.0 min range. Treatment in reducing conditions allowed establishing that they are S-derivatives of N-terminal acetylated cystatin B, namely its S-glutathionyl, S-cysteinyl and S–S dimer. The identification was confirmed by high resolution HPLC–ESI-MS–MS experiments on the intact naturally occurring proteins and their tryptic digests. S-unmodified cystatin B is rarely detectable in whole saliva of healthy adults (5 subjects out of 65) and its percentage does not overcome approximately 20% of total cystatin B (11±9%). In the majority of subjects (60 out of 65) the mean percentages of the S-modified derivatives were S-glutathionyl 53±13%, S-cysteinyl 15±5%, S–S 2-mer 32±13%. Variations of the percentages of these S-modified derivatives of cystatin B could be indicative of oral oxidative stress. As we are aware, this is the first time that S-glutathionylation and S-cysteinylation were described as extensive PTM of a salivary protein and the first time that these PTMs were detected in naturally occurring cystatin B.

1. Introduction

Human cystatin B (or stefin B) is a member of type 1 cystatin family (cysteine proteinase inhibitors), called also stefins [1]. Type 1 cystatins are α/β-type globular proteins differing from type 2 cystatins for size (about 100 amino acid residues in type 1 versus 120 residues in type 2), absence of disulfide bonds (two bonds in type 2 cystatins) and phosphorylation. Even though they have also been detected in sensible amounts in different bodily fluids, type 1 cystatins were commonly considered potent intracellular inhibitors of papain and cathepsins L, S and H [1,2]. Besides this inhibition

Abbreviations: XIC, extracted ion current; TIC, total ion current; TFA, 2,2,2-trifluoroacetic acid; DTT, dithiotreitol; IAM, iodoacetamide

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role, different recent studies outlined for them a very complex set of functions and interplays [3]. Cystatin B is widely distributed inside the cytoplasm of most human cells but was also detected in different bodily fluids and increased levels have been recently described in a variety of malignant tumours [4,5]. Its gene expression is increased in human monocytes stimulated by lipopolysaccharides [6] and it plays important and composite, although not defined, roles in neural stem cells as well as in differentiated neurons and astrocytes, displaying different localization inside the different cell types [7]. Moreover, it is an intracellular modulator of bone resorption in rats [8].

In the last years, our research group has been involved in the proteomic analysis of saliva. The analytical scheme followed is centred in a platform consisting of an HPLC–ESI-MS analysis of the acidic–soluble fraction of whole saliva followed by different enzymatic and chemical treatments in order to establish the structure of the intact, naturally occurring proteins and peptides [9–11]. Utilizing this platform and different samples of human whole saliva, the present study demonstrates that: cystatin B is present in human whole saliva mainly as S-glutathionyl, S-cysteinyl and S–S 2-mer derivatives (at the Cys_3 residue), a novel post-translational modification of this protein. The S-unmodified cystatin B derivative was undetectable in whole saliva of the majority of subjects (at the detection limit of the HPLC–ESI-MS apparatus utilized).

2. Materials and methods

2.1. Ethics statements

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 (according to the instructions of the Declaration of Helsinki). Full written consent forms were obtained from donors and all rules were respected.

2.2. Reagents and apparatus

All common chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba, (Milan, Italy), Merck (Damstadt, Germany) and Sigma Aldrich (St. Louis, MI, USA). The HPLC–ESI–IT-MS apparatus was a Surveyor HPLC system (ThermoFisher, San Jose, CA, USA) connected by a T splitter to a PDA diode-array detector and to an LCQ Deca XP Plus mass spectrometer or to an LCQ Advantage mass spectrometer. Both mass spectrometers were equipped with an ESI source. The chromatographic column was a Vydac (Hesperia, CA, USA) C8 column, with 5 μm particle diameter (column dimensions 150 × 2.1 mm). Some samples of whole saliva were also analyzed by an Ultimate 3000 Nano/Micro HPLC apparatus (Dionex, Sunnyvale, CA, USA) equipped with a FLM-3000-Flow manager module coupled to an LTQ Orbitrap XL apparatus (ThermoFisher). In this case a Biobasic 8 (ThermoFisher) capillary column (3 μm particle diameter; column dimension 180 μm i.d. × 10 cm) was utilized.

2.3. Subjects enrolled

Resting whole saliva was collected from 65 informed adult volunteers (37 ± 18 years old, 38 males, 27 females) with no clinically identifiable oral lesions and no known medical illness.

2.4. Sample collection and treatment

Donors did not eat or drink 2 h before the collection. Resting whole saliva was collected using a soft plastic aspirator between 2 and 4 p.m. in order to ensure consistent parotid secretion, which is at a maximum in the early afternoon. An acidic solution (0.2% 2,2,2-trifluoroacetic acid, TFA) was added to the salivary samples in 1:1 v/v ratio at 4 °C immediately after collection and the solution was centrifuged at 8000 *g* for 5 min (4 °C). The acidic supernatant was separated from the precipitate and either immediately analyzed by HPLC-MS apparatus or stored at −80 °C.

2.5. RP-HPLC–ESI-MS analysis

The following solutions were utilized for the chromatographic separation using the Surveyor-LCQ-Deca-XP apparatus: (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 54% of B in 39 min, and from 54% to 100% of B in 10 min, at a flow rate of 0.30 mL/min. The T splitter addressed a flow-rate of 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 214 and 276 nm. Mass spectra were collected every 3 ms in the positive ion mode. MS spray voltage was 4.50 kV, and the capillary temperature was 220 °C.

High-resolution micro-HPLC–ESI-MS/MS experiments on Ultimate 3000-LTQ Orbitrap XL were performed by using the following eluents: (A) 0.056% aqueous TFA and (B) 0.050% TFA in acetonitrile/H_2O 80/20 (v/v). The applied gradient was 0–4 min 5% B, 4–34 min from 5% to 50% B (linear), 34–64 min from 50% to 90% B (linear), at a flow rate of 4.5 μL/min. High-resolution positive MS/MS spectra were collected in full scan using the lock mass for internal mass calibration (polydimethyl cyclosiloxane, 445.1200 m/z) with the resolution of 60,000 and 30,000, respectively, and m/z range from 350 to 2000. In data-dependent scan mode the three most intense multiply-charged ions were selected and fragmented by using collision induced dissociation (35% normalized collision energy) and spectra were recorded. Alternatively, fragmentation was carried out using the same conditions on selected multiply-charged ions corresponding to specific protein masses. Tuning parameters were: capillary temperature 220 °C, source voltage 2.4 kV, capillary voltage 26 V, and tube lens voltage 245 V.

2.6. Dithiotreitol reduction of S-derivatives of cystatin B

S-Derivatives of cystatin B were collected from whole saliva (8 mL) by preparative RP-HPLC. 50 μL of the enriched fraction containing cystatin B S-derivatives was submitted to reduction of disulfide bonds at 30 °C for 1 h in 50 mM Tris/HCl buffer pH 8.5