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Orosensory Stimulation Effects on Human Saliva Proteome

Katharina Lorenz,[†] Matthias Bader,[†] Andreas Klaus,[‡] Walter Weiss,[‡] Angelika Görg,[‡] and Thomas Hofmann[*,†]

[†]Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner Strasse 34, D-85354 Freising, Germany

[‡]Proteomics Group, Technische Universität München, Am Forum 2, D-85350 Freising, Germany.

ABSTRACT: Saliva flow induced by 6-gingerol (pungent), hydroxy-α/β-sanshools (tingling), and citric acid (sour) was measured, and the time-dependent changes in the whole saliva proteome were analyzed by means of 2D-PAGE, followed by tryptic in-gel digestion and MALDI-TOF-MS peptide mass fingerprint analysis. The proteins showing significantly decreased abundance after oral 6-gingerol stimulation were identified as glutathione *S*-transferase P, the heat shock protein β-1, the heat shock 70 kDa protein 1, annexin A1, and cytoplasmic β-actin, whereas prolactin inducible proteins (PIP), short palate, lung and nasal epithelium carcinoma-associated protein 2 (SPLUNC2), zinc-α-2-glycoproteins (Zn-α-GP), and carbonic anhydrase VI (CAVI) were found with increased abundance. As the effects of this study were observed instantaneously upon stimulation, any proteome modulation is very likely to result from the release of proteins from preformed vesicles and not from de novo synthesis. The elevated levels of SPLUNC2, Zn-α-GP, and CAVI might be interpreted to trigger innate protective mechanisms in mucosal immunity and in nonimmune mucosal defense and might play an important role during the initial stage of inflammation.

KEYWORDS: saliva, taste, salivary proteome, pungency, gingerol, hydroxysanshool, Szechuan pepper, oral care

INTRODUCTION

Human saliva consists of a truly complex mixture of electrolytes, small organic molecules, peptides, and proteins secreted from the major submandibular, sublingual, and parotid glands and minor salivary glands, together with the gingival crevicular fluid, a serum transudate present in the gingival crevice surrounding the teeth, desquamated epithelial cells, and microorganisms, as well as food debris.[1] The average daily saliva flow of healthy human subjects is well-known to vary greatly between 0.6 and 1.5 L, and also the composition of the salivary gland secretions have been reported to be affected considerably by different forms of stimulation, time of day, diet, age, gender, and the use of pharmacological drugs, as well as several disease states.[2]

In the past decade, standardization of saliva sample collection, preparation, and handling procedures as well as recent developments in metabolomics and proteomics opened new opportunities to study the molecular composition of human saliva.[3−5] A series of investigations have been undertaken to separate human saliva by means of 2D-PAGE[6−8] and, more recently, comprehensive proteome analyses have been performed for whole human saliva[9−14] and glandular parotid secretion[14] as well as submandibular/sublingual saliva,[15] respectively. The identification of a total of 1.166 proteins in the ductal secretions collected from parotid and submandibular/sublingual glands of healthy subjects recently confirmed the outstanding complexity of human saliva.[16]

Salivary proteins are reported to be involved in calcium-binding, mineralization of dental hard tissues, oral mucosa protection, and interactions with oral microorganisms.[17−19] Among these proteins, proline-rich proteins as well as mucins, adsorbing to the tooth surface in the form of the acquired pellicle,[20] were found to protect teeth from acid-induced demineralization[21] and provide adhesion sites for some oral microorganisms.[19,22] Moreover, salivary proteins are proposed to play a key role in food mastication and digestion processes; for example, mucins contribute to lubrication and protection of oral epithelial surfaces, support food bolus formation, and ease the swallowing process.[17,19,23,24]

Although the knowledge on the putative role of saliva in taste perception is rather limited, changes in the quantity and composition of saliva seem to affect taste sensitivity during the initial processes of taste stimulation. First, taste molecules must pass through the salivary fluid layer to reach the receptor sites. This process may include solubilization of the tastants in saliva, chemical interactions of the tastants with salivary components, and the diffusion and dilution of the tastants in saliva. Some salivary constituents are known to chemically interact with taste molecules; for example, some proline-rich proteins (PRPs) and histatins were reported to complex and precipitate puckering astringent plant polyphenols,[25−29] thus combating gastrointestinal irritation by tannin-rich food at a very early stage. Moreover, the complexation of polyphenols by salivary proteins was observed to diminish the aversive orosensation induced by these molecules by lowering their free concentration.[30] Also, the intensity of a sour taste is known to be reduced by saliva due to the buffering action of salivary bicarbonate.[31,32] In an electrophysiological investigation in rats, smaller HCl responses were observed when the tongue was stimulated with, or adapted to, saliva, $NaHCO_3$, and $KHCO_3$ than when it was adapted to water, NaCl, and KCl, respectively.[33] Through its action on texture and rheology of starch-based food products, the action of salivary α-amylase has been related to the perception of saltiness.[34] Although not yet functionally confirmed, lingual lipase is hypothesized to be involved in oral fat

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perception by hydrolyzing a small fraction of dietary triglycerides to release free fatty acids, which act as agonists for fatty acid responsive G-protein coupled receptors in taste buds.[35−38]

Another effect of saliva on taste sensation is that some salivary constituents can continuously stimulate some taste receptors, resulting in an alteration of taste sensitivity (adaptation to saliva). In other words, responses to incoming dietary tastants are determined by the sensitivity of the saliva-adapted receptors. For example, the taste detection threshold of sodium chloride is slightly above the salivary sodium level, which sets the baseline of salt perception by continuously passing sodium ions through the $\delta\beta\gamma$-ENaC.[39] With respect to salt taste in humans, psychophysical studies have indicated that salivary sodium levels elevate taste thresholds and decrease suprathreshold intensities of NaCl.[40,41] Any changes in the perireceptor environment such as modulation of the saliva composition might affect taste sensitivity of humans.

Moreover, saliva protects the taste receptor from damage brought about by dryness and bacterial infection and from disuse atrophy via a decrease in transport of taste stimuli to the receptor sites. This is a long-term effect of saliva that may be related to taste disorders. In this context, the saliva proteome was proposed to be an indicator of taste disorders. For example, the abundance of Zn-α-2 glycoprotein (Zn-α-GP), prolactin-inducible protein (PIP), and cystatin SN as well as carbonic anhydrase VI (CAVI) in whole saliva was found to be significantly decreased in taste-impaired patients.[42] Among these proteins, CAVI, a zinc metalloenzyme reversibly catalyzing the conversion of carbon dioxide to hydrogen carbonate and free protons, has been related to taste perception due to its proposed implication in the paracrine modulation of taste function and taste receptor cell apoptosis.[43,44]

The rather dynamic changes in saliva proteome composition raised the question as to whether gustatory stimuli themselves are able to induce changes in the abundance of selected saliva proteins. Recently, significantly elevated levels of amylase were found in mouse saliva after stimulation with polyphenol-rich diets.[45] Moreover, oral stimulation with sweet-tasting glucose, umami-tasting monosodium glutamate, and the bitter-tasting agents calcium nitrate, urea, and quinine, respectively, were observed to individually influence the saliva proteome.[46,47]

As in particular sour, pungent, and tingling compounds are well-known to trigger salivation,[48,49] the objectives of the present investigation were to quantitatively measure salivation induced by citric acid (sour), 6-gingerol (pungent), and hydroxysanshools (tingling) and, in addition, *N*-ethyl-*p*-menthan-3-carboxamide (cooling), to investigate the time-dependent changes in the whole saliva proteome by means of 2D-PAGE/MALDI-TOF analysis.

MATERIALS AND METHODS

Chemicals. The following materials were obtained commercially: 2-D quant kit, 2-D cleanup kit (Ettan Sample Preparation Kits and Reagents, Amersham Biosciences, Uppsala, Sweden), PlusOne acrylamide PAGE, PlusOne methylenebisacrylamide, PlusOne *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED), PlusOne ammonium peroxodisulfate (APS) (Amersham Biosciences); 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), thiourea, glycine, trizma (TRIS, 99.9%), iodoacetamide, agarose type I-B, methanol, acetic acid, ethanol, ammonium bicarbonate (Sigma, Steinheim, Germany); glycerol, orthophosphoric acid, acetonitrile (HPLC grade) (Merck, Darmstadt, Germany); ammonium sulfate, sodium carbonate, silver nitrate, sodium thiosulfate, 1,4-dithiothreitol (DTT), urea (Roth, Karlsruhe, Germany); 2,2′-dithiodiethanol (HED), tris(bathophenanthroline disulfonate) ruthenium(II) sodium salt, formaldehyde (37%), butanol, trifluoroacetic acid (TFA) (Fluka, Neu-Ulm, Germany); sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany); precision plus protein unstained standards (Bio-Rad, München, Germany); Pharmalyte 3−10 for IEF (GE Healthcare, München, Germany); trypsin sequencing grade (modified, Roche Diagnostics, Mannheim, Germany); α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Bremen, Germany). Bottled water (Evian) was used for sensory analysis. Citric acid (Merck), 6-gingerol (Sigma), *N*-ethyl-*p*-menthan-3-carboxamide (WS-3; Symrise AG, Holzminden, Germany), and a Szechuan pepper extract (Synthite, Kerala, India), isolated from *Zanthoxylum piperitum* by means of carbon dioxide extraction and containing hydroxy-α-sanshool and hydroxy-β-sanshool in amounts of 2.7 and 9.1% as determined by HPLC-UV−vis, were purchased from commercial suppliers.

Measurement of Stimulated Saliva Flow. Eight volunteers (4 women and 4 men, ages 26−28 years) were recruited from the Technische Universität München, Germany, without any exclusion parameters besides being in good health, nonsmoking, and not under medication. All volunteers, giving informed written consent to the work, appeared fasting at the morning of the experiment and consumed a standardized breakfast composed of toasted bread with jam and coffee. The study was approved by the Ethical Commission of the Technische Universität München. After breakfast, the subjects brushed their teeth and rinsed their mouths with water (100 mL) and, with the exception of water, were asked not to consume any food and smoking articles, respectively, for 1 h prior to the experiment.

Nonstimulated as well as stimulated whole saliva samples were collected in a sensory room at 22 °C using the following standardized procedure: For the sampling of nonstimulated saliva (control), the subjects were asked to rinse their oral cavities with bottled water (8 mL) and, then, to spit out. After 60 s and swallowing, an aliquot (2 mL) of bottled water was taken up in the mouth and, after performing chewing motions for 15 s, the subjects were asked to expectorate in a preweighed 10 mL cup (prestimulus sample).

For the sampling of stimulated saliva, the subjects were asked to rinse their oral cavities with bottled water (8 mL) and, then, to spit out. After 60 s and swallowing, an aliquot (2 mL) of an aqueous stimulus solution of citric acid (3 g/100 mL), 6-gingerol (60 mg/100 mL), WS-3 (104 mg/100 mL), or a Szechuan pepper extract (100 mg/100 mL), respectively, was taken up in the mouth and, after performing chewing motions for 15 s, the subjects were asked to expectorate in a preweighed 10 mL cup (stimulus sample). Thereafter, the subjects were requested not to swallow, but to take up an aliquot (2 mL) of bottled water in the mouth and, after performing chewing motions for 30 s, the subjects were asked to expectorate in a preweighed 10 mL cup (poststimulus sample 1). Without swallowing in between, the latter part of this experiment was repeated three additional times to give poststimulus samples 2−4.

The corresponding samples collected from eight different subjects at three independent days were pooled and stored at −20 °C until use. The amount of saliva was calculated from the weight difference of the expectorated saliva/water mixture and the aliquot (2 g) of the aqueous stimulus solution used to collect the stimulus sample and the aliquot (2 g) of water used to obtain the prestimulus sample (control) as well as poststimulus samples 1−4.

Collection of Saliva Samples for Proteomics Study. For protein analysis, the experiment detailed above was repeated with three volunteers (two women and one man, ages 26−28 years) using aliquots (2 mL) of an aqueous stimulus solution of citric acid (3 g/100 mL), 6-gingerol (60 mg/100 mL), WS-3 (208 mg/100 mL), and the Szechuan pepper extract (100 mg/100 mL), respectively, but only the prestimulus sample, the stimulus sample, and poststimulus samples 1 and 2 were collected to determine the mean saliva flow increase induced by each stimulant.

Quantitation of Protein Content in Saliva Samples. The protein concentration in the pooled saliva samples was determined by using the 2-D Quant Kit (Ettan Sample Preparation Kits and Reagents,

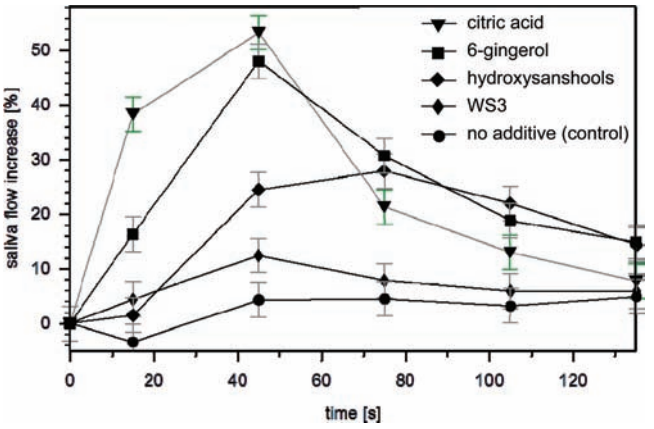

Figure 1. Time course of the saliva flow increase induced by an aqueous stimulus solution (4 mL) containing (■) pungent-tasting 6-gingerol (60 mg/100 mL; 2.1 mmol/L), (▼) sour-tasting citric acid (3 g/100 mL), (◆) a tingling Szechuan pepper extract (100 mg/100 mL), (♦) cooling agent WS-3 (104 mg/100 mL), and (●) no additive (control). The amount of saliva was calculated from the weight difference of the expectorated saliva/water mixture and the aliquot (4 g) of the aqueous stimulus solution used to collect the stimulus sample and the aliquot (4 g) of water used to obtain the prestimulus sample (control) as well as poststimulus samples 1−4, respectively. Least significant difference is visualized (LSD = 6.384).

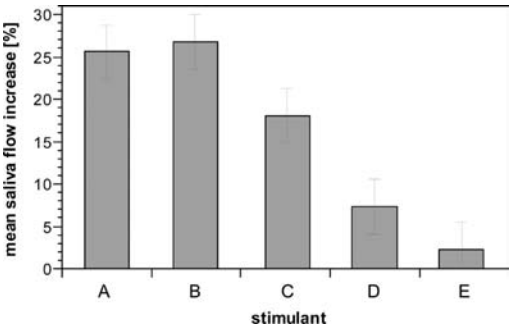

Figure 2. Mean saliva flow increase induced by an aqueous stimulus solution (4 mL) containing (A) pungent-tasting 6-gingerol (60 mg/100 mL; 2.1 mmol/L), (B) sour-tasting citric acid (3 g/100 mL), (C) tingling Szechuan pepper extact (100 mg/100 mL), (D) cooling agent WS-3 (208 mg/100 mL), and (E) no additive (control). The amount of saliva was calculated from the weight difference of the expectorated saliva/water mixture and the aliquot (4 g) of the aqueous stimulus solution used to collect the stimulus sample and the aliquot (4 g) of water used to obtain the prestimulus sample (control) as well as poststimulus samples 1 and 2, respectively. Least significant difference is visualized (LSD = 6.384).

Amersham Biosciences). Aliquots of 25, 35, and 45 μL of saliva were placed in a tube containing the precipitant solution (500 μL) and, after vortexing and incubation at room temperature for 3 min, an aliquot of the coprecipitant solution (500 μL) was added. After vortexing, the tubes were centrifuged (10000g) for 5 min at 4 °C, and the supernatant was separated from the protein pellet, which was then dissolved in a mixture of a copper solution (100 μL) and deionized water (400 μL) upon vortexing. After addition of the working color reagent (1.0 mL), the mixture was left at room temperature for 20 min and was then photometrically analyzed at 480 nm using water as the reference. A standard curve, recorded by plotting the absorbance measured for standard solutions of bovine serum albumin (0, 3.3, 6.7, 10.0, 13.3, and 33.3 μg/mL) against the quantity of protein, showed excellent linearity ($y = -0.0066x + 0.8374$; $R^2 = 0.9991$) within the range of 0−50 μg of protein.

Protein Cleanup. Prior to 2D-PAGE, saliva proteins were treated by means of the 2-D Cleanup Kit (Ettan Sample Preparation Kits and Reagents, Amersham Biosciences) following the supplier's instructions. Aliquots of saliva, corresponding to 100 μg of protein, were mixed with the precipitant (300 μL) and, after vortexing, the mixture was kept on ice for 15 min before the coprecipitant (300 μL) was added. The samples were centrifuged (12000*g*) for 5 min, and the supernatant was separated from the protein pellet and discarded. Another aliquot (40 μL) of coprecipitant was placed on top of the pellet and, after cooling in an ice bath for 5 min, centrifugation for 5 min, and separation of the supernatant, deionized water (25 μL) was added to the pellet and vortexed for 10 s. An aliquot of wash buffer (1 mL) and wash additive (5 μL) was added, the mixture was vortexed for 20 s and, then, incubated at −20 °C for 30 min while vortexing for 20 s once every 10 min. Thereafter, samples were centrifuged (12000*g*) for 5 min, the supernatant was carefully removed and discarded, and the protein pellet was taken up in an aliquot 200 μL of lysis buffer (2 mol/L thiourea, 7 mol/L urea, 4% CHAPS, 2% dithiothreitol, 2% Pharmalyte 3−10) prior to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Two-Dimensional Polyacrylamide Gel Electrophoresis. *Isoelectric Focusing (IEF) in the First Dimension.* Prior to IEF, the IPG dry strips were rehydrated overnight to their original thickness of 0.5 mm using rehydration buffer (500 μL) containing thiourea (2 mol/L), urea (6 mol/L), 1% CHAPS, 0.4% HED, and 0.5% Pharmalyte 3−10 following the instructions of the supplier. IPG 3−11 strips were used for the analytical screening of the saliva proteins, whereas IPG 4−7 strips were used for the MALDI-TOF-MS identification of proteins. After IPG strip rehydration, aliquots (100 μL) of the protein samples dissolved in lysis buffer were applied into disposable silicone rubber cups placed onto the surface of the IPG strip. For analytical 2D-PAGE, an amount of 50 μg of protein was applied and, after electrophoresis, protein spots were subsequently stained with silver nitrate (0.2% in water). For MALDI-TOF-MS analysis of protein spots, aliquots of 150 μg protein were applied on the gels. The latter gels were stained with Coomassie Blue R-250. Isoelectric focusing was performed using Ettan IPGphor II using the following program: 20 °C, 50 μA per strip; step 1, step-n-hold, 1 h, 150 V; step 2, step-n-hold, 1 h, 300 V; step 3, step-n-hold, 2 h, 600 V; step 4, gradient, 1.5 h, 8000 V; step 5, step-n-hold, 25000 Vh (IPG 3−11) or 52000 Vh (IPG 4−7), 8000 V; step 6, step-n-hold, 20 h, 500 V.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) in the Second Dimension. Following a literature protocol,[50] the IPG strips were equilibrated for 15 min with a 100 mL preparation of solution I prepared from 1 g of DTT and 33 mL of an aqueous solution containing 181.66 g/L TRIS, 4 g/L SDS, and 65 mL of hydrochloric acid (25% in water), which was adjusted to pH 8.6 by using NaOH and made up to 100 mL by an aqueous solution of 360 g/L urea, 20 g/L SDS, and 300 g/L glycerol. Thereafter, the IPG strips were incubated for an additional 15 min with a 100 mL preparation of solution II containing 40 g/L iodoacetamide in equilibration buffer. After equilibration, the IPG strips were washed with 2-DE buffer II containing 7.96 g/4 L SDS, 23.2 g/4 L TRIS, and 120 g/4 L glycerol, then placed onto the surface of the SDS-PAGE gels, and covered with a layer of an agarose solution made from agarose (250 mg) and bromophenol blue solution (100 μL) in 50 mL of 2-DE buffer II at 80 °C. The second dimension was done with vertical polyacrylamide gels (T = 13%) on a Ettan DALTwelve System with 2-DE buffer I (7.96 g of SDS, 23.2 g of TRIS, 120 g of glycerol per 8 L) in the lower chamber and 2-DE buffer II in the upper chamber using the following program: 20 °C; 1 h at 5 mA per gel; 1 h at 8 mA per gel; 16 h at 240 mA per gel. The casting vertical SDS gels (16 × 18 cm) were prepared from a mixture of 225 mL of buffer D, 379.8 mL of acrylamide/bisacrylamide solution (300 g of acrylamide, 8 g of bisacrylamide per L), 254.7 mL of Millipore water, 45 g of glycerin, 100 μL of TEMED, and 6.75 mL of 10% APS solution. After the addition of TEMED

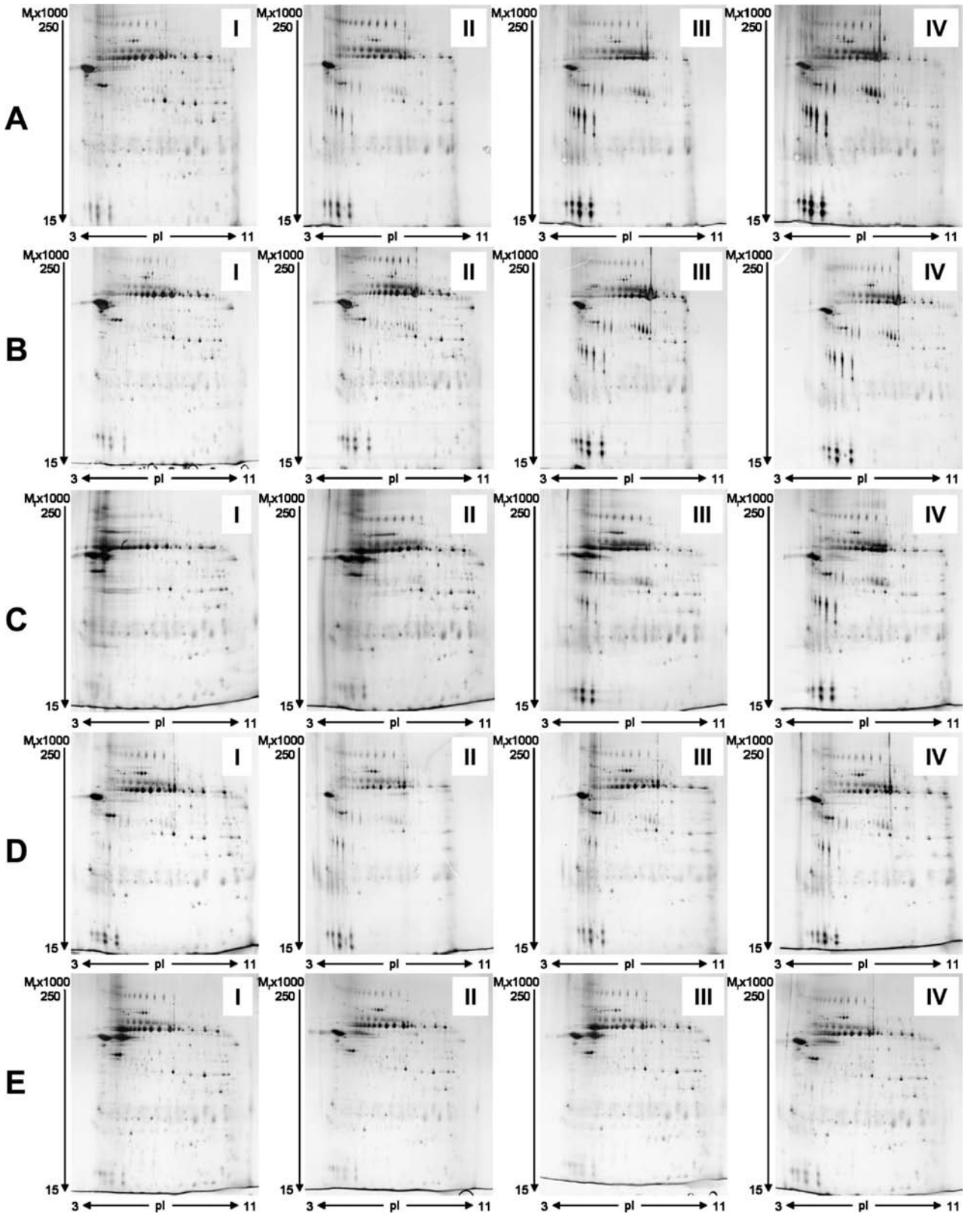

Figure 3. 2D-PAGE mapping (IPG 3−11) of salivary proteins in prestimulus saliva (I) and in stimulus (II) and poststimulus saliva samples 1 (III) and 2 (IV) collected after rinsing the mouth with an aqueous stimulus solution (2 mL) containing (A) pungent-tasting 6-gingerol (60 mg/100 mL; 2.1 mmol/L), (B) sour-tasting citric acid (3 g/100 mL), (C) tingling Szechuan pepper extact (100 mg/100 mL), (D) cooling agent WS-3 (208 mg/100 mL), and (E) no additive (control).

and APS solution, the mixture was stirred again and, then, filled into the casing shortly.

Silver Staining of Protein Spots. Using a literature protocol with some modifications[51] and the IPG strips 3−11, the 2D-PAGE gels were first

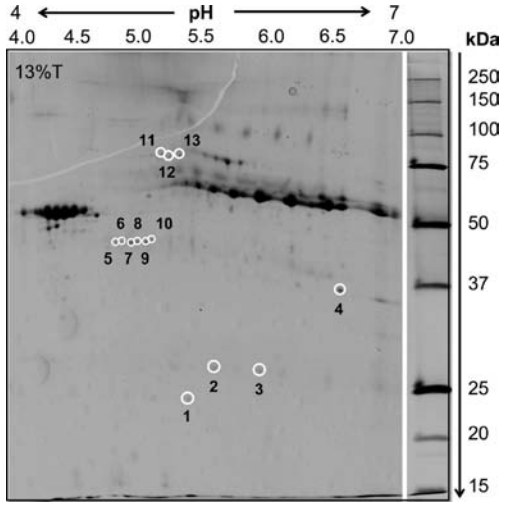

Figure 4. 2D-PAGE gel (IPG 4−7) of prestimulus saliva proteins. Listed spots indicate proteins that decrease in abundance after stimulation with an aqueous solution (2 mL) of 6-gingerol (2.1 mmol/L). Numbering of proteins refers to Table 1.

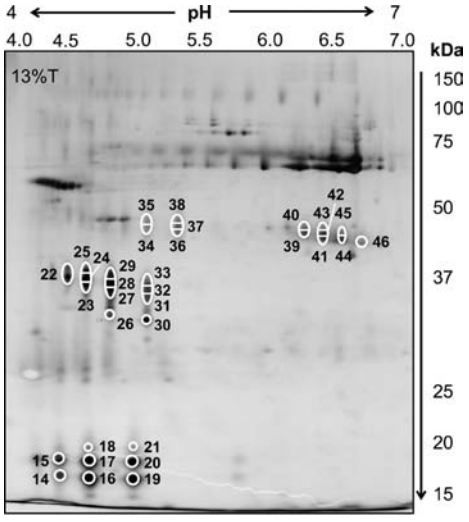

Figure 5. 2D gel (IPG 4−7) of proteins in poststimulus saliva sample 1 after stimulation with an aqueous solution (2 mL) of 6-gingerol (2.1 mmol/L). Protein spots, showing significant increase in abundance when compared to the control, are labeled and numbered according to Table 2.

fixed in a fixing solution (40% ethanol, 10% acetic acid, 50% water) for 4 h and then washed with ethanol/water (30:70, v/v), followed by ethanol/water (15:85, v/v) and water for 20 min each. The washed gels were sensitized for 1 min in a 0.02% aqueous sodium thiosulfate solution and were then washed three times with Millipore water (20 s each). The gels were stained with an aqueous solution of 0.2% silver nitrate and 0.02% formaldehyde (37%), washed three times with water (20 s each), and then treated for 1 min with an aqueous developer solution containing 3% sodium carbonate, 0.05% formaldehyde (37% aqueous solution), and 0.0005% sodium thiosulfate. After staining, the gels were washed once with water and, then, treated with an aqueous solution of glycine (10 g/L) and, finally, washed for 5 min with Millipore water.

Coomassie Brilliant Blue R-250 Staining of Protein Spots. For 2D-PAGE using the IPG strips 4−7, the gels were first treated for 4 h with an aqueous fixing solution containing 40% ethanol and 10% acetic acid, then washed with Millipore water for 30 s, and then treated with the Coomassie Brilliant Blue staining solution for 4 h. The latter staining reagent was prepared by adding G-250 Coomassie Brilliant Blue (1.2 g) to a solution of phosphoric acid (100 mL) and ammonium sulfate (100 g) in Millipore water (200 mL), which was then made up to 800 mL with Millipore water and, after dissolution upon stirring, diluted with methanol (200 mL). The stained gels were equilibrated two times (each for 30 min), first with the aqueous equilibration solution I containing 50 g/L ammonium sulfate and 100 mL/L methanol and, second, with the aqueous equilibration solution II containing 25 g/L ammonium sulfate and 50 mL/L methanol. For destaining, the gels were treated with an aqueous 20% ethanol solution for 5 min prior to MALDI-TOF-MS analysis.

SYPRO Ruby Staining of Protein Spots. Following a literature protocol with some modifications,[52] the 2D gels prepared by using the IPG strips 4−7 were first treated for 4 h with a fixing solution containing 40% ethanol and 10% acetic acid in water, then washed four times (30 min each) with an aqueous 20% ethanol solution, and, finally, incubated for 6 h in a staining solution (1 μmol/L RuBP). The gels were either washed four times for 30 s with Millipore water or destained for 3 h with an aqueous solution of 40% ethanol and 10% acetic acid for a better signal/noise ratio.

Image Analysis of Protein Spots. The images from silver-stained gels were digitized by means of a flatbed scanner (Epson expression 1680 Pro). For spot alignment between 2D gels and differential analysis of protein levels the Progenesis SameSpots software (Molecular Dynamics, Newcastle, U.K.) was used according to the manufacturer's instructions. After matching, background subtraction, and normalization, protein spots were ranked by p value (one-way ANOVA) with maximum fold change based on spot normalized volume. For the prestimulus sample (control) and the stimulus sample, as well as poststimulus samples 1 and 2, only protein spots showing statistically significant spot normalized volumes (p, 0.05) were considered as differentially modulated in the saliva.

Protein Identification by Peptide Mass Fingerprinting. The Coomassie-stained protein spots were manually excised and destained with a water/acetonitrile mixture (70:30, v/v) containing NH_4HCO_3 (50 mmol/L) for 40 min. Thereafter, in-gel digestion was performed overnight with an aqueous solution of trypsin (0.05−0.15 μg/L) and NH_4HCO_3 (10 mmol/L). For mass spectrometric analysis, the peptides were dissolved in a solution of α-cyano-4-hydroxycinnamic acid (CHCA; 10 mg/mL) and aqueous trifluoroacetic acid (TFA; 0.1% in water) in water/acetonitrile (50:50, v/v). MALDI-TOF mass spectra were acquired by Toplab GmbH (Martinsried, Germany) using the 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (AB Sciex) operating in the positive ion reflector mode (detection range m/z 800−4500). The raw spectra were processed with the Data Explorer software (version 4.3; Applied Biosystems). All spectra were externally calibrated using the peptide calibration standard 4700 Cal Mix (AB Sciex). A database search of the monoisotopic masses was performed with MASCOT (Matrix Science, London, U.K.) using the SwissProt (20080115) database with the taxonomy *Homo sapiens*, maximum missed cleavage 1, and peptide mass tolerance ±75 ppm. Additional database searches were performed with ProFound[53] using the NCBI nr (20080407) database and GPSExplorer (Applied Biosystems).

RESULTS AND DISCUSSION

To investigate the influence of sour, pungent, cooling, and tingling compounds on the abundance of proteins in human saliva, first, aqueous solutions of citric acid, ginger's pungent principle 6-gingerol, and the cooling agent *N*-ethyl-*p*-menthan-3-carboxamide, known as WS-3, as well as a tingling Szechuan pepper CO_2 extract were used as oral stimulants to analyze their salivation-enhancing activity over time.

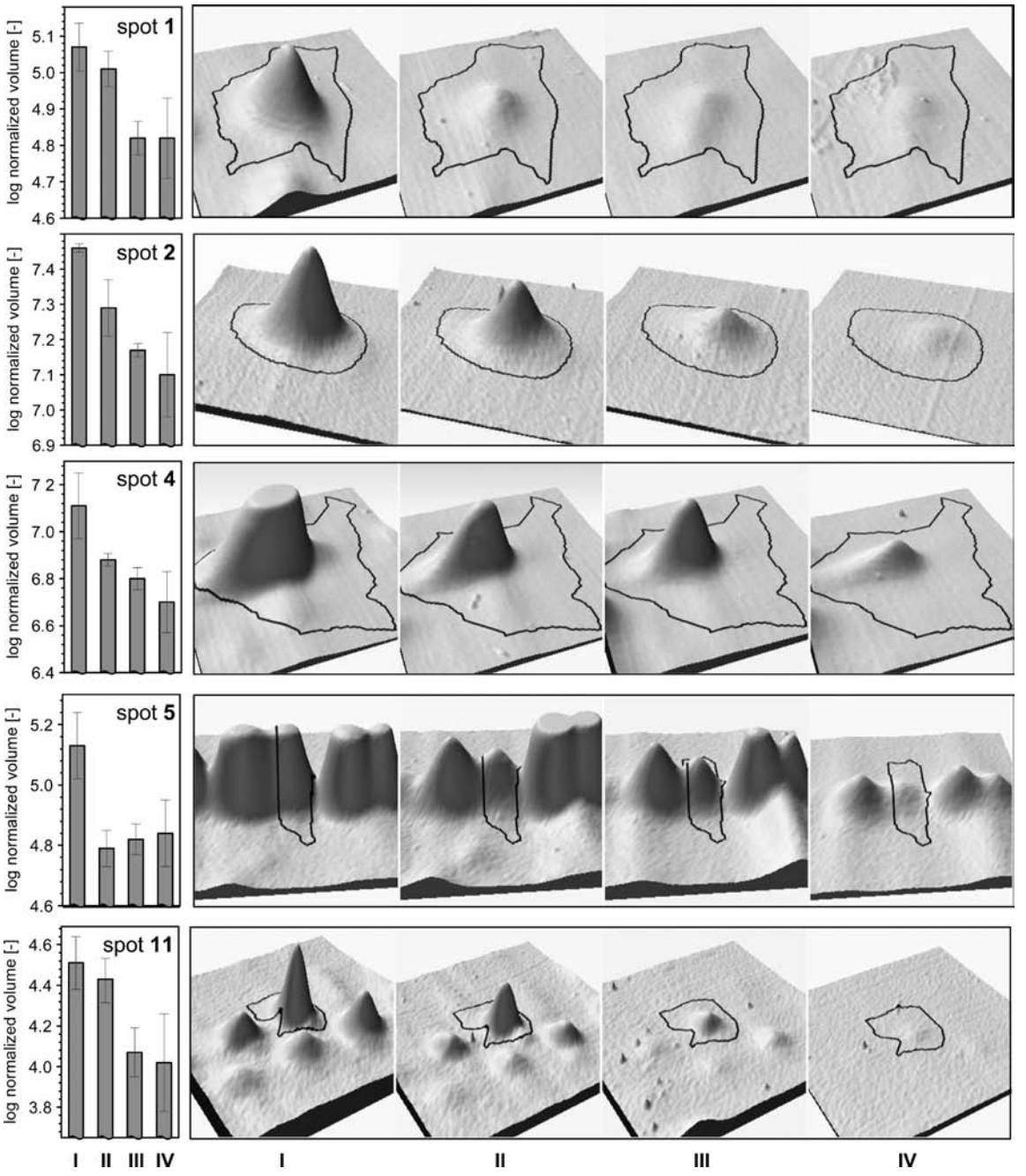

Figure 6. Influence of 6-gingerol (2 mL; 2.1 mmol/L in water) on the abundance of selected salivary proteins 1, 2, 4, 5, and 11 in prestimulus saliva (I), stimulus saliva (II), and poststimulus saliva samples 1 (III) and 2 (IV); zoom of 2D gel (IPG 4−7) after staining with SYPRO Ruby.

Influence of Orosensates on Salivation. After evaluation of appropriate concentrations of each stimulus in preliminary experiments (data not shown), eight healthy volunteers were challenged with aqueous solutions of citric acid (156.0 mmol/L), 6-gingerol (2.1 mmol/L), cooling agent WS-3 (4.9 mmol/L), or Szechuan pepper extracts (1.0 g/L) containing 0.35 and 0.10 mmol/kg hydroxy-α-sanshool and hydroxy-β-sanshool, respectively, and, after performing chewing motions for 15 s, the subjects were asked to expectorate. Thereafter, the subjects were requested not to swallow, but to take up an aliquot of bottled water in the mouth and, after performing chewing motions for 30 s, to expectorate. Without swallowing in between, the later part of this experiment was repeated three additional times to give a total of six collected saliva samples for each person and test stimulus. When compared to a control experiment, which was performed with a blank water sample without any additive, citric acid induced the most rapid effect on saliva production and increased saliva flow by 38 and 53% after only 15 and 45 s, respectively (Figure 1). Also, 6-gingerol and the hydroxy-α/β-sanshool mixture led to a significant enhancement of the saliva flow, but the onset was somewhat lower and the increased saliva flow was more long-lasting when compared to citric acid. In contrast, the saliva flow measured after stimulation with cooling agent WS-3 was only marginally increased when compared to the control (Figure 1).

Influence of Orosensates on Salivary Proteome. To investigate the influence of sour, pungent, cooling, and tingling compounds

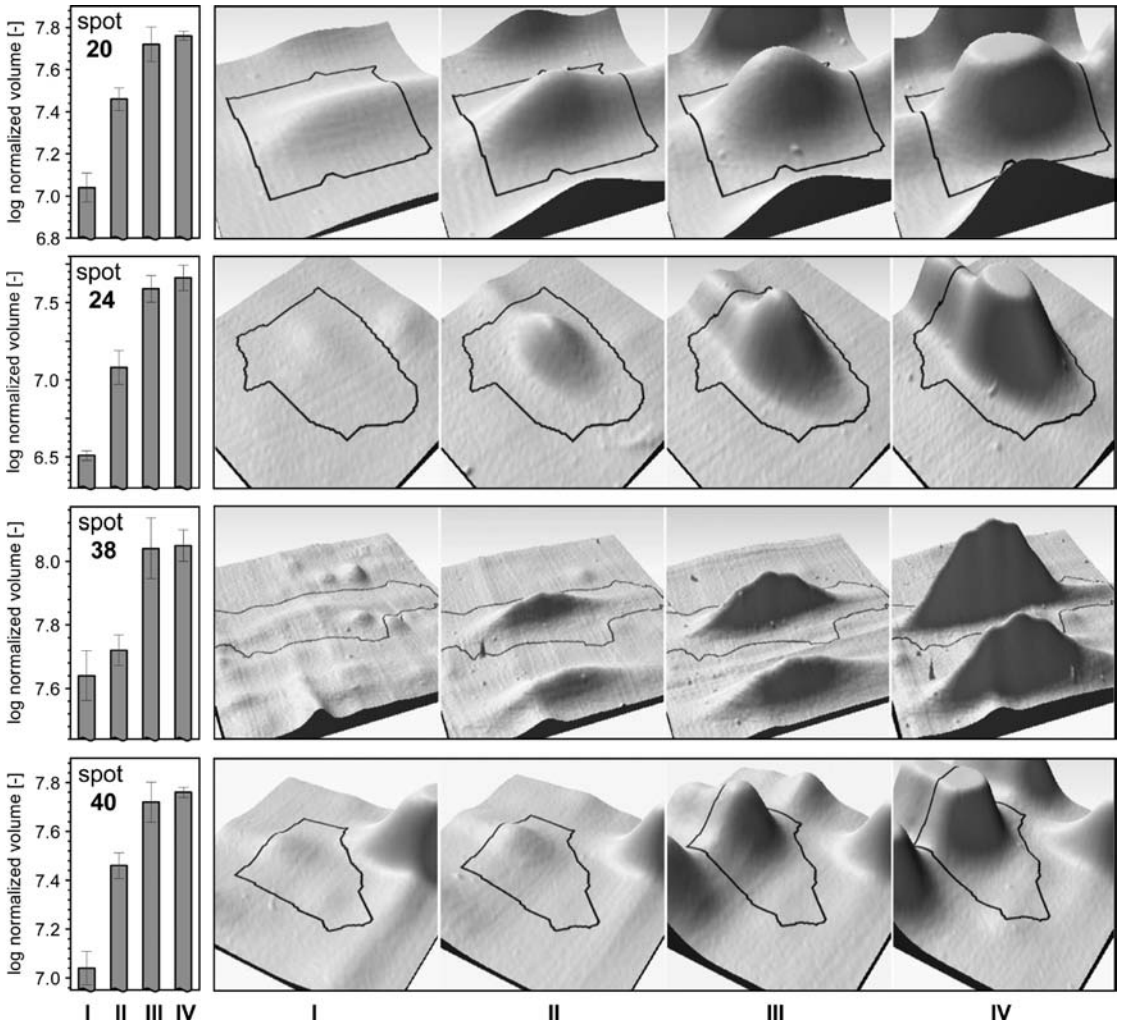

Figure 7. Influence of 6-gingerol (2 mL; 2.1 mmol/L in water) on the abundance of selected salivary proteins 20, 24, 38, and 40 in prestimulus saliva (I), stimulus saliva (II), and poststimulus saliva samples 1 (III) and 2 (IV); zoom of 2D gel (IPG 4−7) after staining with SYPRO Ruby.

on the abundance of proteins in human saliva, saliva samples were again collected from volunteers before and after stimulation with aqueous solutions (2 mL) of citric acid (156 mmol/kg), 6-gingerol (2.05 mmol/kg), cooling agent WS-3 (4.9 mmol/kg), and Szechuan pepper extracts (100 mg/100 mL), containing 0.345 and 0.102 mmol/kg hydroxy-α-sanshool and hydroxy-β-sanshool, respectively, using the standard protocol detailed above. In contrast to the previous experiment, an increased concentration of cooling agent WS-3 (208 mg/100 mL) was used, and only the prestimulus sample, the stimulus sample, and poststimulus samples 1 and 2 were collected to measure the mean saliva flow increase induced by each stimulant (Figure 2).

2D-PAGE mapping (IPG 3−11) of salivary proteins in prestimulus saliva (I) and in stimulus (II) and poststimulus saliva samples 1 (III) and 2 (IV) collected after rinsing the mouth with 6-gingerol (A), citric acid (B), the hydroxy-α/β-sanshool mixture (C), and WS-3 (D), as well as the vehicle (E, control) are displayed in Figure 3. When compared to the control (E), oral stimulation with 6-gingerol (A) induced the strongest impact on the abundance of salivary proteins over the sampling steps I−IV, followed by citric acid (B). Using Progenesis Same Spots software, differences of >1.5-fold increase or decrease in protein abundance between nonstimulated and stimulated saliva, a p value of <0.05 (one-way ANOVA), as well as a power value of >0.8 (multiple-comparison test) was considered to be significant. The relative abundance of totals of 56 (47 increasing, 9 decreasing) and 75 (46 increasing, 29 decreasing) spots was modified upon stimulation by 6-gingerol and citric acid, respectively. Also, the Szechuan pepper extract (C), followed by cooling agent WS-3 (D) induced some changes in the abundance of distinct salivary proteins (Figure 3); for example, the relative abundance of totals of 23 (19 increasing, 4 decreasing) and 9 (6 increasing, 3 decreasing) spots was modified. In comparison, gels I−IV obtained from saliva collected after blank stimulation (E) did not show any significant differences (Figure 3). As 6-gingerol exposure induced the highest number of proteins with increased abundance, the following identification studies were focused on saliva samples collected after 6-gingerol stimulation.

Modulation of Salivary Protein Abundance upon Oral 6-Gingerol Stimulation. As the most proteins showing stimulant-induced increase in abundance (Figure 3) were located in the pH range between 4 and 7, laboratory-made linear IPG strips 4−7 were used to increase resolution in the following 2D-PAGE separations. The 2D gels obtained from prestimulus saliva (I), stimulus (II) and poststimulus saliva samples 1 (III) and 2 (IV) after oral 6-gingerol exposure were stained by SYPRO Ruby for

Table 1. Identified Saliva Proteins Showing Significantly Decreased Abundance after Oral Stimulation with an Aqueous Solution (2 mL) of 6-Gingerol (2.1 mmol/L)

protein (spot no.)[a]	factor of abundance increase	*p* value	coverage[b] (%)	pI[c]	MW[d] (kDa)
GSTP1_HUMAN;[a] glutathione *S*-transferase P (EC 2.5.1.18)					
1	1.8	$1.1e^{-004}$	44	6.0	23.58
HSPB1_HUMAN;[a] heat shock protein β-1, stress-response protein (SRP27)					
2	2.2	$3.0e^{-005}$	36	6.0	22.86
3	2.1	$8.1e^{-005}$	38	6.0	22.83
ANXA1_HUMAN;[a] ANXA1 protein, annexin A1					
4	2.3	$8.5e^{-006}$	43	6.6	38.92
ACTB_HUMAN;[a] actin, cytoplasmic 1 (β-actin)					
5	2.0	$1.9e^{-004}$	27	5.3	42.05
6	2.2	$9.8e^{-005}$	27	5.3	42.05
7	3.7	$0.3e^{-002}$	27	5.3	42.05
8	2.5	$0.3e^{-002}$	27	5.3	42.05
9	2.3	$0.4e^{-002}$	27	5.3	42.05
10	2.3	$0.3e^{-002}$	28	5.3	42.05
HSP71_HUMAN;[a] heat shock 70 kDa protein 1 (HSPA1A)					
11	3.0	$4.2e^{-004}$	18	5.5	70.29
12	2.5	$3.2e^{-003}$	19	5.5	70.29
13	2.7	$7.1e^{-004}$	18	5.5	70.29

[a] Identified proteins using the SwissProt (20080115) database with the taxonomy *Homo sapiens*. Numbering of protein spot refers to Figure 4. [b] Minimum sequence coverage. [c] Isoelectric point. [d] Molecular weight.

quantitative analysis using Progenesis SameSpots software. In addition, gels were stained with Coomassie Blue, followed by picking of individual spots and destaining prior to MALDI-TOF/TOF-MS, respectively. The relative abundance of 46 spots was modulated upon 6-gingerol exposure, among which 13 spots (no. 1−13) decreased in abundance (Figure 4) and 33 spots (no. 14−46) showed increased abundance (Figure 5). Among the group of decreasing spots, the modulation of the abundance of salivary proteins 1, 2, 4, 5, and 11 is exemplified by their decline from the prestimulus saliva (I) over the stimulus saliva (II) to the poststimulus saliva samples 1 (III) and 2 (IV) in Figure 6. In comparison, the increasing relative abundance of selected protein spots 20, 24, 38, and 40 is depicted in Figure 7.

The total of 46 spots were picked from the 2D gels and, after destaining and tryptic in-gel digestion, were analyzed by MALDI-TOF-MS peptide mass fingerprint analysis and identified as the proteins summarized in Table 1 (decreasing proteins) and Table 2 (increasing proteins).

The proteins showing decreasing abundance after oral 6-gingerol stimulation may be devided into four major groups: (i) glutathione *S*-transferase P (spot 1), (ii) a group of chaperones, namely, heat shock protein β-1 (HSPB1, spots 2 and 3) and heat shock 70 kDa protein 1 (HSPA1A, spots 11−13), (iii) annexin A1 (spot 11), and (iv) cytoplasmic β-actin (spots 5−10), respectively (Table 1).

Glutathione *S*-transferase P (GSTP, spot 1 in Figure 4) is known to catalyze the conjugation of glutathione to a wide range of hydrophobic electrophiles and, by doing so, plays a key role in the detoxification of xenobiotica such as lipid peroxidation products and reactive oxygen species (ROS), respectively.[54,55] This enzyme was found to be overexpressed in several tumor varieties and seems to protect tumor cells from apoptosis elicited by cytotoxic agents.[54,56] Moreover, GSTP1 was reported to inhibit lipopolysaccharide-inducible release of pro-inflammatory factors in macrophages.[57]

Protein spots 2 and 3 as well as 11−13 were identified as heat shock protein β-1 (HSPB1) and heat shock 70 kDa protein 1 (HSPA1A), respectively (Figure 4). The expression of these proteins is reported to be induced as a response to environmental stress such as oxidative stress or heat shock and is involved in thermotolerance and other cell functions such as cell growth and differentiation.[58−60] Moreover, HSPB1 was found to be expressed in response to estrogen stimulation in breast cancer (MCF-7) cells.[58] These heat shock proteins are well-known to function as chaperones during protein folding, assembly, and membrane translocation and to prevent aggregation of damaged polypeptide chains in cells.[59]

Protein spot 4 (Figure 4) was identified as annexin A1, a cytosolic calcium-binding protein that has been reported to bind to cellular membranes in the presence of calcium ions.[61] Although its function has not been yet unequivocally defined, it appears to be involved in vesicular trafficking and fusion.[62] Interestingly, annexin A1 has been suggested to exhibit anti-inflammatory properties in vitro[63] and to play a key role in the innate immune defense.[64] It has been induced in lung tumors[65] and during exposure to smokeless tobacco in hamster cheek pouch epithelium cells.[66] Most interestingly, oral stimulation with umami, bitter, and sour taste compounds was reported to induce a relative increase in annexin A1 abundance in human

Table 2. Identified Saliva Proteins Showing Significantly Increased Abundance after Oral Stimulation with an Aqueous Solution (2 mL) of 6-Gingerol (2.1 mmol/L)

protein (spot no.)[a]	factor of abundance increase	*p* value	coverage[b] (%)	pI[c]	MW[d] (kDa)
PIP_HUMAN;[a] prolactin-induced protein (PRP)					
14	3.0	$4.0e^{-007}$	52	8.3	16.85
15	2.8	$1.2e^{-005}$	52	8.3	16.85
16	3.3	$1.5e^{-006}$	55	8.3	16.85
17	3.7	$1.8e^{-006}$	67	8.3	16.85
18	3.5	$1.8e^{-005}$	63	8.3	16.85
19	4.3	$5.5e^{-006}$	67	8.3	16.85
20	5.2	$7.6e^{-008}$	63	8.3	16.85
21	5.2	$2.2e^{-008}$	63	8.3	16.85
SPLC2_HUMAN;[a] short palate, lung and nasal epithelium carcinoma-associated protein 2 (SPLUNC2), parotid secretory protein (PSP)					
22	12.1	$1.7e^{-005}$	30	5.4	27.17
23	4.9	$1.1e^{-004}$	28	5.4	27.17
24	9.0	$3.3e^{-007}$	32	5.4	27.17
25	9.1	$6.0e^{-008}$	30	5.4	27.17
26	6.9	$1.9e^{-008}$	30	5.4	27.17
27	9.4	$2.4e^{-004}$	26	5.4	27.17
28	12.1	$1.7e^{-005}$	26	5.4	27.17
29	9.8	$1.2e^{-006}$	30	5.4	27.17
30	6.1	$1.7e^{-005}$	33	5.4	27.17
31	7.8	$6.5e^{-006}$	28	5.4	27.17
32	10.9	$1.9e^{-005}$	32	5.4	27.17
33	8.7	$9.6e^{-006}$	30	5.4	27.17
ZA2G_HUMAN;[a] zinc-α-2-glycoprotein (Zn-α-2-GP)					
34	1.7	$1.0e^{-002}$	28	5.6	34.08
35	3.1	$0.2e^{-002}$	31	5.6	34.08
36	2.5	$2.5e^{-006}$	31	5.6	34.08
37	4.6	$1.1e^{-005}$	35	5.6	34.08
38	3.7	$5.8e^{-006}$	31	5.6	34.08
CAH6_HUMAN;[a] carbonic anhydrase VI (CAVI)					
39	4.4	$8.9e^{-005}$	25	6.5	35.46
40	4.2	$2.4e^{-004}$	34	6.5	35.46
41	4.6	$1.2e^{-004}$	26	6.5	35.46
42	5.8	$5.1e^{-005}$	31	6.5	35.46
43	5.6	$1.0e^{-003}$	37	6.5	35.46
44	5.7	$3.8e^{-005}$	25	6.5	35.46
45	5.4	$2.3e^{-004}$	25	6.5	35.46
46	5.1	$7.4e^{-005}$	25	6.5	35.46

[a] Identified proteins using the SwissProt (20080115) database with the taxonomy *Homo sapiens*. Numbering of protein spot refers to Figure 5. [b] Minimum sequence coverage. [c] Isoelectric point. [d] Molecular weight.

whole saliva,[46] which is in contradiction to its decreased abundance found after oral 6-gingerol exposure in the present study. Therefore, it might be concluded that the modulation of the salivary proteome is strongly dependent on the type of stimulant.

β-Actin, assigned to protein spots 5−10 (Figure 4), belongs to a highly conserved group of proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. Whereas the α-actins are found as major constituents of the contractile apparatus in muscle tissues of vertebrates, β- and γ-actins are important components of the cytoskeleton of most cell types and function as mediators of internal cell motility.[67−69]

The proteins showing increased abundance after oral 6-gingerol stimulation may be devided into four categories: (i) prolactin-inducible proteins (PIP; spots 14−21), (ii) short palate, lung, and nasal epithelium carcinoma-associated protein 2 (SPLUNC2; spots

22−33), (iii) zinc-α-2-glycoproteins (spots 34−38), and (iv) CAVI (spots 39−46), respectively (Table 2).

Among the protein spots showing increased abundance after oral 6-gingerol stimulation, proteins 14−21 (Figure 5) were identified as prolactin-induced proteins (PIP), which are well-known to exist in many isoforms differing in the glycosylation and phosphorylation status.[10,11,70] These secretory glycoproteins are secreted into human saliva and show high binding affinity toward hydroxyapatite, bacteria, actin, fibronectin, fibrinogen, and keratin, as well as a CD4 receptor, which modulates the immune response during viral infections.[14,71] Being well in line with its hydroxyapatite binding activity, PIP was found as one of the major proteins of the enamel-pellicle, thus suggesting that PIP is involved in the formation of the enamel and has a protective role in the salivary system.[71] Among the bacteria of the human oral flora, PIPs showing highest binding affinities for *Streptococcus* species[72] were observed to inhibit bacterial growth.[71,73,74] The ability of PIP to bind to oral bacteria, keratin, and CD4, as well as the interleukin-induced up-regulation of PIP gene expression found in human breast carcinoma cell lines, suggests that it may play a role in mucosal immunity or in nonimmune mucosal defense.[73,75] These functions would be consistent with the presence of PIP in saliva, tears, submucosal glands of the bronchi, and apocrine glands of the skin.[75] Moreover, PIP expression was reported to be increased by prolactin and steroids and also influenced by interleukins, thus suggesting that the levels of PIP in saliva might reflect activities in the neuroendocrine and neuroimmune systems and raising the possibility that salivary PIP may be a candidate biological stress marker.[10,76]

Protein spots 22−33 (Figure 5) were identified as short palate, lung, and nasal epithelium carcinoma-associated protein 2 (SPLUNC2), also known as parotid secretory protein (PSP). Like the PIPs, this lipid-binding protein is reported to exhibit antibacterial as well as anti-inflammatory activities.[77−79] This protein was identified as a receptor molecule for the SabA adhesions of the gastric pathogen *Helicobacter pylori*.[80] Interestingly, the SPLUNC2 shows high sequence similarity to the family of bactericidal-increasing proteins (BPI), which are able to bind to lipopolysaccharides.[81] Gorr et al.[82] succeeded in preparing the bacteria-agglutinating peptide GL-13 from PSP and demonstrated that this class of antimicrobial peptides does not directly kill bacteria but reduces bacterial adhesion and promotes agglutination, thus leading to increased clearance by host phagocytic cells.[83] These data clearly suggest that PSP may have a role in the innate defense system at the gingival epithelial surface and play an important role during the initial stage of inflammation.[78]

Protein spots 34−38 (Figure 5) were assigned as Zn-α-2-GP, a soluble zinc-containing glycoprotein found in several body fluids and ductal secretions such as serum, sweat, cerebrosphinal fluid, seminal plasma, urine, and saliva, respectively.[83] Similar to lipolytic hormones, this protein triggers fat degradation in adipocytes and induces the extensive fat loss associated with some advanced cancers.[83] On the basis of the amino acid sequence, Zn-α-2-GP is related to the antigens of the major histocompatibility (MHC) complex and is, therefore, postulated to play a role in immune response.[84] When compared to healthy subjects, saliva collected from patients with taste disorders showed decreased abundance of Zn-α-2-glycoprotein, PIP, and CAVI.[42] Furthermore, besides PIP and CAVI, the Zn-α-2-GP is reported as a receptor protein binding to the SabA adhesins of *H. pylori*.[80]

CAVI, assigned to protein spots 39−46 (Figure 5), is reported to be secreted into saliva by serous cells of the parotid and submandibular gland and also locally by the von Ebner's glands.[43] By catalyzing the reversible conversion of carbon dioxide to bicarbonate and protons, this enzyme is involved in pH regulation mechanisms and is reported to ensure protection of the upper alimentary tract from acid injury and of the dental enamel and is, therefore, considered an anticaries protein in saliva.[85−87] This is strengthened by the observation that low salivary CAVI concentrations are associated with increased caries prevalence, particularly in subjects with neglected oral hygiene.[86] Moreover, a clinical disorder formulated as a syndrome of hyposmia (decreased smell acuity), hypogeusia (decreased taste acuity), dysosmia (distorted smell function), and dysgeusia (distorted taste function) is reported to correlate with decreased secretion of parotid saliva CAVI and associated pathological changes in taste bud anatomy.[88] As CAVI is found in humans exclusively in parotid saliva and has been associated with growth and development of taste buds, inhibited CAVI synthesis is suggested to be associated with the development of taste bud abnormalities and, furthermore, by the loss of taste function.[43] CAVI is secreted by the van Ebner's glands directly into the bottom of the trenches surrounding the circumvallate and foliate taste papillae. From a flavor perception point of view, CAVI has long been recognized as playing a central role in taste function[89] and seems to be implicated in the paracrine modulation of taste function and taste receptor cell apoptosis.[43]

In conclusion, the increased salivary abundance of PIP, SPLUNC2, and CAVI after exposure to pungent 6-gingerol seems to trigger innate protective mechanisms in mucosal immunity and in nonimmune mucosal defense and might play an important role during the initial stage of inflammation. As the effects of this study were observed instantaneously upon stimulation, any proteome modulation is very likely to result from the release of proteins from preformed vesicles and not from de novo synthesis, which in pancreatic exocrine cells was shown to take about 30 min to pass from the rough endoplasmatic reticulum to the condensing vacuoles.[90]

On the basis of the data of the present investigation, future studies are needed to answer the question as to how the composition of the salivary proteome as well as our oral defense system is affected by the plethora of taste molecules present in our daily diet.[91] As the concentrations of salivary components of different individuals vary greatly,[47] the individual's taste sensitivity may also fluctuate widely in response to these variations. Therefore, future studies need to be targeted toward a better understanding of the relationship between salivary constituents such as proteins, amino acids, and electrolytes and the individual's taste sensitivity.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49-8161/71-2902. Fax: +49-8161/71-2949. E-mail: thomas.hofmann@tum.de.

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