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Salivary Protein Profiles and Sensitivity to the Bitter Taste of Caffeine

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Abstract

The interindividual variation in the sensitivity to bitterness is attributed in part to genetic polymorphism at the taste receptor level, but other factors, such as saliva composition, might be involved. In order to investigate this, 2 groups of subjects (hyposensitive, hypersensitive) were selected from 29 healthy male volunteers based on their detection thresholds for caffeine, and their salivary proteome composition was compared. Abundance of 26 of the 255 spots detected on saliva electrophoretic patterns was significantly different between hypo- and hypersensitive subjects. Saliva of hypersensitive subjects contained higher levels of amylase fragments, immunoglobulins, and serum albumin and/or serum albumin fragments. It also contained lower levels of cystatin SN, an inhibitor of protease. The results suggest that proteolysis occurring within the oral cavity is an important perireceptor factor associated to the sensitivity to the bitter taste of caffeine.

Key words: bitterness, cystatin, proteolysis, proteome, saliva, two-dimensional electrophoresis

Introduction

Since many poisons are bitter, bitterness is believed to be a negative cue preventing mammals from ingesting potentially harmful food constituents (Chandrashekar et al. 2000). Bitterness therefore influences food choices, including in humans. It is, for example, one of the major causes for the rejection of some vegetables (Drewnowski and Gomez-Carneros 2000). The perception of bitter taste varies greatly between individuals, which has raised questions on the mechanisms underlying this variability. Three main issues are addressed in the literature: specific genetic variation (typically sequence variants of taste receptors), generic genetic variation (e.g., density of taste bud), and environmental factors (diet, health, or hormonal status, etc.). The relative proportions of these factors in perceptive variation differ between bitter tastants. For example, the variation in the perceived intensity is highly heritable for 6-n-propyl thiouracyl (PROP) but more modestly heritable for other compounds including caffeine (Hansen et al. 2006). Such results are in agreement with the current knowledge on human bitter taste receptors, named hTAS2Rs. In particular, polymorphism in the gene encoding for hTAS2R38 results in 3 main variants of the receptor, strongly correlated with the individual bitter sensitivities to phenylthiocarbamide and PROP (Kim et al. 2003; Bufe et al. 2005). In contrast, in a study on the molecular receptive ranges of the 25 human TAS2R bitter taste receptors identified to date, it was demonstrated that caffeine has the ability of activating at least 5 TAS2Rs (Meyerhof et al. 2010). Polymorphism on one hTAS2R would therefore affect less caffeine perception than in the case of PROP. Together with the lower heritability of sensitivity to this molecule, this suggests that perireceptor events (e.g., salivary parameters) and environmental factors in the broad sense contribute substantially to the variability in caffeine perception.

Apart from water, proteins are the main constituents of human whole saliva. In whole saliva, proteins are secreted by major and minor salivary glands but originate also from nonglandular sources, such as the gingival crevicular fluid, mucosal secretions, etc. Salivary proteins are involved in diverse functions, including digestion and protection of the oral cavity (Humphrey and Williamson 2001). In addition, some saliva proteins play a role in food sensory perception. For instance, proline-rich proteins and histidine-rich

proteins are involved in the sensation of astringency through their binding to polyphenols (Bajec and Pickering 2008), and responses to astringent stimuli are dependent on one subject's saliva protein pattern stability (Dinnella et al. 2009, 2010). Another well-documented example is the central role of carbonic anhydrase VI (CAVI) in taste perception. Thus, a deficiency in CAVI is associated with an overall decreased taste perception (Shatzman and Henkin 1981). More generally, differences in saliva protein patterns have been evidenced in taste-impaired patients compared with healthy subjects (Igarashi et al. 2008). In this context and given the fact that saliva proteome is highly variable between individuals (Quintana et al. 2009), the objective of the present study is to test whether variability in saliva proteome composition is related to variability in sensitivity to the bitterness of caffeine and if so, to formulate mechanistic hypotheses on the link between saliva proteome and taste sensitivity.

Materials and methods

Subjects

Twenty-nine healthy male volunteers, aged 25–45, completed the study consisting of four 1-h sessions. They were nonsmokers and presented no overt sign of oral pathologies, such as gingivitis or dental decay. Their body mass index (BMI) ranged from 19 to 30. Subjects were asked to refrain from drinking or eating during the hour preceding the sessions. The entire procedure, including saliva sampling, was approved by the local ethical committee (Comité de Protection de Personnes Est I Bourgogne). Information about the study was communicated, and written informed consent was obtained from the participants.

Detection threshold determination for caffeine

Food-grade caffeine was purchased from Jerafrance. All solutions were prepared with Evian bottled water. The same water was used as the blank stimulus. Sensory testing took place in a specialized facility comprising of computerized individual booths equipped with the FIZZ software. Detection thresholds were determined following ISO 13301:2002 "General guidance for measuring odor, flavor, and taste detection thresholds by a 3-alternative forced-choice (3-AFC)." Briefly, the method consists in presenting to subjects series of 3 glasses, 2 glasses containing water, and 1 glass containing a solution of caffeine. Subjects have to identify which one is different from the others. At the first session, 10 caffeine concentrations were presented in ascending order, ranging from 0.05 to 8.7 mM in 0.25 log steps. Results of the first session were used to determine a subject's best estimated threshold (BET). In the following 2 sessions, caffeine concentrations were adapted individually: 5 concentrations were presented, namely the BET concentration and 2 higher/2 lower concentrations separated by 0.125 log steps. In total, subjects tested 5 concentrations 6 times. Data were statistically handled as described in ISO 13301:2002 in order to determine individual detection thresholds.

Saliva collection and processing

At rest saliva was collected at the beginning of all 4 sessions. During 5 min and at their own rhythm, subjects spat out the saliva passively accumulating in their mouth into a preweighed ice-chilled vessel. Immediately after collection, samples were weighed, centrifuged at 14 000 × *g* for 20 min at 4 °C, aliquoted, and stored at –80 °C until analysis. This saliva is referred to as "clarified saliva." Salivary flow rate, averaged over the 4 replicates, was expressed in g/min.

Saliva characterization: protein content and proteolytic activity

Protein content was measured in duplicates in clarified saliva using the Bradford method, using bovine serum albumin (BSA) as a standard. Proteolytic activity was measured in duplicates using a commercial kit (Pierce Fluorescent protease assay kit, Thermo Scientific). This kit is based on the detection of fluorescence following proteolytic digestion of fluorescein isothiocyanate–labeled casein (FTC-casein). The protocol was modified as follows: 100 μL of FTC-casein solution, prepared following the manufacturer's instructions, were incubated for 1 h with varying volumes of saliva (0–20 μL), the total volume of the assay being adjusted to 150 μL with Tris-buffered saline. Fluorescence emitted was plotted versus the volume of added saliva. The proteolytic activity was defined as the slope of the resulting linear curve. It was expressed in arbitrary unit (AU)/mL of saliva and converted in AU/mg of protein.

Two-dimensional electrophoresis analysis

Based on the sensory analysis results, 2 groups of 6 subjects (6 hyposensitive and 6 hypersensitive, noted S– and S+, respectively) were selected, and their saliva was used for 2D electrophoresis analysis. For each subject, three 1 mL aliquots sampled on 3 different days were thawed at room temperature and pooled. Clarified saliva was utralfiltered at 15 000 g for 30 min at 10 °C using filtration devices with a 5 kDa cutoff in order to concentrate the proteins. The protein content of the resulting extracts was measured using the Bradford method. The first dimension was performed on a Protean IEF cell (Bio-Rad). Protein extracts containing 500 μg of proteins were included in 400 μL of a rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.1% dithiothreitol (DTT), and 1% Ampholytes Bio-Lyte (4 °C). These were loaded onto 17 cm 3-10NL IPG strips (Bio-Rad). Strips were rehydrated at 0 V for 13 h and 50 V for 8 h, and isoelectric focusing was carried out at a final voltage of 8000 V up to 60 kVh. Strips were then equilibrated successively in 2 solutions containing 6 M urea, 1.5 M Tris–HCL pH 8.8, glycerol, 10% sodium dodecyl sulfate (SDS), and either 1% DTT or 2.5% iodoacetamide for 15 and 20 min, respectively. They were

finally placed onto 12% SDS polyacrylamide gels and sealed with agarose. Electrophoresis was performed at 25 mA per gel for 5 h on a Protean II Multicell (Bio-Rad). Analytical gels were stained with Flamingo fluorescent gel stain (Bio-Rad) following the manufacturer's instructions, whereas gels prepared for identification of proteins were stained using colloidal Coomassie blue following the protocol described by Candiano et al. (2004). Gel images were acquired on a Pharos FX imaging system (Bio-rad) for fluorescently stained gels or on a GS-800 densitometer (Bio-Rad) for colorimetrically stained gels. Images were analyzed using Samespots software (NonLinear Dynamics). Spot volumes were normalized within one gel by the total volume of all valid spots for that particular gel and log transformed.

One-dimensional electrophoresis and immunoblotting

Western blotting was used in order to verify one finding of special interest evidenced previously by 2D electrophoresis, namely underexpression of cystatin SN in hypersensitive subjects. SDS–polyacrylamide gel electrophoresis was performed on 14% acrylamide gels using the Mini-Protean-2 device (Bio-Rad) according to the method of Laemmli (1970). Ten micrograms of protein were loaded per lane, and electrophoresis was carried out at 15 mA per gel for 90 min. Proteins were then transferred onto 0.45 μm nitrocellulose membranes at 10 V for 20 min using a Transblot SD cell (Bio-rad). Membranes were saturated in 8% skimmed milk powder in 9‰ NaCl and incubated for 1 h with a 1:200 dilution of the primary antibody (anti-cystatin SN: sc-73885, Santa Cruz Biotechnology Inc.) in 9‰ NaCl/5% BSA. After washing in phosphate-buffered saline (PBS)-Tween 0.05%, membranes were blocked in 8% skimmed milk powder in 9‰ NaCl and incubated for 1 h with a 1:400 dilution of goat anti-mouse Horseradish peroxidase (HRP)-conjugated antibody in 9‰ NaCl/5% BSA. Membranes were finally washed in PBS-Tween and colorimetrically revealed using the HRP substrate kit (Bio-Rad). Images of blots were acquired on a GS-800 densitometer (Bio-Rad). Semiquantification of bands was performed using the software Quantity One (Bio-rad). Results were expressed in AU.

Statistics

Statistics were performed using Statistica software (StatSoft). The correlation between the subjects' detection thresholds and their BMI and salivary flow rate was evaluated using the Spearman correlation test. The choice of a nonparametric test was guided by the presence of 3 censored values for the calculated thresholds (see Results). The difference in BMI, salivary flow rate, protein content, proteolytic activity, and spot abundance between the hyposensitive and the hypersensitive subjects was tested by one-way analysis of variance. Underexpression of immunologically detected cystatin SN in hypersensitive subjects was statistically tested using a one-tailed Student test. The level of significance was set at 5%.

Protein identification by mass spectrometry

Spots of interest were manually excised from the gels and placed in a 96-well plate. They were then successively washed twice with 0.1 M NH_4HCO_3 and 100% acetonitrile (ACN) for 10 min. Gel pieces were successively incubated 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 2 min, and in ACN for 8 min. Digestion was performed in 15 μL of a solution of 40 mM NH_4HCO_3/10% ACN containing 10 ng/μL of trypsin (V5280, Promega). Ten microliters of trypsin were removed, and 5 μL of 40 mM NH_4HCO_3/10% ACN was added before incubation at 37 °C for 2 h and 30 min. Peptides extraction was performed by successive incubation in 1 μL of 0.5% TFA and 10 μL of ACN for 8 min twice.

For the analysis in MS and MS/MS mode, 1 μL of matrix (3.5 mg/mL HCCA in a solution of ACN/0.5% TFA, ref 201344, Bruker Daltonics) per 0.5 μL of the digest was deposited on a ground steel target plate. Analysis was conducted using a MALDI-TOF/TOF UltrafleXtreme Bruker Daltonics in automatic mode. The database search was performed on SwissProt and restricted to Human entries. Two missed cleavages were allowed. Carbamidomethyl modification of cysteine was accepted as a stable modification and methionine oxidation as a variable modification. Mass deviation tolerance was set at 30 ppm in MS mode and 0.6 Da in MS/MS mode. Systematic reinterrogation on the list of unmatched peaks was performed until no new protein was identified.

Results

Detection thresholds for the bitter taste of caffeine

Detection thresholds for caffeine were calculated for 26 subjects and ranged from 0.38 to 6.04 mM. For the remaining 3 subjects, their detection thresholds were below the minimum concentration of caffeine presented to them in the second and third session (0.5, 0.20, and 0.37 mM, respectively). Detection thresholds for caffeine had a median value of 1.33 mM (n = 29) and a mean value of 1.83 mM (n = 26). No significant correlation was observed between detection thresholds and subjects' basic physiological recordings (BMI, salivary flow).

Six subjects hypersensitive to caffeine (<0.5 mM) and 6 subjects hyposensitive to caffeine (>2.8 mM) were selected (Figure 1). Again, there was no significant difference between the 2 groups in terms of BMI (means S–: 25.3; S+: 23.4) or salivary flow (means S–: 0.60; S+: 0.60 g/min).

Protein content and proteolytic activities

The mean protein contents in the S– and S+ groups were 0.90 and 0.50 mg/ml, respectively, and the difference between the 2 groups was marginally significant (P = 0.07). Proteolytic

activity was on average 784 and 1425 AU/μg of protein in the S– and S+ groups, respectively. However, due to high between-subject variability, the difference between the 2 groups was not significant.

Saliva electrophoretic patterns

Overall, 255 spots were detected, matched across gels, and retained for the analysis. These spots spanned the approximate molecular weight (MW) range 10–55 kDa. The expression levels of 26 spots were significantly different between the S– and S+ groups (Table 1), with 3 spots overexpressed in hyposensitive subjects and 23 overexpressed in hypersensitive subjects. Within-group coefficients of variation for individual spots ranged from 9% to 115%. Positions of these spots are shown in Figure 2.

Twenty-two of the 26 spots were successfully identified by mass spectrometry. Details of identification parameters are provided in Table 2. The identified spots corresponded to different forms of 4 major salivary proteins, namely salivary amylase, serum albumin, immunoglobulins, and cystatin SN.

Cystatin SN was represented mainly by a spot of great abundance (spot 17) at an apparent MW close to the theoretical one. Despite its presence also in a fainter spot (spot 54) at a lower MW, it can be confidently postulated that cystatin SN levels were reduced in hypersensitive subjects. As to amylase and quite expectedly since spots over 55 kDa were not resolved in our conditions, all the identified forms were at MW lower than that of the full-length protein. Mass spectrometry data (Figure 3) suggested that spots 2, 11, 54, 153, 14, 18, 121, and 84 were N-terminal fragments of the protein because the detected peptides were grouped in that region. Amylase peptides detected in spots 9, 57, 64, and 55 were more scattered on the protein sequence. Those spots may therefore correspond to internal fragments or variants of amylase. At the exception of spots 2 and 11, all spots containing amylase were overexpressed in hypersensitive subjects. Concerning serum albumin, examination of mass spectrometry data revealed that spot 15 may contain the full-length isoform2 of serum albumin, whereas spots 13 and 12 were most likely C-terminal fragments of the protein (data not shown). All were overexpressed in hypersensitive subjects. Finally, out of the 6 forms of Ig kappa chain C region, 5 had an apparent molecular approximately twice higher than the theoretical value, which may result from comigration or polymerization of the protein. All spots containing Ig kappa and Ig alpha were more abundant in hypersensitive subjects.

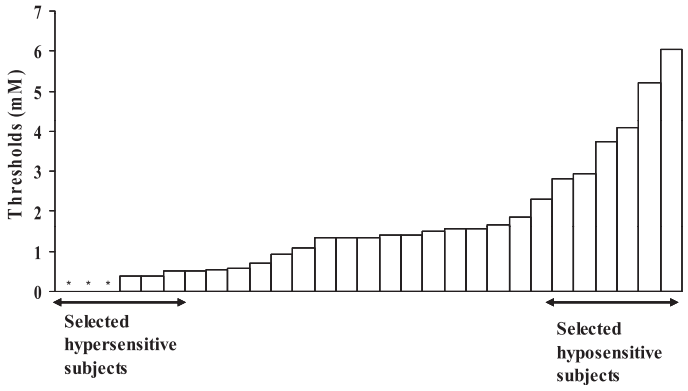

Figure 1 Individual detection thresholds for the bitter taste of caffeine (n = 29). For 3 subjects, indicated by *, thresholds were below the minimum concentration of caffeine presented to them in the second and third session (0.5, 0.20, and 0.37 mM for subjects 18, 32, and 50, respectively).

Table 1 Relative quantities (normalized volumes expressed in ppm) and coefficients of variation (CVs expressed in percent) of the 26 spots significantly different ($P < 0.05$) between the hyposensitive and the hypersensitive subjects

Spots	Hyposensitive subjects		Hypersensitive subjects		P
	Relative quantity (ppm)	CV (%)	Relative quantity (ppm)	CV (%)	
2	2877	115	912	51	0.046
17	64 896	45	34 973	45	0.043
11	3593	33	2206	28	0.024
10	567	59	947	36	0.035
92	3159	43	5475	18	0.017
54	2209	49	3790	27	0.041
9	3544	47	7085	31	0.013
13	3429	36	6460	20	0.005
12	3246	35	5846	22	0.007
153	2077	40	2960	9	0.039
14	1216	25	1790	22	0.021
88	4311	32	6532	26	0.036
61	3777	27	5649	29	0.036
19	5024	18	7072	10	0.002
118	8472	15	11 951	31	0.037
18	5886	13	7938	21	0.020
121	1755	12	2457	23	0.015
84	2047	25	3335	24	0.005
57	2049	35	3075	29	0.043
15	2697	25	4327	12	0.003
64	4236	18	6736	23	0.003
55	2354	39	4198	48	0.035
76	3119	34	5029	34	0.039
43	359	21	697	46	0.009
60	936	38	1709	35	0.014
6	435	30	903	51	0.040

The 3 first spots are underexpressed in S+ subjects.

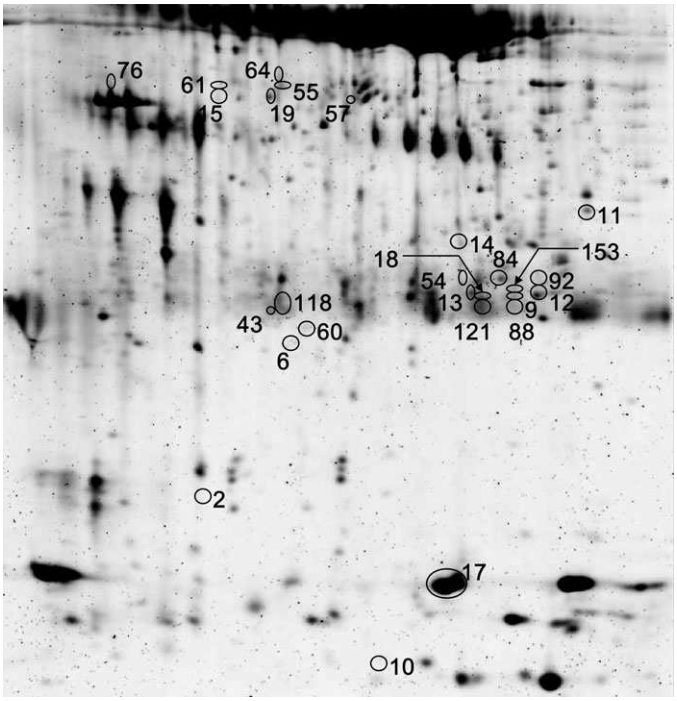

Figure 2 Two-dimensional electrophoretic protein pattern in resting whole saliva. This representative gel corresponds to a hyposensitive subject. Spots differentially expressed between subjects hyposensitive and hypersensitive to the bitter taste of caffeine are numbered.

Since Ig kappa (immunoglobulin light chain) can belong to any isotype of immunoglobulins but only Ig alpha heavy chains were identified, this most likely indicates that IgA specifically was overexpressed in hypersensitive subjects.

Abundance of cystatin SN

The primary antibody used is raised against full-length cystatin SN, and it was indeed detected as a single band of approximately 14 kDa. Semiquantification of bands confirmed that full-length cystatin SN was significantly ($P = 0.04$) overexpressed in hyposensitive subjects (15.74 AU vs. 10.79 in hypersensitive subjects). The fold-change between the 2 groups was 1.46 (Figure 4).

Discussion

The present study aimed at investigating the link between saliva proteome composition and gustatory sensitivity to bitterness, choosing caffeine as a bitter tastant and opting for a differential approach. The mean detection threshold we found for caffeine (1.83 mM averaged over 26 subjects, excluding 3 subjects with very low values) is in reasonable agreement with values previously reported, for example, 1.20 mM for young predominantly female tasters (Keast and Roper 2007) or 1.30 and 1.99 mM for young and elderly tasters, respectively (Schiffman et al. 1994). Based on these results, we selected 2 groups of subjects with clearly contrasted thresholds and found that overall saliva of hypersensitive subjects contained higher levels of amylase variants and/or fragments, immunoglobulin, and serum albumin and/or serum albumin fragments and lower levels of cystatin SN.

It is noteworthy that the proteins differentially represented between the 2 groups originate from different sources. Thus, amylase is secreted predominantly from the parotid gland, serum albumin is a plasmatic protein, immunoglobulins (in particular IgA) are secreted by major and minor glands (Marcotte and Lavoie 1998), whereas cystatin SN is predominantly produced by the submandibular and sublingual glands (Baron, DeCarlo, et al. 1999). In other words, the differences we observed were not merely the result of variability in the contribution of one source of proteins relatively to the others.

We did not observe significant reduction of CAVI proportions in hyposensitive subjects, as reported, for example, in parotid saliva of patients with hypogeusia (Shatzman and Henkin 1981). Our results are also at first sight contradictory with Igarashi et al. (2008) who reported a significant lower proportion of electrophoretically separated cystatin SN in patients suffering from taste disorders. However, no description of the taste disorders was provided, and this term can apply to many situations (hypogeusia, dysgeusia, or sensation of an unpleasant taste even in the absence of a stimulus). In contrast, some of the results described here corroborate data reported on a group of patients complaining from taste aberrations with reduced taste acuity (Hershkovich and Nagler 2004). In particular, compared with controls, such patients were characterized by a similar flow rate but significantly higher total protein content.

Taken individually, the proteins differentially expressed do not point out at a straightforward mechanism that could explain differences in sensitivity to the bitter taste of caffeine. For example, amylase overrepresented in hypersensitive subjects is one of the most abundant salivary proteins. It is represented as many isoforms in whole saliva (Hirtz et al. 2005). Its enzymatic activity consists of cleaving the glucosidic bonds of complex carbohydrates. It is suggested to initiate starch digestion in-mouth and may even retain some activity down to the small intestine (Valdez and Fox 1991). It, however, possesses many other functions. It plays, for example, an antibacterial role through binding mechanisms (Rudney et al. 1995), and it has an antiinflammatory action (Hirtz et al. 2006). Salivary amylase is upregulated by psychosocial stress (Nater et al. 2006) and in the case of several pathologies including periodontitis (Wu et al. 2009) or type I diabetes (Hirtz et al. 2006).

The second protein that was found more abundant in hypersensitive subjects is serum albumin. Serum albumin in saliva comes from plasma, and it enters the oral cavity through the gingival crevicular fluid or through epithelial permeability, the latter increasing during inflammation. Increased levels of serum albumin are therefore associated with conditions, such as gingivitis or periodontitis (Henskens

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

Spots	Proteins identified	Theoretical/ Estimated MW[a] (kDa)	No of unique peptides (MS mode)	Percentage of coverage	Mascot score[b] in MS mode	No of unique peptides (MS/MS mode)	Mascot score[c] in MS/MS mode	MSDB reference
2	Salivary alpha-amylase	57.7/16.4	—	—	—	2	142.89	AMYS_HUMAN
17	Cystatin SN	16.3/13.2	8	53.9	78.9	2	209.27	CYTN_ HUMAN
11	Salivary alpha-amylase	57.7/32.4	18	39.53	145	—	—	AMYS_HUMAN
10	Ig kappa chain C region	11.6/11.0	—	—	—	2	122.31	KAC_ HUMAN
92	Not identified							
54	Cystatin-SN	16.3/27.7	—	—	—	2	116.29	CYTN_ HUMAN
	Ig alpha-1 chain C region	37.6/27.7	—	—	—	2	115.89	IGHA1_ HUMAN
	Salivary alpha-amylase	57.7/27.7	—	—	—	2	174.66	AMYS_HUMAN
9	Salivary alpha-amylase	57.7/26.5	—	—	—	3	132.86	AMYC_HUMAN
13	Serum albumin[d]	69.3—47.4/26.7	—	—	—	3	139.39	ALBU_ HUMAN
12	Serum albumin[d]	69.3—47.4/26.6	—	—	—	2	145.71	ALBU_ HUMAN
153	Salivary alpha-amylase	57.7/27.0	12	29.35	65.4	—	—	AMYS_HUMAN
14	Salivary alpha-amylase	57.7/30.3	11	27.59	96.4	—	—	AMYS_HUMAN
88	Ig kappa chain C region	11.6/25.8	—	—	—	2	80.43	KAC_ HUMAN
61	Ig alpha-1 chain C region	37.6/44.1	9	35.41	77	2	218.59	IGHA1_ HUMAN
	Ig alpha-2 chain C region	36.5/44.1	8	30	64.3	—	—	IGHA2_ HUMAN
19	Ig alpha-2 chain C region	36.5/44.1	7	30	76.1	—	—	IGHA2_ HUMAN
118	Ig kappa chain C region	11.6/26.0	5	82.08	62.7	2	253.98	KAC_ HUMAN
18	Ig kappa chain C region	11.6/26.5	6	86.79	65	2	136.65	KAC_ HUMAN
	Salivary alpha-amylase	57.7/26.5	—	—	—	2	120.09	AMYS_HUMAN
121	Ig kappa chain C region	11.6/25.8	—	—	—	2	176.06	KAC_ HUMAN
	Salivary alpha-amylase	57.7/25.8	16	34.64	94.3	—	—	AMYS_HUMAN
84	Salivary alpha-amylase	57.7/27.7	16	34.05	113	—	—	AMYS_HUMAN
57	Salivary alpha-amylase	57.7/42.6	5	14.29	60.1	2	76.52	AMYS_HUMAN
15	Serum albumin[d]	69.3—47.4/42.8	15	26.6	64.5	2	103.7	ALBU_ HUMAN
64	Ig alpha-1 chain C region	37.6/44.7	—	—	—	2	159.03	IGHA1_HUMAN
	Salivary alpha-amylase	57.7/44.7	—	—	—	2	96.01	AMYS_HUMAN
55	Salivary alpha-amylase	57.7/44.1	22	42.47	143	—	—	AMYS_HUMAN
76	Not identified							
43	Ig kappa chain C region	11.6/25.8	—	—	—	2	179.49	KAC_ HUMAN
60	Not identified							
6	Not identified							

[a]The reported theoretical MW is that of the entire chain (including signal peptides).
[b]In MS mode, the identification is significant if Mascot score > 60.
[c]In MS/MS mode, the identification is significant if Mascot score > 20.
[d]Serum albumin is expressed as either isoform 1 (69.3 kDa) or isoform 2 (47.4 kDa).

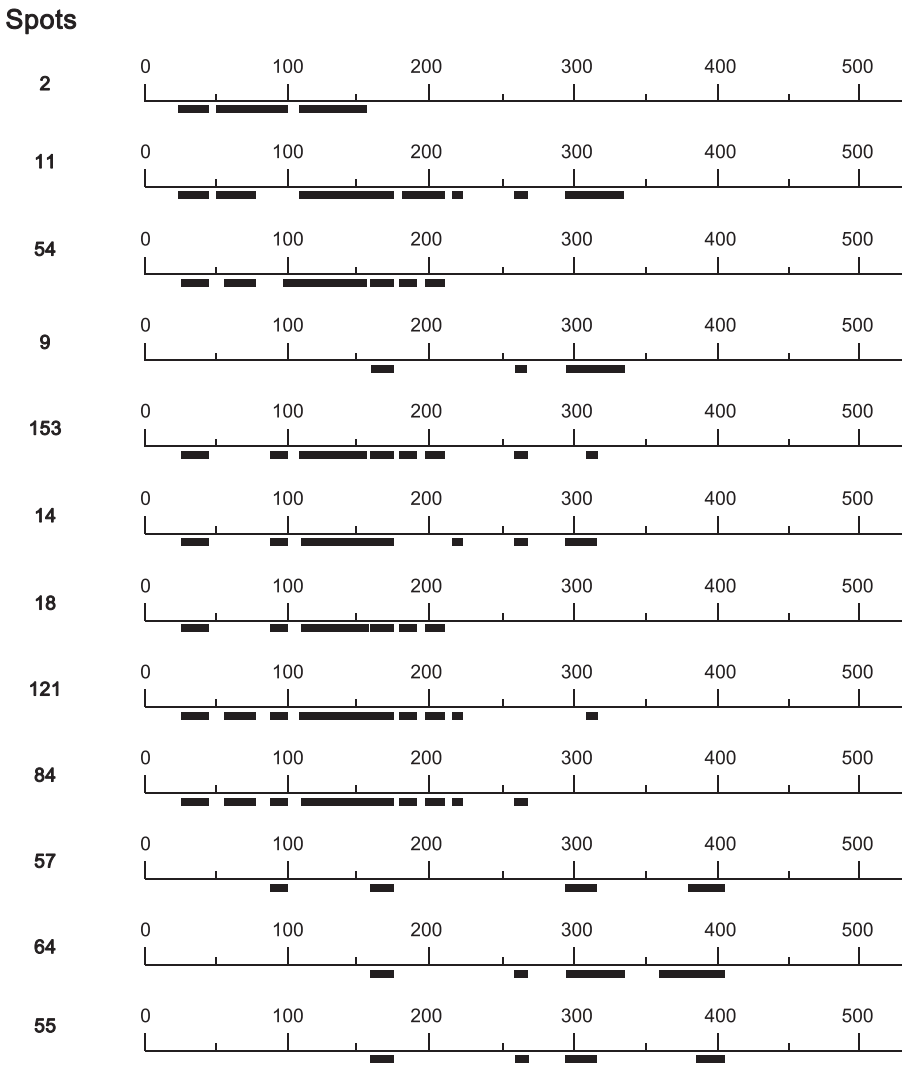

Figure 3 Coverage of α-amylase sequence (511 amino acids) by the peptides identified by MALDI-TOF MS (indicated by black bars) in spots containing amylase. The entire α-amylase sequence includes a signal peptide (amino acids in position 1–15).

et al. 1993; Gonçalves et al. 2010). Also overrepresented in hypersensitive subjects are IgA, which are the most abundant immunoglobulins in whole saliva (Teeuw et al. 2004). A main function of IgA is to limit microbial adhesion as well as antigen penetration in the mucosa (Marcotte and Lavoie 1998). In adults, levels of IgA are transiently raised by an acute stress challenge but downregulated by prolonged stress (Hucklebridge et al. 1998). Levels of IgA can be modified by oral diseases. For example, IgA1 and IgA2 are increased in oral lichen planus, and IgA2 is increased in acute recurrent aphtous ulceration (Sistig et al. 2002) or in generalized aggressive periodontitis (Wu et al. 2009).

At this point, it is interesting to note that all the 3 proteins more abundant in hypersensitive subjects have been described to be jointly overexpressed in patients with periodontitis, and in fact, profiles of such patients are also characterized by a lesser abundance of cystatin SN (Wu et al. 2009; Gonçalves et al. 2010). A targeted study of cystatins had also reported the depletion of cystatin SN in periodontally diseased patients (Baron, Gansky, et al. 1999). In our study and although subjects declared that they were free of oral pathologies, differences of profiles between hyposensitive and hypersensitive subjects may therefore indicate discreet differences in inflammatory statuses. Inflammation often results in taste dysfunction, through interferon-induced taste bud apoptosis and altered gene expression (Wang et al. 2007), but these conclusions apply mainly to acute viral and bacterial infections. For the subjects included in the present study, the inflammation levels would be only modestly different between the 2 groups.

In our view, one of the most striking results was the underrepresentation of full-length (therefore most probably functional) cystatin SN in hypersensitive subjects. One reason which drew our attention to this finding is that it is consistent with another study (Morzel M, Palicki O, Chabanet C, Schwartz C, Nicklaus S, unpublished data), where we found

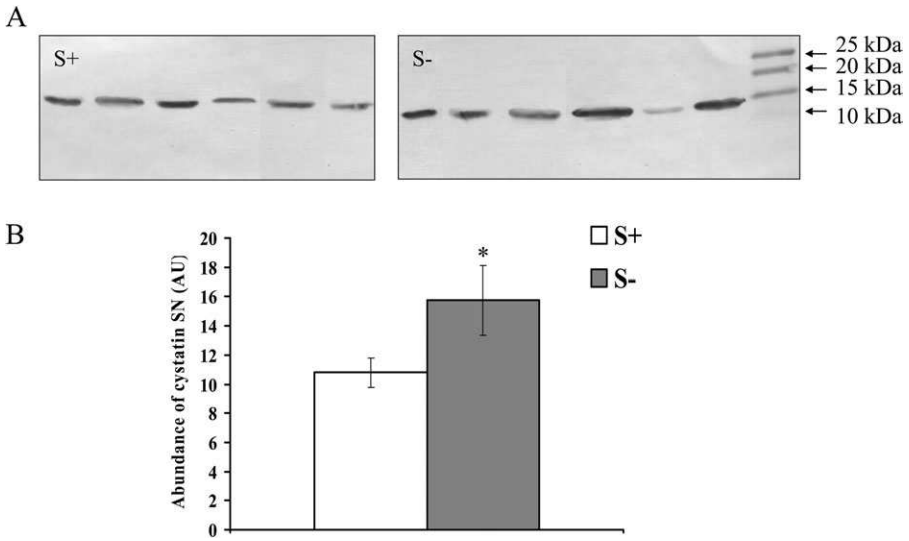

Figure 4 Abundance of cystatin SN in hypersensitive (S+) and hyposensitive (S−) subjects. **(A)** Western blotting patterns of 6 hypersensitive subjects and 6 hyposensitive subjects. **(B)** Semiquantification of immunoreactive bands (mean ± standard error of the mean) in hypersensitive and hyposensitive subjects. *indicates a significant difference ($P < 0.05$).

that salivary cystatins were overexpressed in saliva of infants who readily accepted a bitter solution thereby possibly presenting a reduced sensitivity to bitterness. Cystatin SN is specific to saliva, where its main function is the inhibition of cysteine proteases, for example, the lysozomal cathepsins B, L, and H (Baron, DeCarlo, et al. 1999). Cystatin SN thus plays an important role in the control of in vivo proteolytic events (Dickinson 2002). Here, overrepresentation of amylase fragments in hypersensitive subjects was associated to lower cystatin SN abundance, in accordance with Gonçalves et al. (2010) who attributed enhanced amylase proteolysis in periodontitis patients to cysteine protease activity. Furthermore, although the difference between the 2 groups was not statistically significant, hypersensitive subjects showed higher proteolytic activity values. We therefore propose that proteolytic events probably constitute an important perireceptor factor contributing to the differences of sensory sensitivity. Interestingly, it was previously reported that therapeutics protease inhibitors used, for example, for the treatment of HIV infection modify taste perception and in particular block or blunt the perception of quinine bitterness (Schiffman et al. 1999). The mechanism remained, however, unclear. Here, the proportion of the particular protease inhibitor cystatin SN was also increased in hyposensitive subjects but within physiological values. Again, there is no obvious explanation for this observation, but one can note that amylase, cystatins, and serum albumin are constituents of the oral salivary pellicles (Bradway et al. 1992; Siqueira et al. 2007). Mucosal pellicles are formed by absorption of salivary components onto epithelial cells. They cover all oral surfaces and have various functional properties including control of bacterial colonization, moisture retention, lubrication, and barrier protection (Bradway et al. 1989). Although never formally investigated, one can hypothesize that this barrier function may have an impact on the accessibility of tastants to receptors. A thinner or looser pellicle would then be associated with a facilitated tastant/taste receptor interaction, thereby increased sensitivity. In our case, reduced cystatin abundance and the subsequent enhanced proteolysis of amylase and serum albumin may impair mucosal pellicle formation or stability. Proteolysis of pellicle proteins would in turn favor bacterial colonization and growth (Wickström et al. 2009), which is consistent with the resemblance in salivary profiles between hypersensitive subjects and periodontitis patients.

To summarize, salivary profiles differed between subjects hyposensitive and hypersensitive to the bitterness of caffeine and suggested that enhanced in-mouth proteolysis is a key perireceptor factor associated to a higher gustatory sensitivity to this compound. The perspective of this work is to test this hypothesis on a new larger set of subjects.

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