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Discovery of Salt Taste Enhancing Arginyl Dipeptides in Protein Digests and Fermented Fish Sauces by Means of a Sensomics Approach

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S Supporting Information

ABSTRACT: As enzymatic digests of fish proteins were recently reported to enhance salt taste, the fish protein protamine was digested by chymotrypsin and trypsin and subsequently screened for candidate salt taste modulating (STM) peptides. To achieve this, first, a two-step sensory assay was developed and demonstrated to be a rather suitable tool for the detection of salt taste enhancers and the "quantitation" of their salt taste enhancing activity on the basis of isointensities with reference solutions. By means of activity-guided fractionation using ultrafiltration, gel permeation chromatography, and hydrophilic liquid interaction chromatography in combination with the sensory assay for STM activity assessment, a series of arginyl dipeptides, with RP, RA, AR, RG, RS, RV, VR, and RM being the most active, as well as L-arginine were found as salt taste enhancing molecules in fish protamine digests. For the first time, HPLC-MS/MS analysis on a PFP and a HILIC stationary phase, respectively, enabled the quantitative analysis of the arginyl peptides in a series of commercial and laboratory-made protein hydrolysates as well as fermented fish sauces.

KEYWORDS: sensomics, salt taste, taste enhancer, arginine, peptides, protein hydrolysates

INTRODUCTION

As food choice crucially depends on taste quality, the content of salt (NaCl) directly determines sensory acceptance of savory foods, such as soups, sauces, dressings, ready-to-eat meals, and snacks, but also bakery products including sweet cookies and cakes. Epidemiological, intervention, and migration studies and animal experimentation showed evidence for a direct correlation of salt intake and hypertension, cardiovascular diseases, and diet-associated diseases.[1−7] Furthermore, intervention studies also showed that reduced dietary sodium intake lowered blood pressure significantly in both normotensive and hypertensive people[8] and decreased the incidence of hypertension.[5]

Currently, the daily sodium intake is twice as high as the amount of 2 g of sodium/day recommended by the World Health Organization.[9,10] Despite this recommendation, significant sodium reduction in foods is hampered because it results in poor savory taste and increased bitterness, leading to less tasty products.[11] This problem could be overcome by salt taste modulators which, although being tasteless on their own, would be able to bring back the perceived saltiness of sodium-reduced foods to levels expected for foods containing normal sodium levels.

Salt taste seems to be mediated by at least two pathways: one is amiloride sensitive and selectively responsive to sodium, the other is nonselective for a wide range of mineral ions and was shown to be modulated in rodents by *N*-cetylpyridinium chloride.[12−18] Whereas the epithelial sodium channel (ENaC) was found to play the key role in murine salt taste perception and was suggested to be a candidate sodium sensor involved in human salt perception,[19−21] its precise role has not been established yet. Recordings from rodent Chorda tympani (CT) taste nerve upon oral stimulation with NaCl demonstrate that the amiloride-sensitive responses account only for 60−70% of the entire salt-induced signal.[22,23] The remaining 30−40% of the CT signal appear to be mediated by cetylpyridinium-sensitive channels that have not been definitely identified.[22,23]

Due to the current uncertainty about the true salt taste receptors, cell-based assays have not shown any success in the identification of salt taste enhancers. On the other hand, a vast number of empirical studies evaluated food additives for their ability to replace salt or modulate salt taste. The known salt replacers, potassium and ammonium chloride, exhibit severe off-tastes that restrict their use in foods.[24,25] Also, the salt taste enhancing amino acids L-lysine and L-arginine[26,27] and the disaccharide trehalose[28] are associated with fishy and amine-like off-flavors or have too little sensory impact, preventing their use in foods. Moreover, the salt-like taste or saltiness enhancement of a series of synthetic molecules including the peptides L-ornithyl taurine and L-ornithyl-β-alanine has been disputed.[29−32] In addition, bretylium tosylate,[33] TRPV1 agonists,[34,35] *N*-alkylamides,[36] and

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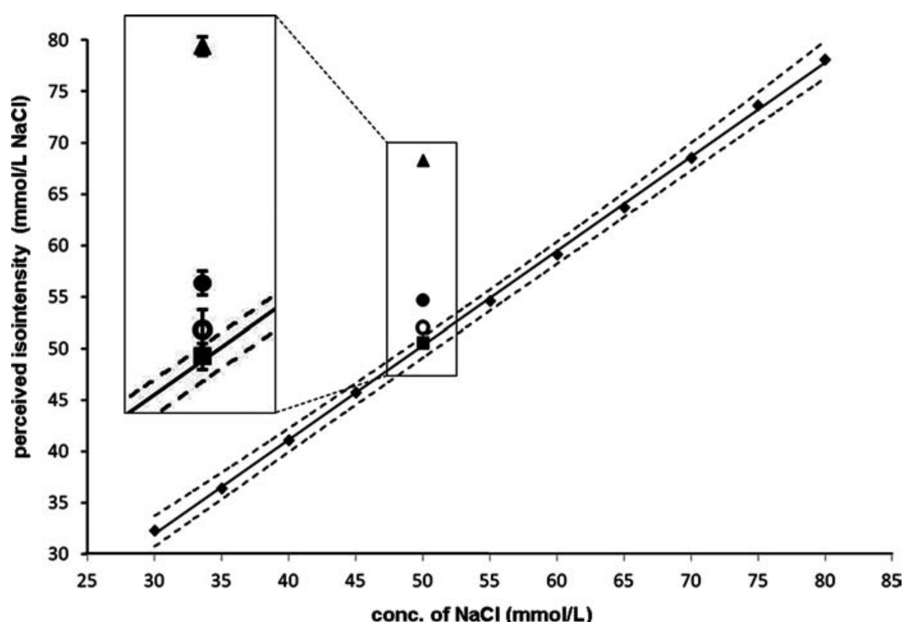

Figure 1. Salt taste modulatory (STM) activity of 0.1% (■), 0.3% (○), 1.0% (●), and 3.0% (▲) of an enzymatic protamine digest in model broth containing a total of 50.0 mmol/L NaCl . The diamonds (◆) indicate the perceived salt intensity of the NaCl reference solutions, and dashed lines display the 95% confidence interval. The error bars in the highlighted excerpt represent the standard deviation of the isointensity measurements.

Maillard reaction products including argpyridaine[37] were found to modulate sensitivity of saltiness in human subjects. However, the synthesis and challenging purification of argpyridaine limit its applicability in food products.

For centuries, fermentation has been used to increase the savory taste and enhance the palatability of foods. Starting with the use of fermented fish sauce in ancient Greece and Rome, it is common practice today in Asia to make savory dishes more delicious with soy sauce. In addition, in Japan seaweed and bonito are added to make soups tastier. In France meat and fish are combined with vegetables to produce more flavorful stocks, whereas in Italy Parmesan cheese, anchovies, and fully ripened tomatoes are cooked with seafood to produce a much tastier dish.[38−41] For most of these taste-enhancing products, L-glutamate is claimed to be the key ingredient,[42] but recent studies on Gouda cheese undoubtedly identified the amino acid L-arginine as a functional salt taste enhancer along with a series of γ-glutamyl dipeptides as kokumi enhancers produced enzymatically upon cheese maturation.[43,44] In addition, peptides from Vietnamese fish sauce were found to influence the perception of sweet, acidic, bitter, and umami taste if NaCl was present.[45] Moreover, enzymatic digests of fish proteins were recently reported to enhance salt taste, although the active principles have not yet been identified.[46]

Aiming at the discovery of the taste-active principles in foods, the so-called sensomics approach has been introduced and further developed over recent years.[42] The combination of liquid chromatographic separation techniques and analytical sensory tools enabled the successful identification of key taste-modifying molecules in red wine,[47] matured Gouda cheese,[43,44] dried morel mushrooms,[48] beef broth,[49] edible beans,[50] chicken broth,[51] stewed beef juice,[52] yeast autolysates,[53] and thermally processed avocado.[54]

In the present report we use the sensomics approach to identify candidate salt taste modulating (STM) peptides in an enzymatic digest of fish protein. Moreover, we evaluate the sensory impact of the identified STM peptides and validate their occurrence in commercial fish sauces.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: sodium L-glutamate monohydrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, acetone, hydrochloric acid, trifluoroacetic acid, formic acid (Merck KGA, Darmstadt, Germany); sodium chloride, maltodextrin (DE 16.5−19.5), protamine sulfate, lysozyme from chicken egg white, α-chymotrypsin from bovine pancreas (type II), trypsin from porcine pancreas, ammonium acetate, L-arginine (Sigma-Aldrich, Steinheim, Germany); yeast extract Gistex XII LS (DSM Food Specialties Savory Ingredients, Delft, The Netherlands); dipeptides RT, TR, SR, RC, CR, RN, DR, QR, ER, RH, HR, YR (EZBiolab Inc., Carmel, IN); and dipeptides RG, GR, RA, AR, RS, RP, PR, RV, VR, RL, LR, RI, IR, RD, RK, KR, RQ, RE, RM, MR, RF, FR, RR, RY, RW, WR (Bachem, Bubendorf, Switzerland). The following protein hydrolysates were obtained commercially: rice protein hydrolysate Remypro N80+ (BENEO-Remy NV, Leuven-Wijgmaal, Belgium), pea protein hydrolysate Pisane C9 (Cosucra, Warcoing, Belgium), pea protein hydrolysates Nutrallys F85 F and F85 M (Roquette Frères, Lestrem Cedex, France), fava bean protein hydrolysate CPX 55 (GEMEF Industries, Paris, France), soy protein hydrolysate Kerrypro 900 (Kerry Group plc, Tralee, Ireland), soy protein hydrolysate SUPRO 670 IP (Welding, Hamburg, Germany), soybean hydrolysates SOLPRO 958 and SOLPRO 940 (Solbar, Ashdod, Israel), soybean hydrolysates SUPRO PLUS 651 IP and SUPRO XT 219D IP (Solae Europe S.A., Le Grand-Saconnex, Switzerland), and soy protein hydrolysate S-005 (Paninkret, Pinneberg, Germany); and gluten hydrolysate from maize, peptone from soybean, casein hydrolysate (Aldrich, Steinheim, Germany). Samples of Lien Ying fish sauce (Rila Feinkost-Importe, Stemwede-Levern, Germany), squid brand fish sauce (Thai Fishsauce Factory Co. Ltd., Bangkok, Thailand), oyster sauce (Tra Maekrua, Samutprakarn, Thailand), fish sauce oyster brand (Heuschen & Schrouff OFT B.V., Landgraaf, The Netherlands), LiBai China fish sauce (Finora Feinkost GmbH, Melle,

Germany), and Exotic Food Authentic Thai Fishsauce (Exotic Food Co. Ltd., Sriracha, Thailand) were obtained from a local supermarket. Water used for chromatography was prepared by means of a Milli-Q Gradient A 10 system (Millipore, Schwalbach, Germany), and bottled water was used for sensory studies (Evian, Danone, Wiesbaden, Germany). For sensory studies, an aqueous model broth stock solution was prepared by dissolving monosodium L-glutamate monohydrate (1.9 g/L), maltodextrin (6.375 g/L), sodium chloride (2.92 g/L), and Gistex XII LS yeast extract (2.1 g/L) in water and adjusting the pH value to 6.5 with traces of formic acid (0.1% in water) in water.

Analytical Sensory Experiments. *Panel Calibration and Training.* Twelve assessors (seven females, five males, ages 23−32 years), who gave informed consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, participated for at least two years in weekly sensory training sessions using the following two-step procedure: In a first ranking experiment, the panelists were asked to arrange four three-digit random-coded test solutions containing NaCl in concentrations varying between 30 and 80 mmol/L (in 5.0 mmol/L steps) according to the perceived salt intensity. On the basis of the rank sum of each sample calculated from the rank positions assigned by the individual panelists, the so-called Friedman value according to ISO 8587 was calculated.[55] By comparison with tabulated critical values, rank orders with significant differences were identified, whereas by means of a direct comparison test significant margins in salt taste enhancing activity were determined.

In a second set of experiments, the salt intensity of the samples of the ranking test were individually compared to that of a dilution series of NaCl (30−80 mmol/L, in steps of 5.0 mmol/L) in water or model broth solution, respectively. To achieve this, the panelists were asked to identify the NaCl concentration of the dilution series showing isointense saltiness when compared to the test sample. In addition, the sensory assessors were allowed to quote NaCl concentrations for the test samples which lay between the concentrations of two dilution steps. The panel data obtained for each concentration in triplicates were averaged and are given as "salt taste isointensity" expressed in mmol/L NaCl.

Determination of the Salt Taste Enhancing Activity of the Protamin Digest and Arginyl-Peptides. Prior to sensory analysis, each test sample was analyzed for its sodium content by means of ion chromatography. To judge the protamine digest as well as the individual peptides for their salt taste enhancing activity using the two-step evaluation procedure detailed above, two of the four test samples contained various amounts of the protein digest (0.1, 0.3, 1.0, 3.0% w/v; Figure 1) or individual peptides (12 mmol/L), respectively, in model broth adjusted to a total of 50 mmol/L NaCl, whereas the other two solutions (controls) contained various NaCl levels between 40 and 65 mmol/L and were used to test the accuracy and reproducibility of the sensory panel. If these control solutions were not correctly identified within the 95% confidence interval (Figure 1), the experiment was repeated. In another set of experiments, water was used as matrix instead of the model broth. The averaged isointensity values of the panel were used to express the salt taste enhancement in percent.

Protein Hydrolyses. *Enzymatic Hydrolysis.* A solution of chymotrypsin (1.0 mg/mL) in KH_2PO_4 buffer (0.01 mmol/L; pH 7.5; 10.0 mL) was added to a solution of the target protein (1.0 g) in KH_2PO_4 buffer (0.01 mmol/L; pH 7.5; 78.9 mL), the mixture was incubated for 24 h at 37 °C and, then, kept at 80 °C for 10 min to terminate digestion. After cooling to room temperature, an aliquot (11.1 mL) of a trypsin solution (1 g/L phosphate buffer; pH 7.5) was added, and the mixture was maintained at 37 °C for 48 h, followed by a thermal inactivation for 10 min at 80 °C. To ensure that the peptides identified in the hydrolysates were not impurities of the proteins or enzymes used, control experiments were performed in the absence of the protein or the enzyme, respectively. The digests were lyophilized and stored at −20 °C until used for further analysis.

Acidic Hydrolysis. The target protein (1 g) was thermally treated in the presence of hydrochloric acid (1.5 mol/L; 100 mL) for 12 h at 100 °C. After cooling, the hydrolysates were lyophilized and used for LC-MS/MS analysis.

Ultrafiltration. An aliquot (1.5 g) of lyophilized protamine digest was dissolved in water (100 mL) and separated by means of ultrafiltration using a Vivacell 250 filtration system equipped with a Vivacell 250 insert with a 5 kDa cutoff membrane (Sartorius, Göttingen, Germany) and operated under nitrogen atmosphere (3.5 bar) on a Typ 3005 laboratory shaker (GFL, Burgwedel, Germany; 150 rpm). The low molecular weight (LMW) fraction (<5 kDa; yield = 84.7%) and the high molecular weight (HMW) fraction (≥5 kDa; yield = 15.3%) were lyophilized and kept at −20 °C until further used.

Gel Permeation Chromatography (GPC). An aliquot (1.5 g) of the LMW fraction was dissolved in water (15 mL) and was separated on a 100 × 5 cm XK50/100 column (GE Healthcare Bio-Science AB, Uppsala, Sweden) filled with a slurry of Sephadex G-15 (GE Healthcare Bio-Science AB, Uppsala, Sweden) in 1% formic acid as stationary phase. Using a P-1 type peristaltic pump (GE Healthcare Bio-Science AB), a UV 2075 Plus detector (Jasco, Groß-Umstadt, Germany) operating at a wavelength of 220 nm, and an Ultrarorac Fraction Collector 2070 II (LKB, Bromma, Sweden), chromatography was performed with aqueous formic acid (1% in water) as mobile phase (2 mL/min). Seven GPC fractions, I (yield = 1.6%), II (yield = 14.7%), III (yield = 35.8%), IV (yield = 32.1%), V (yield = 9.3%), VI (yield = 6.5%), and VII (yield = 2.1%) (Figure 2), were collected, freeze-dried, and kept at −20 °C until used for sensory analysis as well as UPLC-TOF-MS analysis.

Hydrophilic Interaction Liquid Chromatography/Evaporative Light Scattering Detection (HILIC/ELSD). For HILIC-ELSD analysis, the HPLC apparatus (Gilson International, Limburg-Offheim, Germany) was equipped with a type 321 HPLC pump, a 506C type system interface module, a 234 type autoinjector unit, an UV−vis-156 type detector (Gilson International), and a Sedex 85 type evaporative light scattering detector (LT-ELSD, Sedere S.A., Alfortville Cedex, France), which was operated at 40 °C with air as operating gas (3.5 bar). Analytical separations were performed on a 150 × 4.6 mm i.d., 3 μm, TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) with a flow rate of 1.0 mL/min. For semipreparative separations, a 300 × 7.8 mm i.d., 10 μm, TSKgel Amide-80 column (Tosoh Bioscience) was used and operated with a flow rate of 6.0 mL/min, which was split 1:8 into the Sedex 85 type evaporative light scattering detector and the GX-281-type fraction collector (Gilson International). Chromatography was performed with a solvent gradient using acetonitrile containing 0.1% formic acid (solvent A) and an 0.1% aqueous formic acid solution (solvent B). The gradient started with 5% solvent B for 5 min, was increased to 100% within 40 min, was kept constant for further 5 min, and, finally, was decreased to 5% solvent B within 5 min, followed by an equilibration time of additional 5 min.

Ultraperformance Liquid Chromatography−Time-of-Flight Mass Spectrometry (UPLC-TOF-MS). TOF-MS analysis was performed on a Waters Synapt G2 HDMS mass spectrometer (Waters, Bedford, MA), which was either operated in the flow injection mode or coupled to an Acquity UPLC core system (Waters, Manchester, U.K.). Aliquots (1 μL) of aqueous solutions of the analyte sample were injected into the UPLC/TOF-MS system equipped with a 150 × 2 mm, 1.7 μm, BEH C18 column (Waters, Manchester, U.K.) operated with a flow rate of 0.3 mL/min and kept at a temperature of 40 °C. Chromatography was performed with a solvent gradient using acetonitrile containing 0.1% formic acid (solvent A) and an 0.1% aqueous formic acid solution (solvent B). Starting with 1% solvent A for 1 min, the content of solvent A was increased to 95% within 3 min, then kept constant for 1 min, and, thereafter, decreased again to 1% within 0.2 min, followed by an equilibration time of another 0.3 min. The scan time for the MS^E method (centroid) was set to 0.1 s. Measurements were

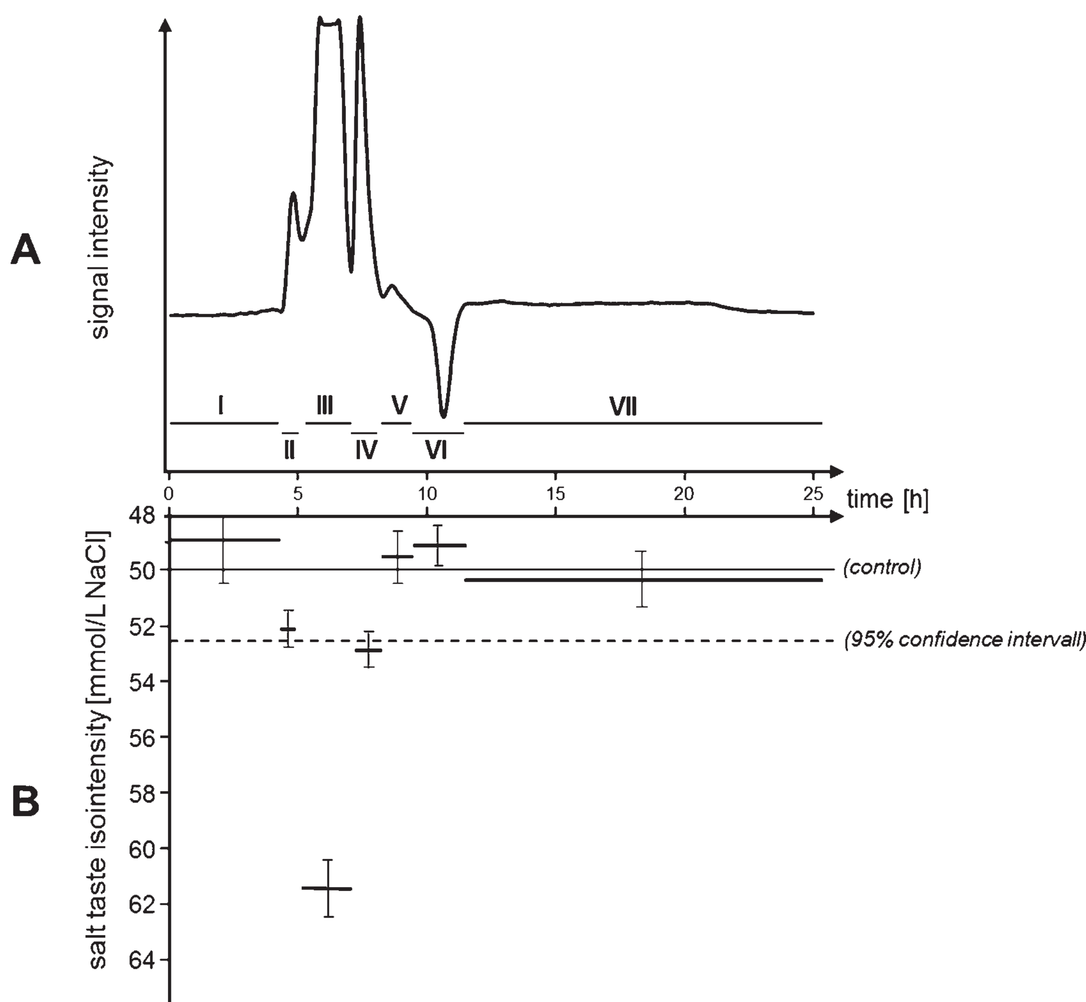

Figure 2. (A) GPC chromatogram (λ = 220 nm) of the LMW fraction (<5 kDa) isolated from an enzymatic protamine digest; (B) salt taste enhancing activity of GPC fractions I–VII (fraction III, $p < 0.001$).

performed using positive electrospray ionization and the ion source parameters for the capillary voltage (2.0 kV), sampling cone (20 V), extraction cone (4.0 V), source temperature (150 °C), desolvation temperature (450 °C), cone gas (30 L/h), and desolvation gas (850 L/h) given in parentheses. Data processing was performed by using MassLynx 4.1 (Waters) and the elemental composition tool for determining the exact mass. QuanLynx (Waters) was used for peak detection and integration of the exact masses of arginine (m/z 175.1201), arginyl-glycine/glycyl-arginine (RG/GR, m/z 232.1413), arginyl-serine/seryl-arginine (RS/SR, m/z 262.1517), arginyl-proline/prolyl-arginine (RP/PR, m/z 272.1724), arginyl-glutamine/glutaminyl-arginine (RQ/QR, m/z 303.1851), and arginyl-arginine (RR, m/z 331.2182) with a chromatogram mass window of 5 mDa. All data were lock mass corrected on the pentapeptide leucine enkephaline (Tyr-Gly-Gly-Phe-Leu, m/z 556.2615, $[M - H]^+$) in a solution (2 ng/μL) of acetonitrile and 0.1% aqueous formic acid (50:50, v/v). Scan time for the lock mass was set to 0.3 s, and an interval of 15 s and 3 scans to average with a mass window of ±0.3 Da. Calibration of the Synapt G2 in the range from m/z 50 to 600 was performed using a solution of sodium formate (5 mmol/L) in 2-propanol/water (9:1, v/v).

Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS). For HPLC-MS/MS analysis of the protein hydrolysates, an API 4000 QTRAP LC-MS/MS system (AB Sciex, Darmstadt, Germany) was coupled to an HPG-3400SD pump, a Solvent Rack SRD-3400 degasser, a WPS-3000TSL autosampler, and a TCC-3000SD column oven (UltiMate 3000 series, Dionex, Idstein, Germany). The mass spectrometer was operated in the ESI^+ mode, nebulizer gas was zero-grade air (45 psi), and curtain gas was nitrogen (25 psi). Analysis was done by means of the multiple reaction monitoring (MRM) mode. After collision-induced dissociation, the transition from the pseudo-molecular ion $[M + H]^+$ to a main fragment was recorded. Declustering potential (DP), cell exit potential (CXP), and collision energy (CE) were optimized for each substance by a special tuning process (Table S1, Supporting Information). The dwell time for each mass transition was 15 ms. The entrance potential was 10 V. The quadrupoles operated at unit mass resolution. Instrumentation control and data collection were performed by using Analyst 1.5 (AB Sciex, Darmstadt, Germany).

Sample aliquots (2.0 μL) were injected onto a 150 × 2 mm, 3 μm, TSKgel Amide-80 column (Tosoh Bioscience) to analyze for the target peptides (Table S1, Supporting Information). Chromatography was performed with gradient elution at a flow rate of 0.2 mL/min using aqueous ammonium acetate (5.0 mmol/L; pH 6.5) as solvent A and a mixture of acetonitrile and water (95 + 5; v/v) containing 5 mM ammonium acetate as solvent B. Starting with 5% solvent A for 5 min, solvent A was linearly increased to 50% within 25 min and, then, to 100% within an additional 5 min and, thereafter, decreased to 5% within 5 min, followed by an equilibration for another 15 min at 5% solvent A. The second chromatography system used a 150 × 2 mm, 3 μm, Luna PFP

column (Phenomenex, Aschaffenburg, Germany) and was operated at a flow rate of 0.2 mL/min. After sample injection (2 μL), chromatography was performed with a gradient using aqueous formic acid (1.0% in water) as solvent A and acetonitrile containing 1% formic acid as solvent B. Starting with 100% solvent A for 5 min, solvent B increased to 10% within 5 min and then to 100% within another 4 min, followed by an isocratic elution step for further 5 min. Thereafter, solvent A increased to 100% within 2 min, followed by an equilibration time of another 9 min.

Quantitative Analysis of Arginine and Dipeptides in Protein Hydrolysates and Fermented Fish Sauces. For quantitation of arginine and dipeptides, aliquots of the protein hydrolysates (20.0 mg) or the fish sauces (500 μL) were taken up with a quaternary mixture (2.0 or 1.5 mL) of water/methanol/acetonitrile/acetone (1:1:1:1, v/v/v/v) and, after centrifugation (12000 rpm) at 4 °C, the supernatant was separated from solvent under vacuum. The residue was dissolved in water (1.0 mL), and an aliquot (2.0 μL) was injected into the LC-MS/MS system. Quantitative analysis was performed by means of external standard calibration with 1:20, 1:50, 1:100, 1:200, 1:500, and 1:1000 dilutions of an aqueous stock solution containing arginine and arginyl peptides (1.0 mmol/L). Spiking experiments at three different concentration levels, followed by LC-MS/MS analysis, revealed recovery rates between 86.0 and 95.0% (Table S1, Supporting Information).

Ion Chromatography. For the analysis of sodium ions, samples (1−5 mg) were dissolved in 1 mL, and the solution was membrane filtered (0.45 μm) and diluted with water to obtain a sodium concentration between about 1 and 80 mg/L sodium ion. Sample aliquots (2 μL) were analyzed by means of an ICS-2000 Ion Chromatography System (Dionex, Sunnyvale, CA) equipped with an AS autosampler, a CSRS300 suppressor (2 mm) operated in autosuppression recycle mode (4.0 mA), and a methanesulfonic acid eluent cartridge (EGC II MSA, Dionex). The samples were eluted on a 250 × 2 mm IonPac CS18 column equipped with an 50 × 2 mm IonPac CG18 guard column (Dionex). Chromatography was performed with methanesulfonic acid (5.0 mmol/L) as solvent at a flow rate of 0.3 mL/min. Quantitation was performed using an external standard calibration with standard solutions ranging from 0.1 to 100 mg/L (six-point calibration). System control and data acquisition were accomplished using Dionex Chromeleon version 6.8 software.

Hierachical Cluster Analysis. Data analysis was performed within the programming and visualization environment R (version 2.10.1).[56] The sensomics heatmap was calculated using the heatmap.2 function of R based on the concentration data determined by means of LC-MS/MS (for exact data, see Table S2, Supporting Information). The dendrogram was constructed by means of an agglomerative linkage algorithm proposed by Ward[57] specifying the distance between two clusters as the increase in the error sum of squares after two clusters have been fused into a single cluster and seeking a minimum distance at each clustering step.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR measurements were performed on a 500 MHz Advance III spectrometer (Bruker, Rheinstetten, Germany). Samples were solved in D_2O and analyzed at 308 K. Data processing was performed using Topspin version 1.3 (Bruker), and data interpretation was performed using MestRe-Nova 5.0 software (Mestrelab Research, La Coruña, Spain).

RESULTS AND DISCUSSION

Analytical Sensory Assay for Salt Taste Assessment. To search for STM activity in chymotrypsin and trypsin digests of protamin, a suitable sensory assay had to be developed first. The assay must enable us to reliably and reproducibly assess differences in saltiness of NaCl solutions in the absence and presence of added protamine hydrolysates and fractions thereof.

On the basis of preliminary sensory studies, a two-step protocol was developed to accurately determine the salt taste intensity of NaCl-containing solutions. First, a ranking experiment was performed with four test solutions containing NaCl at concentrations between 30 and 80 mmol/L. The panelists had to rank correctly the saltiness of the sorted test samples in increasing intensity to those. Next, they compared the salt taste intensities of the test samples to those of a dilution series of NaCl between 30 and 80 mmol/L in water or model broth solution. The panelists were asked to identify those NaCl solutions showing the same saltiness as the test samples or those having saltiness between those of two solutions. After statistical analysis of the panel data of triplicates, "salt taste isointensity" values were determined and expressed in mmol NaCl/L. The 95% confidence interval, shown in Figure 1, demonstrates that the taste panel evaluated the salt taste intensity of a given solution with good accuracy. In addition, regression analysis of the panelists' responses revealed that the perceived salt taste intensity is linearly correlated with the NaCl concentration (coefficient of determination = 0.9996).

Using this two-step evaluation procedure we examined the protamine digest for its STM activity. To this end we offered the panelists four test samples of aqueous model broth containing 50 mmol/L NaCl with various amounts of protein digest (0.1, 0.3, 1.0, 3.0% w/v). The other two solutions (controls) had random NaCl levels between 40 and 65 mmol/L and were used to test the accuracy of the sensory panel. The averaged isointensity values of the panel were used to express the salt taste enhancement in percent. Hydrolysates with isointensity values outside the 95% confidence interval (Figure 1) have been proposed as salt taste modulators. As displayed in Figure 1, the protamine digest showed significant effects on the salt taste of the model broth at concentrations of 1.0 and 3.0%. It enhanced the salt taste such that a 50.0 mM NaCl solution was perceived as if it contained 54.7 or 68.3 mM NaCl, corresponding to salt taste enhancements of 9.4 and 36.6%, respectively.

Fractionation of the Protamine Digest by Means of Ultrafiltration and Gel Permeation Chromatography. To gain first insight into the molecular weight of the taste-active molecules, we separated the protamine digest by means of ultrafiltration with a 5 kDa cutoff, with subsequent lyophilization into a LMW fraction of 84.7% and a HMW of 15.3%. Both fractions were analyzed for their sodium content by means of ion chromatography and then dissolved in model broth according to their relative proportions, that is, at concentrations of 2.54% for LMW and 0.46% for HMW. Finally, both solutions were adjusted to a total of 50 mmol/L NaCl. After analytical confirmation of the correct NaCl concentration, sensory analysis was performed using our two-step procedure for salt taste assessment. The presence of the LMW fraction raised the salt taste intensity of the test broth to that of a model broth containing 68.0 mmol/L. This value is in good agreement with the STM activity found for the total protamine hydrolysate at a spiking level of 3.0%. Application of the HMW fraction did not show any salt taste enhancement in the model broth (data not shown).

To further resolve the STM compounds, the LMW fraction was separated by means of GPC on Sephadex G-15. Monitoring the effluent at 220 nm by means of UV−vis detection, the LMW fraction was separated into GPC fractions I−VII (Figure 2), which were individually freeze-dried and, then, used for sensory evaluation. To this end, the individual GPC fractions were analyzed for their sodium content, then dissolved in model broth according to their relative proportion matching those of the 3.0% total protamine hydrolysate, and, finally, adjusted to a concentration of 50 mmol/L NaCl. After analytical confirmation of the sodium adjustment, sensory analysis was performed using the procedure reported above. Interestingly, fraction III showed the

highest STM activity and led to a perceived saltiness of the model broth corresponding to 61.4 mmol/L NaCl and 23% salt enhancement (Figure 2). The same procedure showed weak saltiness enhancements of 4.2 and 5.6%, respectively, for model broths spiked with fractions II and IV.

Screening for STM Peptides in GPC Fraction III. Next, we aimed at the identification of STM peptides in GPC fraction III using TOF-MS (Figure 3). On the basis of their exact mass and comparison to those of reference compounds, several highly abundant ions could be assigned to pseudomolecular ions of arginine (*m/z* 175.1201, $C_6H_{15}N_4O_2$, ion a), arginyl-glycine and/or glycinyl-arginine (*m/z* 232.1413, $C_8H_{18}N_5O_3$, ion b), arginyl-serine and/or seryl-arginine (*m/z* 262.1517, $C_9H_{20}N_5O_4$, ion c), arginyl-proline and/or prolyl-arginine (*m/z* 272.1724, $C_{11}H_{22}N_5O_3$, ion d), arginyl-glutamine and/or glutaminyl-arginine (*m/z* 303.1851, $C_{11}H_{23}N_6O_4$, ion e), and arginyl-arginine (*m/z* 331.2182, $C_{12}H_{27}N_8O_3$, ion f).

As previous investigations demonstrated the STM activity of L-arginine,[26,43] we considered these arginine-containing dipeptides as potential salt taste enhancers. To identify additional arginyl peptides in the protamine digest, we next subfractionated GPC fraction III by means of HILIC using ELSD after splitting the effluent (Figure 4). The effluent was separated into seven fractions, III_1-III_7, which were collected individually and analyzed by means of UPLC-TOF-MS for the presence of additional arginine-containing dipeptides. Besides L-arginine in fraction III_1, a total of 24 arginyl peptides were identified in fractions III_2-III_6 by comparing the elemental composition and retention times with those for corresponding reference substances (Figure 4). The abundance of these arginyl dipeptides is well in line with the high amounts of up to 70% arginine in protamine.[58]

Salt Taste Modulatory Activity of Arginine-Containing Dipeptides. On the basis of the finding that L-arginine enhances salt taste,[21,26,43] the following experiments aimed at characterizing the STM activity of all single arginine-containing dipeptides using the two-step sensory procedure reported above. Prior to sensory analysis, we confirmed the purity and identity of all dipeptides with sequence RX and XR by HPLC-TOF-MS and ^1H NMR spectroscopy. Next, we determined the STM activity of each peptide (12 mmol/L) in aqueous NaCl (50 mmol/L) and model broth solution containing 50 mmol/L NaCl, respectively (Figure 5). For a total of 17 peptides we determined significant salt taste enhancement in one or the other matrix solution, which increased the saltiness of 50 mmol/L NaCl to values exceeding that of a 52.7 mmol/L NaCl solution ($p = 0.05$).

For most of the peptides investigated, the STM activity was not significantly influenced by the matrix. For instance, the arginyl dipeptide RD increased the saltiness of the 50 mmol/L NaCl solution to that of a 54 mmol/L NaCl solution (8.0% salt enhancement) and

Figure 3. LC-TOF mass spectrum (ESI$^+$, flow injection) of GPC fraction III isolated from enzymatic protamine digest. Pseudomolecular ions a−f were assigned to (a) arginine, $C_6H_{15}N_4O_2$, (b) arginyl-glycine and/or glycinyl-arginine, $C_8H_{18}N_5O_3$, (c) arginyl-serine and/or seryl-arginine, $C_9H_{20}N_5O_4$, (d) arginyl-proline and/or prolyl-arginine, $C_{11}H_{22}N_5O_3$, (e) arginyl-glutamine and/or glutaminyl-arginine, $C_{11}H_{23}N_6O_4$, and (f) arginyl-arginine, $C_{12}H_{27}N_8O_3$, respectively, by means of exact mass analysis and comparison with reference compounds.

Figure 5. Salt taste modulatory (STM) activity of arginyl peptides (12.0 mmol/L) in water (red bars) or model broth (blue bars) adjusted to 50 mmol/L NaCl. Matrix solution contained 50 mmol/L NaCl (black line) [confidence interval ($p = 0.05$, dashed lines]. Error bars represent standard deviation. Among the peptides tested, RP exhibited a sweet off-taste, YR, RY, FR, and RF showed a bitter off-taste, and RW as well as WR showed a strong bitter taste, which did not allow reliable sensory evaluation.

III_1: R
III_2: RI, IR, RL, LR, RM, MR, RP, PR, RF, RW
III_3: RV, VR
III_4: RY, YR
III_5: RE, ER, RA, AR, RG, GR, RD
III_6: RR, RQ, RS

Figure 4. HILIC-HPLC/ELSD chromatogram of GPC fraction III isolated from enzymatic protamine digest. Individual peptides in subfractions III_1-III_6 were unequivocally identified by TOF-MS and cochromatography with reference compounds.

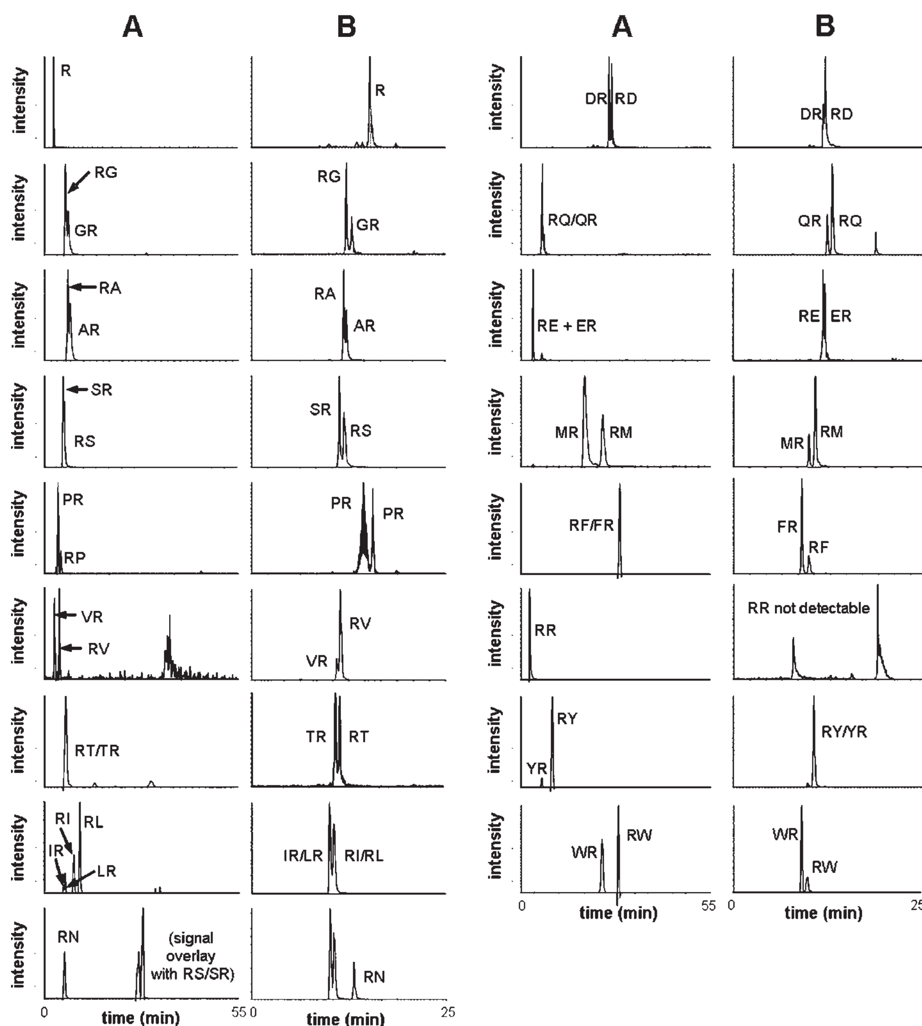

Figure 6. Influence of the stationary phase on the analysis of L-arginine and arginyl dipeptides by means of LC-MS/MS (MRM, ESI$^+$). Stationary phases: (A) pentafluorophenyl column (Luna PFP); (B) hydrophilic liquid interaction chromatography column (TSKgel Amide-80).

of the model broth solution to that of a broth solution containing 54.3 mmol/L NaCl (8.6% salt enhancement). However, for some arginyl dipeptides, the sensory effects were dependent on the matrix. For example, RR did not show any effect in aqueous NaCl (50 mmol/L), but showed significant salt taste enhancement in model broth containing 50 mmol/L NaCl (Figure 5). The biggest difference between both matrices was found for the peptide RP. Whereas it enhanced the perceived salte taste of the model broth by 21%, it did not exhibit any STM activity in the aqueous NaCl solution. Other peptides showed stronger effects in the broth relative to aqueous NaCl. The peptide AR, for example, increased the salt taste intensity of 50 mmol/L NaCl by 14% in the aqueous solution and by 19% in model broth. The matrix dependence might be explained by the pronounced sweet taste of RP observed in water, which could decrease the perceived saltiness of the test solution by mixture suppression.[59] Similar salt-suppressing effects were observed for the peptides RY, YR, RF, and FR, which exhibited a pronounced bitter taste. The intense and long-lasting bitter taste of RW and WR did not allow any reliable evaluation of saltiness enhancement in both the aqueous NaCl and the broth solution.

Quantitative Analysis of Arginine and Arginine-Containing Dipeptides in Protamine Hydrolysates. Quantitative analysis of arginine and arginine-containing peptides was performed by means of HPLC-MS/MS in the enzymatic digest obtained after chymotrypsin/trypsin treatment of protamine as well as in the acid hydrolysate of protamine. After a series of preliminary experiments on various stationary phases, arginine and 13 dipeptides were successfully separated on a pentafluorophenyl (PFP) stationary phase, whereas a TSKgel Amide-80 HILIC column allowed the analysis of additional 18 dipeptides (Figure 6; Table S1, Supporting Information). Using both optimized chromatographic systems, calibration curves were recorded for each analyte from 1.0 to 50 μmol/L to compensate for differences in ionization parameters and intrinsic instrumental background noise of the respective MRM trace and to assess linearity of the method. To analyze the target arginyl peptides with high selectivity by using tandem mass spectrometry operating in the MRM mode, solutions of the analytes were individually infused into the ESI source of the MS/MS system with a constant flow by means of a syringe pump to optimize ionization parameters and collision-induced fragmentation. Typically the most intensive mass transition was used for quantification (Table S1, Supporting Information).

By means of external standard calibration, the enzymatic digest and the acidic hydrolysate of protamine were then analyzed for the content of arginine and arginyl peptides by means of LC-MS/MS. Among the 33 analytes, arginine and 11 arginyl peptides were unequivocally identified and quantified in the hydrolysates (Figure 7).

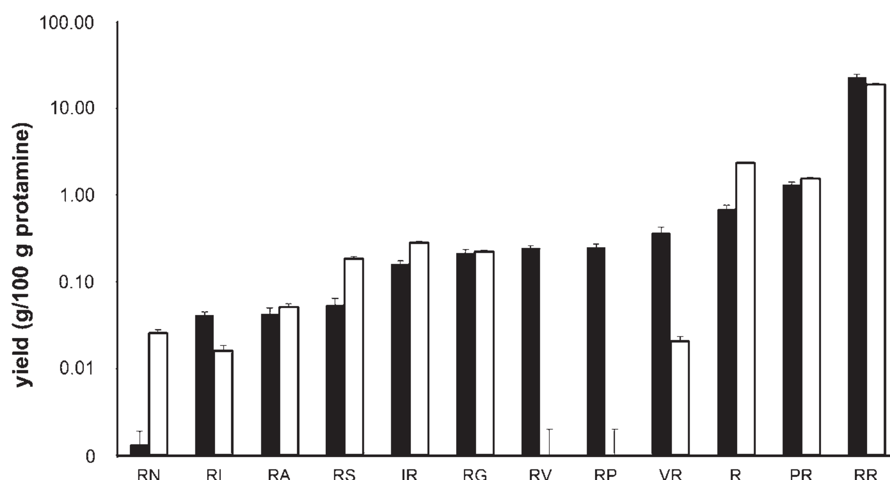

Figure 7. Concentrations of L-arginine and arginyl dipeptides in enzymatic (black bars) and acidic (white bars) protamine hydrolysates. Data are given as the means of triplicates; error bars indicate standard deviation.

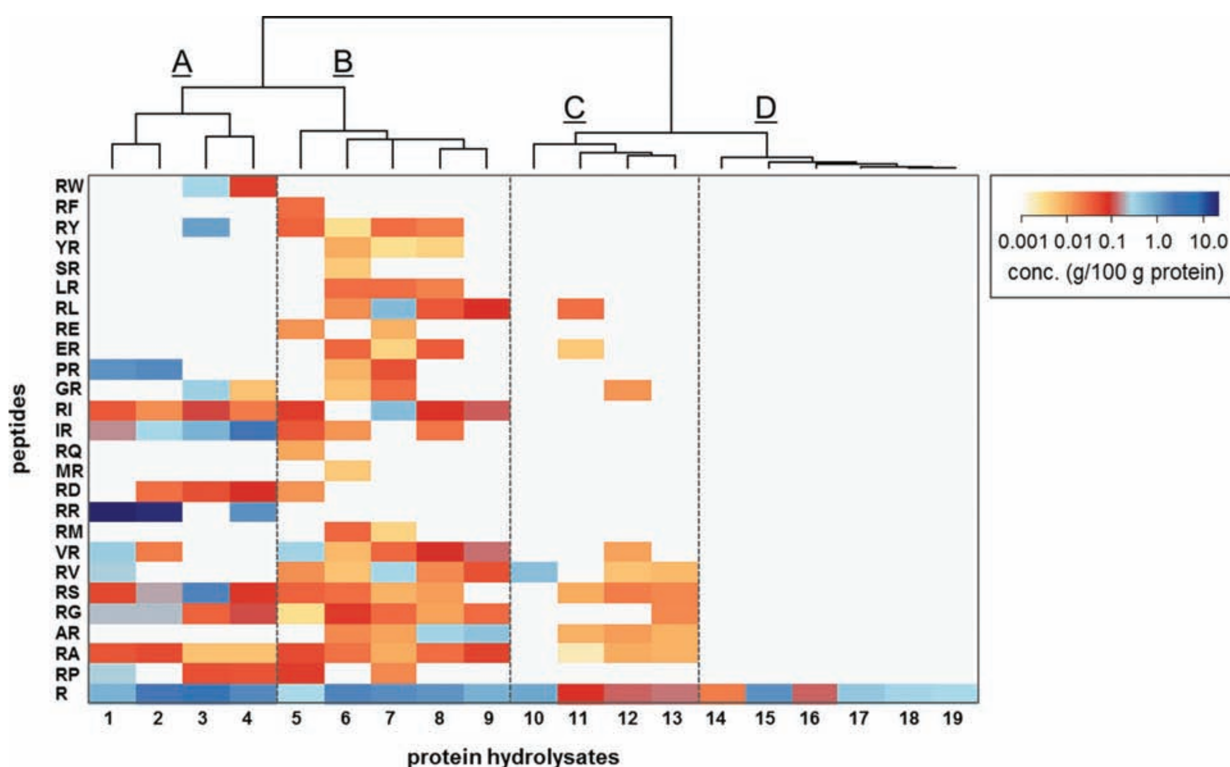

Figure 8. Sensomics heatmapping of arginine and arginyl dipeptides in selected protein hydrolysates: enzymatic protamine hydrolysate (**1**), acidic protamine hydrolysate (**2**), enzymatic lysozyme hydrolysate (**3**), acidic lysozyme hydrolysate (**4**), commercial gluten hydrolysate (**5**), commercial peptone hydrolysate (**6**), commercial casein hydrolysate (**7**), acidic soy protein hydrolysates (**8, 9**), commercial soy protein hydrolysates (**11−16**), fava bean protein hydrolysate (**10**), rice protein hydrolysate (**15**), and pea protein hydrolysates (**17−19**).

Arginyl-arginine (RR) was found as the predominant peptide in both samples, with yields of 23 and 18% in the enzymatic and acidic protamine hydrolysate, respectively. Prolyl-arginine (PR) and arginine were released from protamine in yields of 1.3 and 0.7% (w/w) when using the enzyme cocktail or in yields of 1.5 and 2.3% (w/w) when using hydrochloric acid. All other peptides were found in amounts of <0.4% (w/w). Interestingly, arginyl-valine (RV) and arginyl-proline (RP) were detectable only in the acidic hydrolysate.

Sensomics Mapping of Arginine and Arginyl Dipeptides in Protein Hydrolysates. To screen the hydrolysates of other food grade proteins for the presence of salt taste enhancing arginyl peptides, arginine and total of 32 arginyl peptides were screened by means of PFP-HPLC-MS/MS and HILIC-MS/MS in 8 commercially available protein hydrolysates, enzymatic digests made from chicken lysozyme, salmon protamine, and protein isolates from rice, fava bean, pea, and soy. In addition, acidic hydrolysates were prepared from lysozyme and protamine (Table S2, Supporting Information).

Table 1. Concentration of L-Arginine and Arginyl Dipeptides in Fermented Fish Sauces

	concentration (μmol/L)[a]	
compound	median	range
R	148.4	15.0−1045.7
RG	1.2	1.0−1.4
ER	43.2	0.2−60.4
GR	13.0	2.9−24.2
RP	3.4	0.6−6.1
AR	3.5	0.6−5.6
RE	3.8	2.4−4.0
RS	1.7	1.4−1.8
TR	0.9	0.1−1.2
RA	0.4	0.2−0.7
RT	0.5	0.5−0.6
SR	0.3	0.3
RI	0.2	0.2

[a] The concentrations are given as the mean of triplicates analyzed for six fish sauces.

Hierarchical cluster analysis grouped the samples to give four clusters (A−D) on the basis of their peptide composition (Figure 8). Cluster A comprised the enzymatic and acidic hydrolysate of the basic proteins protamine (**1**, **2**) and lysozyme (**3**, **4**). Within this cluster, RR and PR predominated in both protamine hydrolysates. In comparison, the enzymatic chicken lysozyme digest (**3**) contained major amounts of RY and RS besides arginine, whereas the acidic hydrolysis (**4**) of the same protein favored the release of IR and RR. The samples in cluster B, containing hydrolysates of gluten (**5**), pepton (**6**), casein (**7**), and two soy protein samples (**8**, **9**), showed rather high levels in arginine (0.27−1.75%, w/w) and exhibited a rather widespread distribution of arginyl peptides with yields between 0.003 and 0.537% (w/w) with predominant levels of VR in the gluten hydrolysate, RV, RI, and RL in the casein hydrolysate and AR in soy protein hydrolysate. The hydrolysates of fava bean protein (**10**) and the three soy protein samples (**11**−**13**) in cluster C contained only minor levels of arginyl peptides and arginine (0.081−0.138%, w/w). The hydrolysates of two soy protein samples (**14**, **16**), rice protein (**15**), and three pea protein samples (**17**−**19**), grouping in cluster D, did not contain any significant amounts of arginyl peptides and contained only free arginine (0.021−1.387%, w/w).

Considering the pronounced STM activity of arginine and the peptides RP, RA, AR, RG, RS, RV, VR, and RM (Figure 5), the basic, arginine-rich proteins such as protamine and lysozyme are favored precursors for the enzymatic and/or acidic generation of salt taste enhancing preparations. As fermented fish products were expected to contain these salt taste enhancing peptides, a selection of commercial fish sauces was screened for arginine and arginyl dipeptides in the following.

Quantitative Analysis of Arginine and Arginyl Dipeptides in Fermented Fish Sauces. Six samples of commercially available fish sauces, typically manufactured by fermentation of salted fish over months,[60] were analyzed for arginine and arginyl peptides by means of PFP-HPLC-MS/MS and HILIC-MS/MS, respectively. Besides major amounts of L-arginine ranging from 15.0 to 1045.7 μmol/L, 12 arginyl peptides were detected, among which ER, GR, and RP were found in the highest concentrations of up to 60.4, 24.2, and 6.1 μmol/L (Table 1), respectively. In comparison, the other dipeptides were present in somewhat lower amounts of <6.0 μmol/L.

On the basis of the data obtained, it might be concluded that the two-step sensory test developed is a rather suitable tool to detect natural salt taste enhancers in foods and to "quantificate" their salt taste enhancing activity. Besides the amino acid L-arginine, a series of arginyl dipeptides, namely, RP, RA, AR, RG, RS, RV, VR, and RM, are reported for the first time as salt taste enhancing molecules in fish protamine digests and fermented fish sauces. As the slightly fishy off-flavor of L-arginine does limit the use of this free amino acid in many food applications, studies toward the targeted enzymatic release of the arginyl peptides RA, AR, RG, RS, RV, VR, and RM, none of which were found in the present study to exhibit any off-flavor in aqueous solution, are currently in progress to increase their yields and applicability as natural salt taste enhancing preparations in foods.

■ ASSOCIATED CONTENT

Supporting Information. Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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