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Determination of the human salivary peptides histatins 1, 3, 5 and statherin by high-performance liquid chromatography and by diode-array detection

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method with diode-array detection for the quantification of several human salivary peptides is described. Sample pretreatment consisted of the acidification of whole saliva by phosphate buffer. This treatment produced precipitation of mucins, α-amylases and other high-molecular-mass salivary proteins, simultaneous inhibition of intrinsic protease activities and reduction of sample viscosity. Direct HPLC analysis by diode-array detection of the resulting acidic sample allowed one to quantify histatin 1, histatin 3, histatin 5, statherin, as well as uric acid, in normal subjects. Moreover, the groups of peaks pertaining to proline-rich proteins and cystatins were tentatively identified. The method can be useful in assessing the concentration of salivary peptides from normal subjects and from patients suffering oral and/or periodontal diseases. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Human salivary protein arrangement is characteristic and complex. In fact, among the more than 50 proteins and peptides found in human saliva to date, most are distinctive in their structure and role [1]. About 50–60% of protein quantity is represented by proline-rich proteins (PRPs) and about 20% is constituted by α-amylase isoforms. The remaining 20–30% consists mainly of histatins (histidine-rich proteins), statherin, cystatins and mucins. Their dimensions vary from small polypeptides to proteins with a molecular mass greater than 1 000 000 (high-molecular-mass mucins) [2]. Unlike other biological fluids, human saliva contains strongly basic and acidic proteins and hence the isoelectric point is characterized by great variability. Furthermore, apart from individual differences, it is well known that

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composition of salivary proteins largely depends on the secretion gland, circadian rhythm, sex, age and diet, as well as general health. Due to this intricate situation, an analytical method devoted to the quantification of several salivary components may be very useful. A contemporaneous characterization of many salivary proteins is partially achieved by electrophoresis and techniques deriving from it, such as isoelectrofocusing [3], but results must be regarded as semi-quantitative, due to the distinctive staining ability of the different groups of proteins. The use of hydrophobic-interaction high-performance liquid chromatography (HPLC) was directed towards the analysis of rat parotid salivary proteins, even though separation principally permitted the distinguishment of different classes of PRPs [4]. Similarly, anion-exchange HPLC was utilized for the characterization of individual PRPs, allowing the identification of PRP polymorphism and phenotypes [5]. Other analytical methods were developed for the determination of selected groups of salivary proteins. Thus, HPLC methods [6] and capillary electrophoretic (CE) methods [7] have been utilized for the characterization of histatins, even though they require time-consuming specimen treatments. Accordingly, statherin quantification has been carried out using dedicated HPLC methods [8]. HPLC separation of whole saliva samples permitted the detection of a long form of cystatin [9], but the complex HPLC pattern was not utilizable for analytical purposes. Immunochemical detection is obviously directed towards specific proteins, and even when characterized by good performance often cannot distinguish similar components. In this respect, enzyme-linked immunosorbent assay (ELISA) determined only total histatin concentration [8] and cannot evaluate the concentration of each histatin component.

We focused on the use of a relatively new HPLC stationary phase characterized by particle diameters of 3 μm aided by diode-array detection (DAD), in order to obtain rapid contemporaneous information on the concentration of several salivary peptides, such as the principal histatins and statherin. Moreover, the HPLC method was facilitated by acidic pre-treatment of the sample, which caused the precipitation of mucins, α-amylase and other high-molecular-mass proteins. This sample treatment contemporaneously ensured satisfactory inhibition of intrinsic protease activities and a decrease in solution viscosity.

2. Materials and methods

2.1. Reagents and instruments

All reagents were analytical grade purchased either from Merck (Darmstadt, Germany), Carlo Erba (Milan, Italy) or Sigma–Aldrich (St. Louis, MO, USA). Chromatographic eluents were from Carlo Erba. Standard of histatin 5 was purchased from Sigma–Aldrich. Standards of uric acid and tyrosine were obtained from Merck. The HPLC apparatus was a Beckman (Palo Alto, CA, USA) Gold 125S solvent module equipped with a diode array 168 detector and Gold Noveau software. The chromatographic column was a Hewlett-Packard (Palo Alto, CA, USA) Hypersil BDS-C_{18} with 3 μm particle diameter (column dimensions 100×4 mm) protected by a guard column of ODS Hypersil resin (5 μm; 20×2.1 mm). Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed by a PE-Biosystems Voyager System 1167 (PerSeptive Biosystems, Foster City, CA, USA).

2.2. Methods

2.2.1. Sample collection

The informed donor was advised regarding a common standardized specimen collection procedure. Collection time was established at approximately 4 p.m. About 30 min before collection, the donor was invited to clean his/her teeth with a common toothbrush and commercial toothpaste. The donor was invited to hold up his/her tongue and after about 1 min to drain the saliva collected at the base of the tongue with a small plastic aspirator. The saliva was immediately collected in a plastic tube. After centrifugation (10 s, 1000 *g*), 80 mmol/l phosphate buffer, pH 2.5 was added to the sample (1:1, v/v). The pH was brought to 2.2–2.5 by adding few drops of phosphoric acid (diluted 1:10, from phosphoric acid concentrated solution, 85%, w/w, density 1.71 g/ml). The sample was immediately centrifuged at 8000 *g*, the precipitate was discharged

(except for the electrophoretic analysis, see following sections) and the upper solution was either immediately analyzed by HPLC or stored at −20°C.

2.2.2. HPLC and MALDI-TOF-MS analysis

The following solutions were utilized: (eluent A) 80 mmol/l phosphoric acid, pH 2.50; (eluent B) acetonitrile–eluent A (80:20, v/v). Gradient development was as follows: 0% B for 1 min, linear gradient from 0 to 75% B in 45 min, linear gradient from 75% to 100% B in 1 min, 100% B for 8 min, linear gradient from 100% to 0% B in 1 min. The flow-rate during the whole gradient development was 1.0 ml/min. The DAD window was established between 190 and 350 nm. The volume injected corresponded to 50 μl for analytical purposes and 100 μl for semi-preparative purposes. The peaks attributed to histatins 1, 3, 5 and statherin, according to spectral properties and polarity criteria, were collected, lyophilized and submitted to MALDI-TOF-MS analysis. The mass determination confirmed peptide attribution within the limits of MALDI-TOF-MS experimental error.

The quantification of histatins 1, 3, 5 and statherin was carried out using the peak area at 276 nm in comparison to standards of tyrosine and on the knowledge of the tyrosine content of salivary peptides [10]. In order to verify the bias connected to this procedure, different concentration of histatin 5 standards were used. Results showed that quantification error based on the tyrosine content on salivary samples was less than 5%. The quantification of uric acid was carried out using standard solutions at different concentrations.

2.2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under reducing conditions according to the classical procedure [11], using a total acrylamide percentage of 15%. The samples analyzed were: (i) whole human saliva, (ii) supernatant obtained after acidic precipitation and (iii) acidic precipitate. Acidic precipitate was dissolved under vigorous stirring in the SDS–PAGE sample solution. Gels were stained with Coomassie Blue R-250 omitting organic solvent from the destaining step, in order to develop the pink–violet metachromatic stain of PRPs [12].

3. Results

The treatment of salivary samples with acidic buffer provided: (i) precipitation of several proteins, (ii) a decrease in sample viscosity and (iii) inhibition of intrinsic protease activity. The fraction precipitated by the acidic treatment was characterized by SDS–PAGE. Fig. 1 shows the typical SDS–PAGE pattern of a whole sample (Wh) compared to the acidic supernatant (Sp) and precipitate (Pr). On the SDS–PAGE pattern PRPs were identified on the basis of their pink–violet metachromatic staining [12]. The acidic precipitate consisted of mucins, the isoforms of α-amylase and other salivary components, probably including lactoferrin, immunoglobulins, carbonic anhydrase, albumin and several salivary enzymes. All the PRPs were soluble in the acidic solution, such as subsequently confirmed by

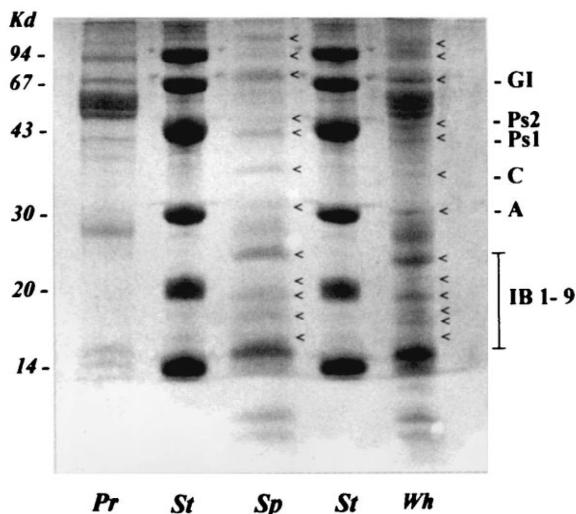

Fig. 1. Typical SDS–PAGE pattern of a salivary sample from healthy donor. The two lanes of molecular mass standards are called St. The other three lanes pertain to whole saliva (Wh), supernatant (Sp) and proteins precipitated (Pr) by acidic treatment of whole saliva, respectively. The electrophoretic bands which appeared pink–violet after Blue Comassie R250 staining (PRP proteins), are indicated by a small arrow. The nomenclature adopted by Beeley [12] for PRPs is reported on the right part of the figure.

HPLC analysis. The nomenclature of PRPs reported by Beeley [12] is shown on the right of the figure. IB1–IB9 are basic PRPs, GI is the main glycosylated PRP. Two other non-glycosylated basic PRPs are Ps1 and Ps2, whereas C and A correspond to the major acidic groups of PRPs. On the lane pertaining to the acidic supernatant (Sp) a band with a molecular mass of 14 000 is present. According to the following HPLC analysis, it was attributed to cystatins. The small basic salivary peptides, histatins and statherin, according to their structure, were also present in solution and probably corresponded to the bands evidenced on the bottom of the gel.

It is well-known that salivary components are subjected to the action of intrinsic proteases. The pH of the precipitating solution prevented protease action as assessed by HPLC pattern stability after sample storage. In fact, the sample stored at 4°C did not show significant qualitative and quantitative differences when submitted to the HPLC analysis within 24 h. Moreover, these experiments allowed us to establish that intra-assay variation was less than 6%. The decrease in specimen viscosity allowed direct HPLC injection. A further advantage is that, after phosphate treatment, sample is dissolved in a medium having a composition similar to that of the solutions used in the HPLC separation.

Separation of the saliva-acidified solution was obtained by a column of Hypersil BDS-C_{18} with a stationary phase of 3 μm. Detailed conditions of separation are reported in Section 2.2. A typical separation obtained on a human saliva sample of a healthy donor is reported in Fig. 2, where on the top the chromatogram obtained at 214 nm and on the bottom that obtained at 276 nm are shown. Computation of the number of theoretical plates per meter (N/m) on statherin peak provided a value of about 10^6, that is a very high value for a chromatographic separation. Moreover, this value was still maintained after 50 analyses, without changing of the guard column, showing that conditions used did not deteriorate column performance. The gradient was

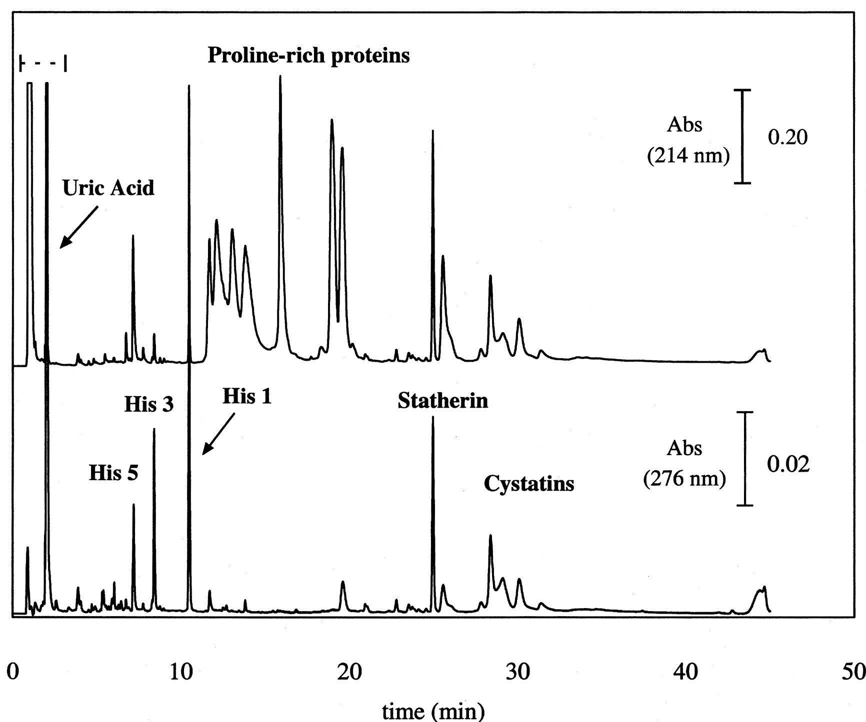

Fig. 2. HPLC pattern obtained at two different wavelengths by DAD analysis of acidified saliva from a healthy donor. On the top the pattern obtained at a wavelength of 214 nm and on the bottom that obtained at 276 nm are reported, in order to show the different absorbancies of salivary components at the two different wavelengths. Detailed chromatographic conditions are reported in Section 2.2.

chosen, after different tests, in order to reach a satisfactory compromise between peak resolution and analysis time.

Separation was assisted by DAD in the 190–350 nm range with a band width of 1 nm. Salivary peptides and proteins are characterized by a very peculiar amino acid content. Histatins 1, 3, 5 and statherin contain in their sequence a variable number of tyrosines (5, 4, 2 and 7, respectively), with tryptophan being absent, cystatins contain both tyrosine (6) and tryptophan (2 or 3, depending on cystatin component), whereas proline-rich proteins are lacking in these aromatic amino acids. Tyrosine and tryptophan are the only two amino acids responsible for a sensible protein absorbance over 260 nm [10]. The maximum of tyrosine absorbance is at 272 nm, whereas that of tryptophan is 280 nm, with a molar extinction coefficient that, under the HPLC conditions used, is approximately four-times higher than that of tyrosine. Hence, the accurate analysis of peak spectra allowed to attribute the major highlighted peaks to histatins, statherin and cystatins. Moreover, using appropriate standard, the peak pertaining to uric acid was also identified. The peaks of PRPs were obviously detectable only at the nonspecific 214 nm wavelength (Fig. 2, top). Histatins 1, 3, 5 and statherin were identified on the basis of polarity criteria, computed according to peptide sequence [10]. The peaks identified were collected and submitted to MALDI-TOF-MS analysis, confirming the molecular mass of these peptides, within the experimental error characteristic of this analysis [$M-H^+$ values: (histatin 5) 3037; (histatin 3) 4063; (histatin 1) 4929; (statherin) 5381].

Quantification of uric acid was carried out using proper standard solutions. The three main histatins (1, 3, 5) and statherin were quantified according to their tyrosine content, using the 276 nm chromatogram. The use of standard of histatin 5 confirmed that this procedure did not generate a relevant analytical error, as reported in experimental. The concentration of these peptides and of uric acid determined on salivary samples from healthy donors ($n=14$) under non-stimulated conditions is reported in Table 1. Values obtained were in satisfactory agreement with other values reported in the literature (see Discussion).

To investigate the possible correlation between the concentration of histatins, statherin and uric acid, a regression analysis on the data obtained from the 14 salivary samples was performed. Correlation coefficients obtained are reported in Table 2, and those considered significant are labeled with an asterisk ($R>0.742$, $P<0.001$; R between 0.623 and 0.742, $P<0.01$; $R<0.623$, not significant). A highly significant statistical correlation was found among histatin concentrations.

Table 2
Correlation coefficients obtained between concentrations of different peptides in human salivary samples ($n=14$) obtained by statistical correlation analysis from the HPLC data

	Histatin 5	Histatin 3	Histatin 1	Statherin
Histatin 3	0.6817 **	–		
Histatin 1	0.7192 **	0.7570 ***	–	
Statherin	0.0923	0.2113	0.0349	–
Uric acid	0.3692	0.3075	−0.0131	0.4066

$P<0.01$: **; $P<0.001$: ***.

Table 1
Concentration (μmol/l; in parentheses the respective values in μg/ml) of peptides and uric acid in healthy human saliva determined by DAD–HPLC ($n=14$)

	Histatin 5	Histatin 3	Histatin 1	Statherin	Total histatin	Uric acid
Median	15.3 (45.9)	11.4 (46.0)	17.7 (85.1)	9.38 (49.7)	44.4 (176.9)	189 (31.7)
Range						
(Min.)	5.82 (17.5)	3.86 (15.6)	3.42 (16.4)	2.71 (14.4)	15.9 (60.0)	103 (17.4)
(Max.)	32.1 (96.4)	25.7 (104.1)	48.6 (233.5)	16.5 (87.3)	102.1 (421.0)	294 (39.4)

4. Discussion

The first mandatory step in performing a satisfactory contemporaneous quantification of several salivary peptides is the easy, rapid and reliable sample collection. To fulfill this requirement, we used a method consisting of direct saliva collection followed by rapid precipitation with acidic phosphate buffer. Acidic treatment of salivary samples was already utilized by other researchers for analytical purposes [13]. The phosphate buffer chosen in this study did not introduce relevant contamination to the sample, since phosphate is present in salivary samples at high concentrations. Acidic treatment provided the advantage of stabilizing the sample for HPLC analysis, by preventing the action of intrinsic salivary proteases. The procedure can also be easily applied under field conditions, and the stabilized sample can be subsequently analyzed. Thus, HPLC analysis can be considered representative of the composition of the saliva at the time of collection. Moreover, acidic treatment simplified sample composition, eliminating α-amylase, mucins and other proteins and decreased the viscosity, in such a way that the sample can be directly injected into the HPLC apparatus.

Histatins are salivary peptides whose name derives from their high histidine content. They have, inter alia, a defensive role against salivary pathogens. At least 12 histatins were detected [14], but the most abundant are histatin 1, histatin 3 and histatin 5. Histatins 1 and 3 are expressed by specific genes, while histatin 5, as well as the other minor histatins, probably derives from protease action on the two genetically-expressed ones [15,16]. Due to the high column performance, the three principal histatins were identified and quantified at 276 nm. Analysis was performed at 276 nm wavelength due to the presence of unidentified substances absorbing at 214 nm (Fig. 2), which may interfere in the detection and quantification of the three major histatins in this not specific wavelength range. During the first period of chromatographic gradient, many minor peaks were detected and their attribution was difficult. On the basis of DAD analysis, many of them could correspond to minor histatins, showing that probably the degradation products are more than 10. Since the preparation method caused inhibition of proteases, the very low concentration found for these minor histatins, with respect to the three principal ones, supported the hypothesis that the minor ones are protease products of the former.

Quantification of histatins 1, 3, 5 was based on their tyrosine content on the supposition that the absorbance of this amino acids was not significantly influenced either by peptide sequence or by the structural environment. Since this assumption is not strictly correct, quantification could be slightly biased. However, based on experience with many other peptides and protease maps [10], this bias should be on the same order of magnitude of other experimental errors deriving from sample dilution and/or treatment. This assumption is corroborated by experiments performed using standards of histatin 5, the unique major salivary peptide commercially supplied. Moreover, the use of this standard allowed us to verify that, after the acid treatment of salivary sample, the recovery of this peptide is greater than 97%. Due to the high similarity in structure and polarity, a similar recovery for histatins 1, 3 and statherin may be assumed.

The values obtained in the analysis of healthy normal samples were in satisfactory agreement with those reported in literature [8]. In fact total histatin concentration is reported to be between 14 to 47 μg/ml in resting salivary secretion by measurements performed throughout ELISA. Instead, values obtained by CE proved to be from 55 to 425 μg/ml [7]. Our results ranged from 60 to 421 μg/ml, with a median of 177 μg/ml. About 50% of this mass pertain to histatin 1, whereas histatins 3 and 5 accounted for about 25% each. Samples in this study were obtained from non-stimulated donors, but the collection time approximately coincided with the hour of maximal salivary flow-rate, which should correspond to maximal histatin secretion.

Statherin is a salivary peptide linked to calcium phosphate precipitation [17]. Like histatins, its high tyrosine content can be utilized for its identification and quantification. Statherin probably derives from a gene related to histatins [18]. Reported statherin concentration in stimulated donor [8] utilizing reversed-phase (RP) HPLC separation ranged between 26 to 233 μg/ml, in agreement with our findings (non-stimulated, from 14 to 77 μg/ml, median value of 49.7 μg/ml).

Whereas the biosynthetic pattern of histatins has been studied, including packaging in acinar cells and a spread distribution throughout the granule [19], that of statherin is unknown. In this respect, the strict correlation between the salivary concentrations of the three major histatins suggested a common secretion route. On the contrary, a lack of correlation between histatin and statherin concentrations could be indicative of different packaging processes.

To our knowledge, a significant connection between the concentration of these salivary peptides and systemic and/or oral pathological states has not been evidenced up to now, although a function on dentin formation, relationship on caries development and a link with oral cavity infections have been hypothesized [14]. Hence, we confide that the HPLC procedure described in this study may be helpful to assess a clinical significance of these salivary components.

On the basis of DAD analysis, the group of peaks eluting approximately between histatins and statherin are quite completely lacking of aromatic amino acids. This group of peaks were hence attributed to PRPs, that are characterized by almost total lack of absorbance over 260 nm wavelength.

Proline-rich proteins are a large family of salivary proteins, characterized by genetic and structural variability [20]. More than 20 PRP components have been separated and divided into three major classes: acidic PRPs, basic PRPs and glycosylated PRPs. The role of acidic PRPs is linked to the dental mineralization process [21], whereas glycosylated PRPs have probably lubricating properties [22]. Although the role of basic PRPs is not completely understood, all PRPs bind tannin and can defend the organism from its deleterious effect [23]. The complexity of the PRP pattern is evident from Fig. 2, top. It is worthwhile to mention that the chromatographic patterns largely changed not only among different subjects, but also among salivary samples delivered at different periods from the same subject. Thus, in order to eliminate at least variability deriving from circadian rhythms, we decided to perform the collection of the sample always at 4 p.m. In any case, the satisfactory resolution obtained by the HPLC method described can be useful in the discrimination among different human phenotypes [5].

After the statherin peak a group of peaks which show by DAD spectral analysis the contemporary tyrosine and tryptophan absorbance is eluted. This group may be attributed to cystatins. Cystatins have been characterized as inhibitors of cysteine proteases. Although their exact function in the oral cavity has not been established, a role in preventing exogenous microbial infections has been hypothesized [24]. The first component characterized was initially named cystatin SAP-1 [25] and later renamed cystatin S [26]. This salivary cystatin S probably consists of three isoforms, with different phosphorylation degrees [27], called S (non-phosphorylated), S1 and S2. In human saliva two other non-phosphorylated cystatins were detected, a neutral one, cystatin SN [28] and a acidic one, cystatin SA [29]. The chromatographic pattern pertaining to this range shows more than five unresolved peaks, demonstrating that to date the identification of cystatins is probably not completed.

Finally, DAD–HPLC permitted determination of uric acid in saliva. Its presence may be related to its antioxidant properties and therefore rapid chromatographic determination could be indicative of antioxidant salivary deficiencies. The concentration of uric acid is not statistically related to that of histatins and statherin, suggesting a different role in the oral cavity.

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