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Short-Term Modification of Human Salivary Proteome Induced by Two Bitter Tastants, Urea and Quinine

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Abstract Salivary proteome patterns of healthy volunteers (n=12) were compared before and after they tasted bitter solutions made of either urea (0.36M) or quinine-hydrochloride (40 μM). Relative abundance of 22 and 18 spots was modified 15 min after stimulation by urea and quinine, respectively. Only two spots were common to both tastants, indicating a molecule-specific response. Proteins, relative quantity of which was altered, were agents of the oral cavity defense (e.g., thioredoxin, cystatin, parotid secretory proteins, etc.) and markers of inflammation (transthyretin and transferrin) or enzymes. In particular, the relative abundance of carbonic anhydrase VI, a protein previously described as crucial to taste function, declined after tasting the urea solution.

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Abbreviations

ACN	Acetonitrile
CAVI	Carbonic anhydrase VI
CHAPS	Cholamidopropyl dimethylammonio-propanesulfonate
CHCA	Cyano-4-hydroxycinnamic acid
DTT	Dithiothreitol
GA3PDH	Glyceraldehyde 3-phosphate dehydrogenase
IPG	Immobilised pH gradient
LC-IT	MS/MS liquid chromatography–ion trap tandem mass spectrometry
MALDI–TOF	Matrix-assisted laser dissociation ionization–time of flight
PIP	Prolactin-inducible protein
PRPs	Proline-rich proteins
PSP	Parotid secretory protein
SDS	Sodium dodecyl sulfate
TCEP	Tris(2-Carboxyethyl) phosphine
TFA	Trifluoroacetic acid
2D	Two-dimensional

Introduction

Saliva has a variety of fundamental biological functions. Lubrication and protection of oral tissues are some of the functions that have been thoroughly investigated in dental medicine. Saliva also plays a role in the sense of taste (Spielman 1990; Matsuo 2000).

In terms of composition, apart from water, saliva is mainly composed of ions, amino acids, peptides, and proteins. The

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proteome complexity of this oral fluid was recently exposed by the identification of 1,166 proteins from the ductal secretions of parotid and submandibular/sublingual glands of healthy individuals (Denny et al. 2008). Whole saliva is expected to be even more complex because it additionally contains proteins secreted by the minor salivary glands and proteins from the gingival crevicular fluid, a serum transudate present in the gingival crevice surrounding the teeth (Lamster and Ahlo 2007).

A limited number of studies showed the possible influence of salivary proteins in flavor perception. Hence, PRPs and histatins can bind and precipitate plant polyphenols in the oral cavity, thereby leading to the sensation of astringency (Williamson 1994; Yan and Bennick 1995). Through its action on the rheology of starch-based food products, α-amylase activity has been related to the perception of saltiness (Ferry et al. 2006). The hypothesis that lipase may be involved in fat perception has also emerged, linked to the fact that it can break down in the oral cavity a small fraction of dietary triglycerides (Kawai and Fushiki 2003). Apart from this direct interaction between salivary proteins and food constituents, saliva proteome may be an indicator of taste disorders. For example, Zn-alpha-2 glycoprotein, prolactin-inducible protein, and cystatin SN were significantly reduced in taste-impaired patients (Igarashi et al. 2008) as well as carbonic anhydrase VI (CAVI), a Zn-protein also called gustin. This protein has been previously related to taste perception due to its role in taste bud development and function (Henkin et al. 1999).

Saliva is a dynamic fluid, the composition of which may vary under the effect of many factors. In the context of investigating the link between saliva proteins and taste perception, it is of interest to know whether gustatory stimuli themselves induce changes in saliva protein composition. However, data on the subject are scarce. Thus, a significant variation in the content of amylase in saliva after stimulation with sucrose was reported (Newbrun 1962). It was also recently shown that saliva proteome can be modified by taste stimulation in different proportions, depending on the taste stimulus (Neyraud et al. 2006), or that tannin-rich diets increase levels of amylase in mice saliva (Da Costa et al. 2008). In this context, the purpose of the study was to investigate the effect of two different bitter molecules (urea and quinine) on the salivary proteome.

Materials and Methods

Chemical Materials

Bitter solutions were made of pharmacological-grade urea (Jerafrance, Jeufosse, France) and quinine hydrochloride (Cooper, Meulun, France) dissolved in Evian® mineral water. Concerning chemicals used for 2D electrophoresis, 1.5 M Tris–HCl pH 8.8 buffer, Tris/glycine/SDS buffer, and Bio-Lyte 3-10 carrier ampholytes were obtained from Bio-Rad (Hercules, CA, USA). Acrylamide 40% and methylenebisacrylamide 2% were purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals were obtained from Sigma (Steinheim, Germany). Concerning chemicals used for mass spectrometry, acetonitrile, trifluoroactic acid, iodocatemide, ammonium bicarbonate, and TCEP were obtained from Sigma (Steinheim, Germany), and CHCA was obtained from Bruker (Bremen, Germany).

Subjects and Sampling Sessions

Twelve volunteers (six women and six men; age range, 26–47 years old; non smokers) were enrolled in this study. These were pre-selected on the basis of their salivary flow rate, where a value lower than 0.5 $g.min^{-1}$ was a criterion of exclusion. All participants provided written informed consent. Two sampling sessions took place at 10:00 A.M. and 3:00 P.M. of the same day. Subjects were instructed to abstain from eating or drinking at least 1 h before the sessions. Two bitter compounds were evaluated, urea (0.36M) and quinine hydrochloride (40 μM), at concentrations above the human recognition threshold values (Meyerhof 2005). In order to avoid any confounding effect of the sampling time on proteome patterns, six subjects were stimulated by urea in the morning and by quinine in the afternoon, while the order was inverted for the other six subjects. In each session, volunteers donated saliva at rest and 15 min after stimulation. Stimulation consisted of drinking 5 ml of the bitter solution in one single sip, which took approximately 2–3 s. At both sampling times (at rest and after stimulation), subjects spat out saliva accumulating freely in their mouth over 2 min. In total, 48 samples were analyzed (12 subjects, two molecule, and two sampling times).

Saliva Processing

Saliva samples were immediately centrifuged at 14,000×*g* for 20 min at 4°C. In order to desalt and concentrate samples, supernatants were ultrafiltered at 15,000×*g* for 30 min at 10°C using spin columns with a 5-kDa molecular weight cutoff (Vivaspin 500, Sartorius AG, Germany). Aliquots of the >5 kDa fraction were stored at −80°C until analysis.

2D Gel Electrophoresis

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. Strips were loaded with 150 µg of protein for analytical gels and 800 µg for preparative gels used for protein identification. Strips were 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 8,000 V for a total of 60 kVh. Thereafter, strips were equilibrated for 15 and 20 min in two consecutive solutions of 6 M urea, 50 mM Tris–HCl pH 8.8, 30% *v*/*v* glycerol, 2% SDS, to which was added DTT at 1% *w*/*v* or iodoacetamide at 2.5% *w*/*v*. The second dimension migration was accomplished on 12% SDS-polyacrylamide gels at 50 mA per gel on a Protean II Multicell (Bio-Rad). The 48 samples were analyzed in a random order. Analytical gels were silver-stained following the protocol of Yan et al. (2000). Staining of preparative gels was done following the protocol of Candiano et al. (2004).

Image and Statistical Analyses

Gel digital images were acquired with the Image Scanner (Amersham Biosciences) and analyzed using SameSpots software v.3.0. Spot quantities were normalized and expressed in parts per million by calculating the ratio of each spot's quantity to the total quantity of valid spots in a gel. This was followed by a natural log transformation, with the intent to make the variance independent of the mean. The difference in composition between at-rest saliva and saliva after taste stimulation was evaluated using paired *t* tests with Statistica software (StatSoft, Tulsa, OK, USA). A spot associated to a p value<0.05 was considered as significant. The sex effect was evaluated for the significant spots after urea and quinine stimulus, respectively, using one way analysis of variance. Classification analyses for the totality of the spots that change after urea and quinine stimulus were done. The spots were grouped by hierarchical cluster analysis using the method of Ward based on Euclidian distances.

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 acetonitrile (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 10ng/µl of trypsin (V5280, Promega, USA). Fifteen microliters of this solution was subsequently removed, and 10 µl of 40 mM NH_4HCO_3/10% ACN was added before incubation at 37°C for 2 h. Peptides were collected in two successive supernatants obtained after addition of 0.5% trifluoroacetic acid (TFA) and ACN (1 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. A volume of 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–TOF (Ultraflex, Bruker Daltonics, Bremen, Germany) used in MS or MS/MS mode. Ionization 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 a maximum accelerating potential of 25 kV in the positive and reflectron modes. The MALDI mass spectra were calibrated using the Peptide Calibration Standards from Bruker Daltonics. The software packages Ultraflex version 3.0, Flex control, Flex Analysis, and Biotools version 3.1 were used to record and analyze the mass spectra. The database search was performed with Mascot in the MSDB database restricted to Human entries. Methionine oxidation was accepted as a variable modification and carbamidomethyl modification of cysteine as a global modification. One missed cleavage was allowed. Mass deviation tolerance was set at 80 ppm in MS mode and 0.5 Da in MS-MS mode.

When identification by MALDI–TOF proved unsuccessful, identification was also attempted using nano LC-IT MS/MS analysis. HPLC was performed with an ultimate LC system combined with Famos autosample and Switchos II microcolumn switching for preconcentration (LC Packings, Amsterdam, The Netherlands). Six microliters of the supernatant containing peptides were loaded on the column PEPMAP C18, 5 µm, 75 µm ID, 15 cm (LC Packings) using a pre-concentration step in a micro-pre-column cartridge (300 µm ID, 1 mm). Supernatants were loaded on the pre-column at 30 µl/min. After 3 min, the pre-column was connected with the separating column, and the gradient was started at 200 nl/min. The buffers were 5% ACN, 0.5% HCOOH in water (A) and 5% H_2O, 0.5% HCOOH in ACN (B). A linear gradient from 10% to 90% B for 45 min was applied. For ion trap MS, a LCQ deca with a nano-electrospray interface (Termofinnigan, Les Ulis, France) was used. Ionization (2.2 kV ionization potential) was performed with a liquid junction and a non-coated capillary probe (New Objective, Cambridge, USA). Peptide ions were analyzed by the data-dependent "triple play" method: (1) full MS scan (m/z 400–1400), (2) zoomscan (scan of the major ion with bigger resolution), and (3) MS/MS of this ion. Identification of peptides was performed with Mascot 2.2,

restricting the taxonomy to *Homo sapiens* (216,961 sequences) in the protein NCBInr 20080912 database. Mass tolerance was set at 0.4 Da. Protein identification was validated when at least two peptides originating from one protein showed significant identification scores.

Results

A total of 509 spots were matched across all gels, which compares very well with previous studies presenting data on silver-stained saliva two-dimensional electrophoretic gels, e.g., 100–120 spots quantified in the study Neyraud et al. (2006) and approximately 300 spots detected in the study Fleissig et al. (2009). Relative abundance of 22 (11 decreasing and 11 increasing) and 18 (ten decreasing and eight increasing) spots was modified for urea and quinine stimulation, respectively. Positions of the spots are indicated in a reference proteome map (Fig. 1). Overall, 20 spots were successfully identified (Table 1). Full details of quality criteria related to protein identification by MS are presented in Tables 2 and 3. The majority of unidentified spots (13 out of 18) were very faintly stained with relative abundance below 1,500 ppm (data not shown). Identification failure may also result from difficulties in ionization of tryptic peptides and/or by the low number of tryptic peptides in low molecular proteins.

Overall, sex had no statistical effect on the representation of significant spots. Only two spots were common to urea and quinine. Looking specifically at proteins common to urea and quinine, although not necessarily corresponding to the same spot, their relative abundance variation was not always comparable: glyceraldehyde-3-phosphate dehydrogenase or GA3PDH (spot 16) and spot 89 decreased and serum albumin (spots 215 and 339) increased after bitter stimulation by urea and quinine, but transferrin (spots 140 and 174) decreased for urea and increased for quinine.

The successfully identified significant spots were enzymes (GA3PDH, CAVI, and amylase), proteins involved in protection of the oral cavity and secreted by salivary glands [PSP, prolactin inducible protein (PIP), and cystatin], and proteins originating from serum (serum albumin, transferrin, transthyretin, and thioredoxin). Cluster representations for significant spots are represented in Fig. 2. For both tastants, two main clusters emerge from the graphics, corresponding naturally to proteins increasing or decreasing in abundance

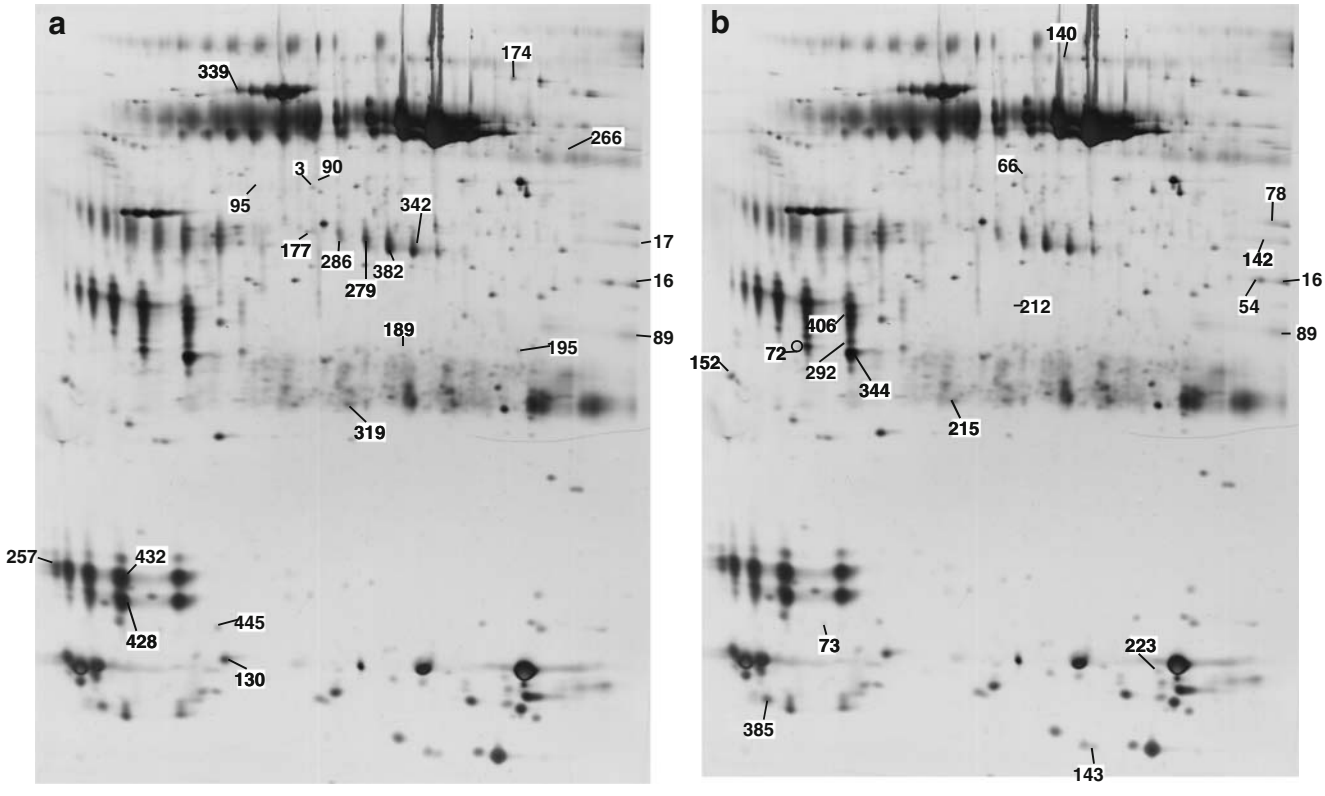

Fig. 1 Typical two-dimensional electrophoresis pattern of human whole saliva (150 μg of protein, pH 3–10, 12% acrylamide gel, silver stained). The significantly modified spots ($p<0.05$) after the bitter taste stimulus by urea (**a**) and quinine (**b**) are represented. *Spots* are labeled by their spot number or identification

Table 1 List of identified spots significantly affected (analysis of variance, $p<5\%$) after bitter stimulation

Spot	Identification	Swiss-Prot entry reference	Δ15min (ppm)	*p* value
Urea				
16	Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH)	P04406	−1,719	0.048
432	Prolactin-inducible protein (PIP)	P12273	−1,932	0.041
428	Prolactin-inducible protein (PIP)	P12273	−1,750	0.006
342	Carbonic anhydrase VI (CAVI)	P23280	−1,768	0.026
286	Carbonic anhydrase VI (CAVI)	P23280	−599	0.037
382	Carbonic anhydrase VI (CAVI)	P23280	−1,932	0.005
279	Carbonic anhydrase VI (CAVI)	P23280	−1,791	0.005
339	Serum albumin	P02768	1,201	0.026
319	Amylase	P04745	746	0.050
195	Amylase	P04745	560	0.0002
445	Transthyretin, chain A	P02766	175	0.0409
257	Prolactin-inducible protein (PIP)	P12273	1,409	0.0156
174	Transferrin	P02787	814	0.0305
Quinine				
215	Serum albumin	P02768	347	0.0336
406	Parotid Secretory Protein (PSP)	Q96DR5	1,380	0.0334
344	Parotid Secretory Protein (PSP)	Q96DR5	570	0.0457
292	Parotid Secretory Protein (PSP)	Q96DR5	242	0.0154
16	Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH)	P04406	−1,186	0.0241
140	Transferrin	P02787	−750	0.0400
385	Thioredoxin	P10599	−480	0.0025
223	Cystatin SN	P01037	−83	0.0390

Values "Δ15 min" are the mean differences ($n=12$) between the spots' quantity after and before stimulation

after the taste stimulus. No obvious factor allows at first sight to differentiate proteins belonging to those two clusters, such as their origin (e.g., glandular vs seric) or their function, neither for urea nor for quinine. However, there are also smaller groups of proteins that share common characteristics. In Fig. 2a, for example, several spots corresponding to CAVI are grouped and clustered with two PIP isoforms (sport 432 and 428), suggesting co-regulation of these proteins. Interestingly, a more acidic isoform of PIP (spot 257) was conversely down-represented after urea stimulation. In Fig. 2b, PSP isoforms are also clustered.

Discussion

The aim of the present study was to document the short-term proteome modification induced by bitter stimulation. Results clearly evidenced that saliva patterns were rapidly modified after the taste stimulus and that the response was molecule-specific.

Although urea and quinine are very distinct in their chemical structure, they both taste bitter to humans. However, individual sensitivity decline with aging (Cowart et al. 1994) differed between the two compounds. Delwiche et al. (2001) later documented covariation in individuals' sensitivities to several bitter compounds, and results supported the hypothesis that different transduction mechanisms are involved in the perception of urea and quinine. Accordingly, bitter taste perception in mammals is mediated through approximately 30 bitter taste receptors, each broadly tuned to many agonists sharing common chemical characteristics (Bufe et al. 2002).

From an opposite point of view, consequences rather than causes, we show in this study that molecule specificity is also expressed in the salivary response to the bitter stimulus. Thus, only three proteins (serum albumin, GA3PDH, and a non-identified protein in spot 89) showed the same general trend after urea and quinine stimulation. GA3PDH is an enzyme of the glycolytic pathway, previously identified in human saliva (Denny et al. 2008). However, because its physiological significance in the oral cavity is largely unknown, it is difficult to interpret its abundance decrease after bitter stimulation. Likewise, the reason for the increase in serum albumin proportion after bitter stimulation is unclear. One should note that the serum albumin increase was observed only in one spot and not the same one for both

Table 2 Details of mass spectrometry results for proteins identified by MALDI-TOF or MALDI TOF-TOF

Spot	Method of identification	Theoretical/ Estimated MW (kDa)	Matched peptides (MS mode)/ Peptides submitted to database search	Percentage of coverage	Mascot score [a]	Matched peptides (MS/MS mode)	Mass (charge) of the precursor ion	Mascot score[b]	MSDB reference
16	MALDI–TOF–TOF	35.9/38.7	9/29	37	80	1	1,763.80 (+1)	85	G3P_HUMAN
432	MALDI–TOF	16.6/16.6	8/30	55	90	n.a.	n.a.	n.a.	SQHUAC
428	MALDI–TOF	16.6/16.0	9/27	58	106	n.a.	n.a.	n.a.	SQHUAC
342	MALDI–TOF–TOF	35.3/42.7	/	/	/	1	1,575.81 (+1)	38	CRHU6
286	MALDI–TOF	35.3/45.3	11 /34	27	99	n.a.	n.a.	n.a.	CRHU6
382	MALDI–TOF–TOF	35.3/42.7	8/18	20	73	1	1,559.82 (+1)	68	CRHU6
279	MALDI–TOF–TOF	35.3/44.7	12/22	35	141	1	1,559.81 (+1)	65	CRHU6
339	MALDI–TOF–TOF	65.7/65.7	42/91	69	298	1	1,910.83 (+1)	74	1AO6A
319	MALDI–TOF–TOF	55.7/27.3	17/53	33	101	1	1427.70 (+1)	56	1SMD
195	MALDI–TOF–TOF	55.7/33.3	27/63	63	187	1	1290.61 (+1)	56	1SMD
445	MALDI–TOF–TOF	13.3/14.7	14/42	96	198	1	1,366.76 (+1)	67	2ROYA
257	MALDI–TOF–TOF	16.6/18.0	9/23	63	125	1	1,995.33 (+1)	60	SQHUAC
174	MALDI–TOF	77.0/72.3	14/30	30	99	n.a.	n.a.	n.a.	TFHUP
215	MALDI–TOF	66.1/28	12/30	22	71	n.a.	n.a.	n.a.	1BJ5
140	MALDI–TOF	77.0/77.0	20/42	36	134	n.a.	n.a.	n.a.	TFHUP
385	MALDI–TOF–TOF	11.7/11.7	6/14	32	67	1	1,479.77 (+1)	50	JH058
223	MALDI–TOF–TOF	16.3/13.3	6/11	58	90	1	1,291.67 (+1)	56	UDHUP2

n.a. not applicable

[a] A Mascot score above 64 is significant ($p<0.05$)

[b] An individual peptide Mascot score above 37 indicates identity or extensive homology ($p<0.05$)

molecules, while many spots on 2D electrophoretic gels correspond to this protein (Hu et al. 2005). Consequently, the relative abundance change of one isoform does not necessarily reflect an overall change in serum albumin levels. This remark also stands for transferrin, another protein present under many forms in saliva, and even more so for amylase. Thus, a vast quantity of amylase variants (140 spots) have been reported to co-exist on salivary 2D gels (Hirtz et al.

Table 3 Details of mass spectrometry results for proteins identified by LC-ESI MS/MS

Spot	Theoretical/ experimental MW (kDa)	Matched peptides	Percentage of coverage	Mass and charge of the precursor ion(s)	Individual peptide mascot scores[a]	NCBI accession number
406	27.1/36.0	6	28	556.78 (+2) 450.63 (+2)	51 42	gi| 16755850
				666..60 (+2)	50	
				551.13 (+2)	84	
				567.70 (+2)	61	
				814.87 (+3)	63	
344	27.1/32.0	6	28	556.64 (+2) 446.02 (+2)	51 37	gi| 16755850
				450.55 (+2)	42	
				666.20 (+2)	50	
				551.06 (+2)	84	
				815.01 (+3)	63	
292	27.1/34.0	2	6	567.62 (+2) 816.08 (+1)	57 35	

[a] An individual peptide Mascot score above 33 indicates significant homology ($p<0.05$) while a score above 41 indicates identity ($p<0.05$)

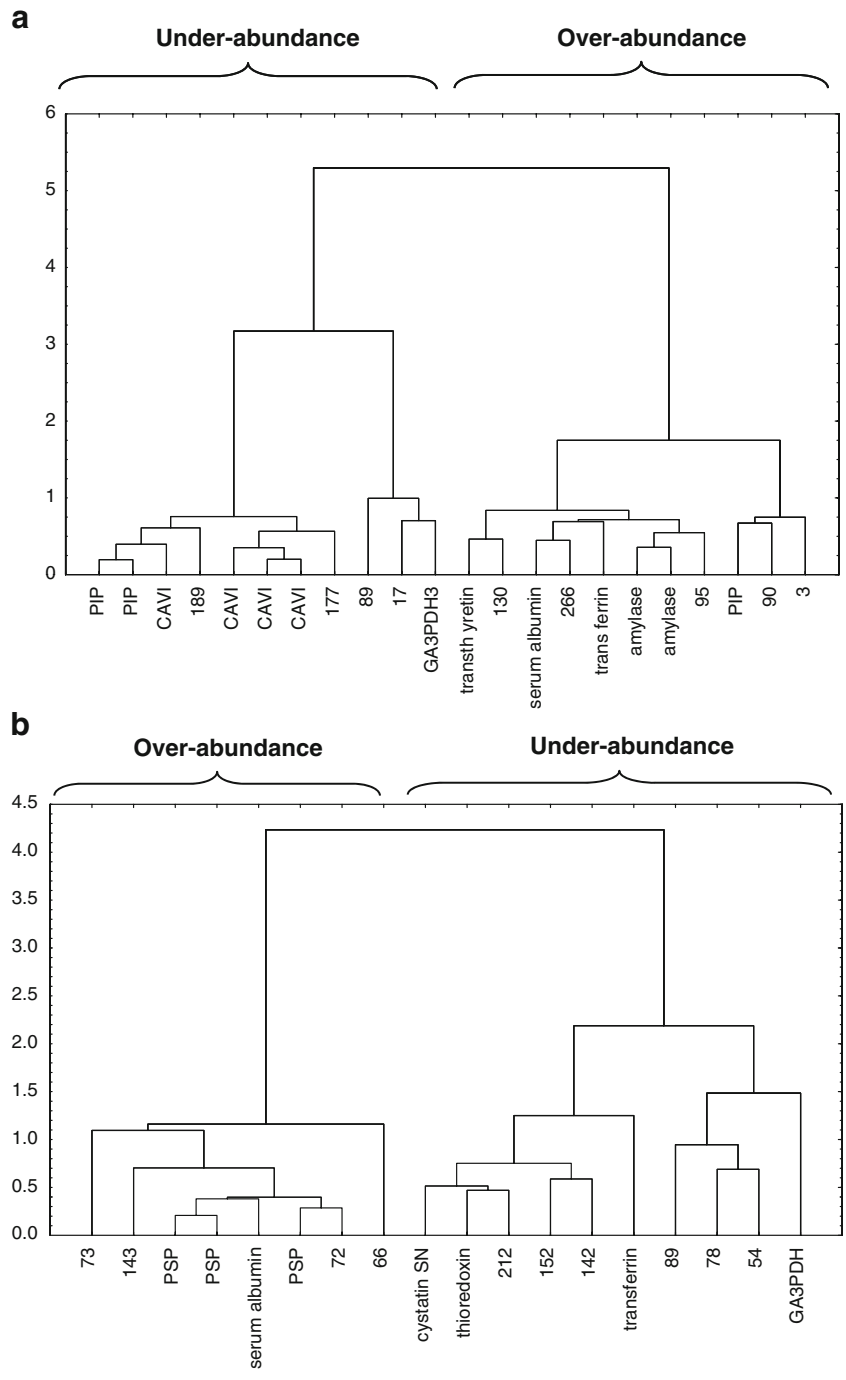

Fig. 2 Clustering (method of Ward based on Euclidian distances) of spots that significantly change ($p<0.05$) after bitter stimulation by urea (**a**) and quinine (**b**)

2005). Variation in two isoforms, although possibly significant in terms of secretory mechanisms, cannot be interpreted as variation in the protein proportion globally.

Keeping in mind these limitations, in our view, results can be considered as of interest when proteins with very specific roles were identified or when proteins repeatedly identified pointed at abundant isoforms.

Starting with stimulation by urea, it thus resulted in under-abundance of CAVI and two isoforms of PIP and over-abundance of one isoform of PIP and transthyretin.

CAVI is secreted into saliva by the serous cells of the parotid and submandibular gland and also locally by the von Ebner's glands (Leinonen et al. 2001). Originally, this protein was called gustin until Thatcher et al. (1998) evidenced that it belonged to the carbonic anhydrase family. In general, carbonic anhydrases catalyze the reversible conversion of carbon dioxide to bicarbonate and protons and are therefore involved in pH regulation mechanisms. In the oral cavity in particular, CAVI is reported to ensure protection of the dental enamel (Kivela et al. 1999) and the

upper alimentary canal (Parkkila et al. 1997) from acid injury. Urea is a weak basic compound that does not increase directly the salivary pH at the concentration used in this study. However, urea present in saliva is rapidly converted to ammonia and carbon dioxide by bacterial ureases from the flora of the oral cavity (Burne and Chen 2000). A mathematical model establishing the link between saliva urea concentration and pH of plaque thus evidenced that, even at normal concentrations (3–5 mM), urea leads to local pH elevation (Dibdin and Dawes 1998). In our case, the pH rise induced by urea could therefore explain the lesser proportion of carbonic anhydrase. From a flavor perception point of view, this is of special interest, since CAVI has long been recognized as playing a central role in taste function (Shatzman and Henkin 1981). A mechanism more recently proposed is that it protects taste buds against apoptosis (Leinonen et al. 2001). The link between saliva pH and how it is modified by food properties on one hand and the abundance of salivary CAVI, evaluated for example by immunological techniques, on the other hand, may deserve further attention.

Concerning PIP, positions of the three significant spots (Fig. 1) indicate that it is specifically the most acidic form of PIP, which rose in intensity while more basic spots saw their abundance drop. PIPs are secreted in saliva in many forms differing by glycosylation (Ghafouri et al. 2003) and phosphorylation (Vitorino et al. 2004) status. The more acidic spots would therefore correspond to more phosphorylated isoforms. Although post-translational modifications are extremely frequent in salivary proteins (Helmerhorst and Oppenheim 2007; Messana et al. 2008), regulation or functionality of more or less phosphorylated PIPs is still unknown (Vitorino et al. 2004). It is therefore unclear why urea would favor phosphorylation of PIPs.

Finally, transthyretin is a traditional marker of inflammatory and nutritional status. For example, Murayama et al. (1999) evaluated and validated its suitability as a salivary marker of protein–energy malnutrition in elderly patients. Reduced quantity in saliva has also been detected in patients suffering from head and neck squamous cell carcinoma (Dowling et al. 2008). Together with transferrin, transthyretin is a negative biomarker of inflammation (Ritchie et al. 1999). In our case, the relative intensity of transthyretin and incidentally of one transferrin spot increased after urea stimulation. Both proteins originate in saliva from serum. However, the time frame used in the study (15 min after ingestion) renders the hypothesis of a regulation in serum levels quite unlikely. Urea may therefore merely increase the proportion of crevicular fluid (containing among other proteins transthyretin, serum albumin and transferrin) in whole saliva. Urea concentration in gingival crevicular fluid was estimated at around 35 mM in healthy adolescents (Ciancio et al. 1977). Consequently, it seems plausible that bathing the gingival crevices in a solution of urea at a higher molarity (360 mM in this study) would favor fluid efflux from gingival crevices.

Considering now quinine-induced modifications of proteome patterns, they were most notably under-abundance of thioredoxin and cystatin SN and over-abundance of PSP. Thioredoxin is a small 12-kDa protein, which is part of the so-called thioredoxin system, including thioredoxin, thioredoxin reductase, and NADPH. Intracellularly, thioredoxin protects against oxidative stress by maintaining proteins in their reduced state (Arner and Holmgren 2000). In saliva, thioredoxin has primarily an antiviral function (Huq et al. 2007). Cystatin SN, and more generally cystatins, also exhibit antiviral/antibacterial functions (Nieuw Amerongen and Veerman 2002). The reduced proportion of these two proteins in saliva 15 min after the quinine stimulus is somehow unexpected, since quinine has no documented antibacterial, antiviral, or antioxidant properties. Finally, PSP is a protein secreted mainly by the parotid glands. It possesses both antibacterial and anti-inflammatory activities, which can be up-regulated by bacteria and humoral factors (Shiba et al. 2005). The over-abundance of PSP in saliva after quinine stimulation does not seem to result from a simple increase of parotid flow, first because no other abundant parotid-specific protein (amylase being the most representative) was more represented and second because quinine intensity in gels was previously demonstrated to have no influence on saliva flow (Neyraud et al. 2005). The PSP over-abundance is therefore a specific response to the molecule itself.

None of the proteins demonstrated by Neyraud et al. (2006) to be quantitatively altered by a bitter stimulus of high intensity (calgranulin A, annexin A1, cystatin S and enolase 1) were found in this study. However, the bitter stimulus was in their study generated by $Ca(NO_3)_2$, and we show in this study that the molecule at the origin of the stimulus has a profound effect on saliva proteome modification.

Conclusions

Two compounds eliciting bitterness were shown to modify the saliva proteome pattern of healthy adults 15 min after ingestion of the solution. Proteins generally involved in the oral cavity defense or in inflammatory processes, which in fact constitute the majority of saliva proteins, were quantitatively affected. More relevant to the chemosensory field, urea also induced variations in CAVI levels, a protein previously linked to taste perception.

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