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Molecular and Sensory Characterization of γ-Glutamyl Peptides as Key Contributors to the Kokumi Taste of Edible Beans (*Phaseolus vulgaris* L.)

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Addition of a nearly tasteless aqueous extract isolated from beans (*Phaseolus vulgaris* L.) to a model chicken broth enhanced its mouthfulness and complexity and induced a much more long-lasting savory taste sensation on the tongue. Gel permeation chromatography and hydrophilic interaction liquid chromatography/comparative taste dilution analysis (HILIC/cTDA), followed by LC-MS/MS and 1D/2D-NMR experiments, led to the identification of γ-L-glutamyl-L-leucine, γ-L-glutamyl-L-valine, and γ-L-glutamyl-L-cysteinyl-β-alanine as key molecules inducing this taste-modifying effect. Sensory analysis of aqueous solutions of these peptides showed threshold concentrations between 3.3 and 9.4 mmol/L for an unspecific, slightly astringent sensation. More interestingly, when added to a savory matrix such as sodium chloride and monosodium glutamate solutions or chicken broth, the detection thresholds of these γ-glutamyl peptides decreased significantly and remarkably enhanced mouthfulness, complexity, and long-lastingness of the savory taste were observed; for example, the threshold of γ-glutamyl-cysteinyl-β-alanine decreased by a factor of 32 in a binary mixture of glutamic acid and sodium chloride. As tasteless molecules inducing mouthfulness, thickness, and increasing continuity of savory foods were coined about 10 years ago as "kokumi" flavor compounds, the peptides identified in raw as well as thermally treated beans have to be considered as kokumi compounds.

KEYWORDS: Kokumi; taste; taste enhancer; mouthfullness; taste dilution analysis; beans; homoglutathione; γ-glutamyl peptides

INTRODUCTION

In many countries all over the world, legumes play an important role in consumer diet and account for approximately one-third of the world's primary crop production (*1*). In particular, peas, beans, lentils, and other podded plants are key ingredients in the typical cuisines of India, South America, the Middle East, and Mexico, respectively. When combined with meat, beans are long-known to enhance the flavor of famous dishes such as the French cassoulet, the Brazilian feijoada, and Mexican chilis.

Many investigations have focused on the chemical composition of pulses. Although the protein quantity and quality were found to vary between different legumes, they contain high levels of proteins when compared with other plant-derived foods such as cereals (*2*). Among the carbohydrates, beans are rich in starch, oligosaccharides, and dietary fiber, accounting for about 50% of the total weight of dried beans. Whereas the nondigestible oligosaccharides have been identified as prebiotic agents (*3*), a lowering of serum total cholesterol in rats was reported for starches of different bean varieties (*4*). Furthermore, saponins present in edible beans were reported to induce a decrease of blood lipids and cholesterol levels and to exhibit anticarcinogenic activity (*5*). Although the mineral content of legumes is rather high, their bioavailability is poor due to the presence of phytate, inhibiting iron absorption by complexation (*6*). Nutritionally beneficial, beans are an excellent source of folates, which in addition to being essential micronutrients are suggested to reduce the risk of neural tube effects (*7*). Among the legumes, soybeans seem to play a unique role due to their bioactive phytochemicals such as isoflavones (*8*).

Although multiple studies were aimed at increasing the knowledge on nutritional benefits and health-promoting effects of legumes, no data are available on the bean ingredients that are responsible for the taste-improving effect when beans such as *Phaseolus vulgaris* L. are used as part of savory-tasting dishes. To discover such taste modulators in complex food products, we have recently developed so-called "sensomics" techniques by combining analytical natural product chemistry and human psychophysical tools such as the taste dilution analysis (TDA) and the comparative taste dilution analysis (cTDA), respectively (*9*, *10*). This approach led to the structural

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and functional characterization of various previously unknown taste compounds such as thermally generated bitter compounds (*9*), astringent taste compounds in black tea infusions (*11*) and roasted cocoa nibs (*12*), the multivalent taste-modulating Maillard reaction product alapyridaine in beef broth (*10*, *13*), umami-enhancing glucosides in morel mushrooms (*14*), and, most recently, bitterness-suppressing molecules such as 1-carboxymethyl-5-hydroxy-2-hydroxymethylpyridinium inner salt (*15*).

To bridge the gap between pure structural chemistry and sensory perception, the aim of the present investigation was to screen for the key compounds inducing the taste-modulating activity of common beans (*P. vulgaris* L.), to isolate and identify the chemical structures, and to determine the sensory thresholds of the compounds found with the highest gustatory response.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: trifluoroacetic acid (Riedel de Haen, Taufkirchen, Germany); sodium chloride and L-glutamic acid (Fluka, Taufkirchen, Germany). All of the reference peptides used within this study were from Bachem (Bubendorf, Switzerland). Acetonitrile was of HPLC grade (Fisher Scientific, Schwerte, Germany). Deuterated solvents were supplied by Euriso-Top (Gif-Sur-Yvette, France). A model chicken broth used as savory matrix for the sensory analyses was freshly prepared by diluting a Gourmetbouillon Huhn type concentrate (Nestlé, Singen, Germany) with bottled water (Evian; Danone, Wiesbaden, Germany) in a concentration of 3.0 g/L. Dried bean seeds (*P. vulgaris* L.) were obtained from a local market.

Preparation of Aqueous Bean Extracts. Dried bean seeds (*P. vulgaris* L., 10 g) were cooked in water (100 mL) for 60 min, minced, and centrifuged. The residue was extracted with bottled water (100 mL) with stirring for 12 h at room temperature. The aqueous layers were combined and, after centrifugation, the clear supernatant was freeze-dried to give the water-soluble extract of the cooked beans (4.45 g, yield = 44.5%). In parallel, dried bean seeds (10 g) were ground in a coffee grinder and then extracted with water (100 mL) for 12 h at room temperature. After centrifugation, the clear supernatant was freeze-dried to give the water-soluble extract of the dried beans (2.78 g, yield = 27.8%).

Gel Permeation Chromatography (GPC). An aliquot (250 mg) of the dry material of the lyophilized extract isolated from the dried beans was taken up in water (10 mL) and, then, applied onto the top of a water-cooled 100 × 5 cm × K 50/100 glass column (Amersham Bioscience, Uppsala, Sweden) filled with a slurry of Sephadex G-10 (Amersham Bioscience) conditioned with water adjusted to pH 4.0 with aqueous formic acid (1 g/100 g). Chromatographic separation was performed using the same solvent at a flow rate of 2 mL/min for 20 h. Monitoring the effluent by means of an L-7490 type RI detector (Merck, Darmstadt, Gemany) produced five fractions (fractions I−V) collected by means of a fraction collector, and the individual fractions were freeze-dried. The residue obtained for each GPC fraction was used for the sensory analysis as well as for chromatographic subfractionation.

Sensory Analyses. *Training of the Sensory Panel.* Nine assessors (five males, four females, ages 22−39 years), who gave informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were trained in sensory experiments at regular intervals for at least 2 years and were, therefore, familiar with the techniques applied. For the training of the individual gustatory modalities, aqueous solutions (2 mL each) of the following reference taste compounds dissolved in bottled water (pH 6.0) were used: sucrose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, NaCl (30 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, sodium L-glutamate (3 mmol/L) for umami taste, tannic acid (0.05%) for puckering astringency, and quercetin-3-*O*-*β*-D-glucopyranoside (0.01 mmol/L) for a velvety astringent, mouth-drying oral sensation. For the training of viscosity, a gelatin solution (0.5% in water) was used; for the training of the activity of mouthfulness enhancement and complexity increase, coined kokumi activity (*16*−*18*), the panel was asked to compare the gustatory impact of the blank model chicken broth (control) with a solution of reduced glutathione (5 mmol/L) in chicken broth (both at pH 6.5). Sensory analyses were performed in a sensory panel room at 19−22 °C in three different sessions using nose clamps.

Pretreatment of Fractions. Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removal of the volatiles in high vacuum (<5 mPa), were freeze-dried twice. GC-MS and ion chromatographic analysis revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used.

Determination of Recognition Threshold Concentrations for Mouthfulness Enhancement (Kokumi) Activity. The kokumi taste thresholds were determined by means of a three-alternative forced-choice test (*19*) using aqueous solutions of sodium chloride (30 mmol/L) or L-glutamic acid (10 mmol/L), a binary mixture of sodium chloride (10 mmol/L) and L-glutamic acid (10 mmol/L), or the model chicken broth as a savory taste matrix, respectively. The pH value of the individual samples and blanks was adjusted to 6.5 by adding trace amounts of formic acid (0.1 mmol/L) and sodium hydroxide solution (1.0 mmol/L), respectively. The samples (4 mL) were presented in serial 1:1 dilutions in order of increasing concentrations to the trained panel in three different sessions using the sip-and-spit method. At the start of each sensory session and before each trial, the subject rinsed with bottled water and expectorated. The samples were swirled in the mouth briefly and expectorated. After indicating which vial contained the taste-modifying compound, the participant received another set of two samples without and one sample with an additive. To prevent excessive fatigue, tasting began at a concentration level two steps below the individual threshold concentration that had been determined in a preliminary sensory experiment. The geometric mean of the last and the second last concentration was calculated and taken as the individual threshold. The threshold value of the sensory panel was approximated by averaging the threshold values of the individuals in three independent sessions (*19*). Values between individuals and separate sessions differed not more than two dilution steps.

Comparative Taste Profile Analysis. The dry material of the lyophilized extracts (50 mg), GPC fractions I− V, as well as the hydrophilic interaction liquid chromatography (HILIC) fractions III/1−III/25 and IV/1−IV/16, respectively, were dissolved in exactly 4.0 mL of the model chicken broth, and the pH value was adjusted to 6.5 using trace amounts of formic acid (0.1 mmol/L) or sodium hydroxide solution (1.0 mmol/L), respectively. These solutions were then presented in a dual test together with the blank model chicken broth (control) to the trained sensory panel, and the intensity of the descriptors bitter, sweet, sour, salty, umami, and mouthfulness/complexity was rated on a scale from 0 (not detectable) to 5 (intensely perceived).

Identification of *γ*-L-Glutamyl-L-leucin and *γ*-L-Glutamyl-L-valine in GPC Fraction III. The lyophilized GPC fraction III was dissolved in aqueous trifluoroacetic acid (0.1% in water; 2 mL) using an ultrasonic bath and membrane filtered (0.45 *μ*m), and aliquots (150 *μ*L) were then separated by HILIC on a 300 × 21.5 mm i.d., 10 *μ*m, TSKgel Amide-80 column (Tosoh Bioscience, Stuttgart, Germany) equipped with a 75 × 21.5 mm i.d., 10 *μ*m guard column (Tosoh Bioscience). Monitoring the effluent by means of an evaporative light scattering detector (ELSD), chromatography was performed at a flow rate of 6 mL/min using aqueous trifluoroacetic acid (0.1% in water) as solvent A, acetonitrile containing 0.1% trifluoroacetic acid as solvent B, and starting with a mixture of 95% B and 5% A for 10 min and then increasing solvent A to 100% within 80 min. The effluent was separated into 25 subfractions, which were collected separately in 15 runs, diluted with the same volume of water, and freeze-dried twice. The HILIC fractions obtained were then dissolved in the model chicken broth and evaluated by means of a comparative taste profile analysis using the blank chicken broth as the control. The sensory evaluation revealed an increased mouthfulness, richness, and complexity (kokumi) in HILIC fractions III/8 and III/9, whereas the other fractions were inactive. Subsequent LC-MS and NMR experiments of the isolates of HILIC fractions III/8 and III/9 led to the identification of the kokumi compounds as *γ*-L-glutamyl-L-valine and *γ*-L-glutamyl-L-leucine, respectively.

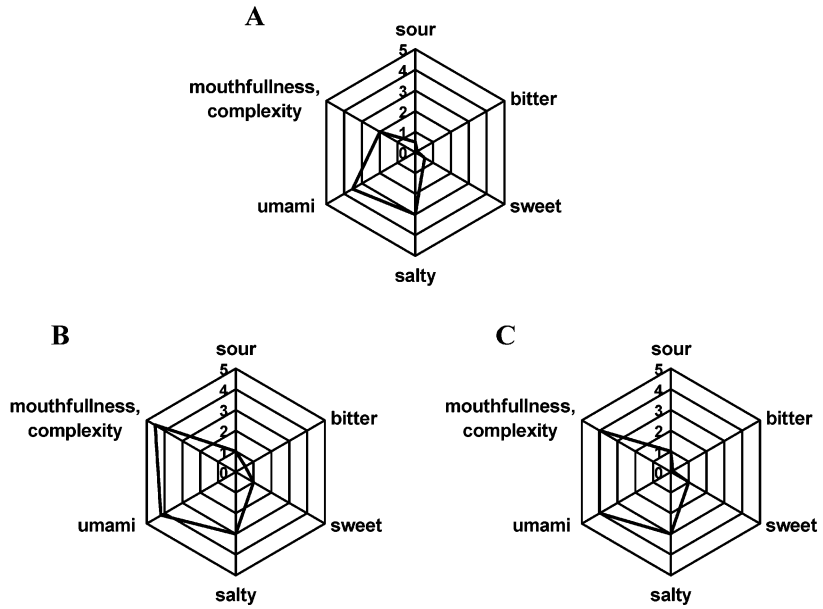

Figure 1. Taste profile analysis of chicken broth without additive (**A**) and with an aqueous extract prepared from cooked (**B**) and noncooked beans (**C**).

γ-L-Glutamyl-L-valine: LC-MS (ESI$^+$), *m/z* 247 (100, [M + H]$^+$), 269 (78, [M + Na]$^+$), 286 (26, [M + K]$^+$); ^{1}H NMR (400 MHz, D_2O; COSY), δ 0.83 [t, 6H, J = 6.2 Hz, H−C(8), H−C(9)], 2.04 [m, 3H, H−C(3), H−C(7)], 2.41 [dt, 2H, J = 2.7 Hz, J = 7.8 Hz, H−C(4)], 3.72 [t, 1H, J = 6.3 Hz, H−C(2)], 4.09 [d, 1H, J = 6.1 Hz, H−C(6)]; ^{13}C NMR (100 MHz, D_2O; HMQC, HMBC), δ 17.2 [C(8)], 18.3 [C(9], 26.2 [C(7)], 29.7 [C(3)], 31.2 [C(4)], 53.7 [C(2)], 59.0 [C(6)], 173.5 [C(1)], 174.8 [C(5)], 176.0 [C(10)].

γ-L-Glutamyl-L-leucine: LC-MS (ESI$^+$), *m/z* *m/z* 261 (100, [M + H]$^+$), 283 (56, [M + Na]$^+$), 299 (19, [M + K]$^+$); ^{1}H NMR (400 MHz, D_2O; COSY), δ 0.82 [d, 3H, J = 5.9 Hz, H−C(10)], 0.86 [d, 3H, J = 5.9 Hz, H−C(9)], 1.58 [m, 3H, H−C(7), H−C(8)], 2.08 [m, 2H, H−C(3)], 2.42 [dt, 2H, J = 2.5, 7.2 Hz, H−C(4)], 3.76 [t, 1H, J = 6.3 Hz, H−C(2)], 4.26 [t, 1H, J = 7.1 Hz, H−C(6)]; ^{13}C NMR (100 MHz, D_2O; HMQC, HMBC), δ 20.6 [C(9)], 22.2 [C(10)], 24.4 [C(8)], 26.2 [C(3)], 31.3 [C(4)], 39.5 [C(7)], 52.0 [C(6)], 53.9 [C(2)], 173.5 [C(1)], 174.4 [C(5)], 177.2 [C(11)].

Identification of γ-L-Glutamyl-L-cysteinyl-β-alanine in GPC Fraction IV. Following the procedure reported above for GPC fraction III, GPC fraction IV was further separated into 16 fractions by means of HILIC. Comparative taste profile analysis using the model chicken broth as savory matrix, followed by LC-MS and NMR experiments, led to the discovery of the kokumi principle in subfraction IV/10 as γ-L-glutamyl-L-cysteinyl-β-alanine, known as homoglutathione.

γ-L-Glutamyl-L-cysteinyl-β-alanine: LC-MS (ESI$^+$), *m/z* 322 (100, [M + H]$^+$), 344 (36, [M + Na]$^+$); ^{1}H NMR (400 MHz, D_2O; COSY), δ 2.09 [m, 2H, H−C(3)], 2.35 [t, 2H, J = 6.8 Hz, H−C(10)], 2.44 [dt, 2H, J = 3.0, 7.6 Hz, H−C(4)], 2.87 [m, 2H, H−C(7)], 3.36 [m, 2H, H−C(9)], 3.69 [t, 1H, J = 6.6 Hz, H−C(2)], 4.37 [t, 1H, J = 6.1 Hz, H−C(6)]; ^{13}C NMR (100 MHz, D_2O; HMQC, HMBC), δ 25.3 [C(7)], 26.1 [C(3)], 31.3 [C(4)], 36.1 [C(10)], 36.5 [C(9)], 54.1 [C(2)], 55.9 [C(6)], 171.7 [C(8)], 173.9 [C(1)], 174.9 [C(5)], 179.7 [C(11)].

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Gilson International B.V., Bad Camberg, Germany) consisted of a type 321 HPLC pump, a type 7725i Rheodyne injector (500 μL loop), and a prepELS type evaporative light scattering detector (ELSD). Chromatographic separations were performed on stainless steel columns filled with carbamoyl-derivatized silica gel (TSKgel Amide 80, Tosoh Bioscience) in either semipreparative (300 × 7.8 mm i.d., 5 μm, flow rate = 1.0 mL/min) or preparative scale (300 × 21.5 mm i.d., 10 μm, flow rate = 6.0 mL/min). After chromatographic separation, the effluent was split in a 1:5 ratio, the smaller aliquot was channeled into the ELSD, and the major aliquot was collected.

Mass Spectrometry (MS). Electrospray ionization (ESI) spectra were acquired on an API 4000 Q-Trap LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) with direct loop injection of the sample (1 μL). The spray voltage was set at −4500 V in the ESI$^-$ mode and at 5500 V in the ESI$^+$ mode. Nitrogen served as curtain gas (25 psi); the declustering potential was set at −20 V in the ESI$^-$ mode and +25 V in the ESI$^+$ mode. The mass spectrometer was operated in the full-scan mode monitoring positive or negative ions. Fragmentation of the pseudo-molecular ions [M − H]$^-$ and [M + H]$^+$ into specific product ions was induced by collision with nitrogen (4×10^{-5} Torr) at a collision energy of −25 V.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR experiments were performed on a Bruker DPX 400 spectrometer (Bruker, Rheinstetten, Germany). Data processing was performed by using Mestre-C software (version 4.8.6.0, Mestrelab Research, Santiago de Compostela, Spain). D_2O was used as solvent and tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

To gain a first insight into the taste-modulatory activity of beans, dried seeds of white, common beans (*P. vulgaris* L.) were soaked in water, cooked for 60 min, and, after cooling to room temperature and mincing, an aqueous extract was prepared. After freeze-drying, an aliquot of the aqueous bean extract was dissolved in water and evaluated by a trained sensory panel. In addition, an aliquot of the bean water solubles was taken up in a model chicken broth to study the taste-enhancing activity of the bean extractables in a savory taste matrix. To achieve this, the trained sensory panel was asked to rate the intensity of the taste qualities sour, bitter, sweet, salty, umami, and mouthfulness/complexity in the different samples on a scale from 0 (not detectable) to 5 (intensely detectable). Whereas the aqueous solution of the bean extract elicited only a slight astringent and faint sweetish taste sensation (data not shown), the addition of an equal amount of bean extractables to the model chicken broth resulted in a strong increase in taste complexity and rich mouthfulness of the broth, whereas the basic taste qualities bitter, sour, salty, sweet, and umami remained mainly unaffected (**Figure 1**). The sensory evaluation revealed an increase of mouthfullness and complexity of the model broth from 2.0 to 4.5 when the bean extract was added and, in addition, the panelists described the taste sensation as much more long-lasting. Molecules inducing that type of mouthfulness and thickness and increasing continuity of food taste perception were coined by the Japanese as "kokumi" flavor compounds (*17*, *18*).

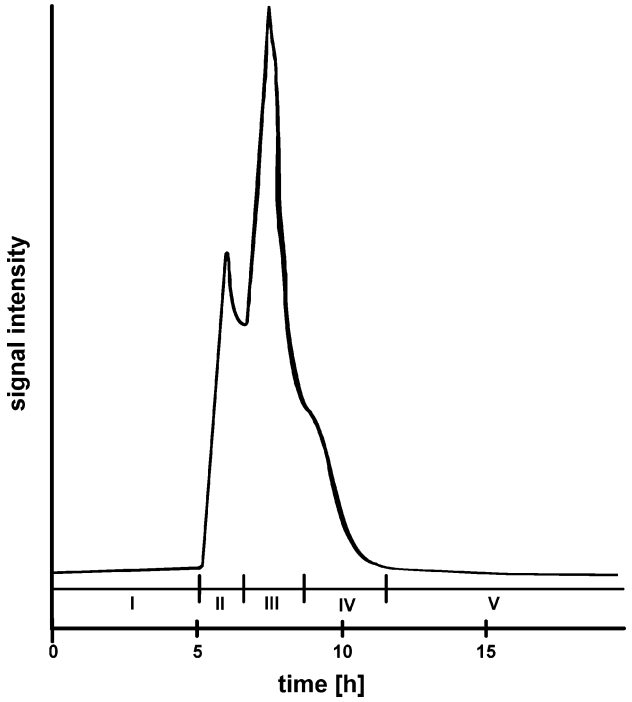

Figure 2. GPC chromatogram (RI detection) of the water-soluble extract prepared from dried common beans.

To investigate whether the kokumi molecules are formed during cooking of the beans or are already naturally occurring in the legumes prior to cooking, an aqueous extract was prepared from nonprocessed beans and used for sensory analysis. Again, a solution of the bean extract in water was evaluated as being nearly tasteless (data not shown). However, when added to the model chicken broth, the perception of mouthfulness/complexity of the savory base was found to increase strongly from 2.0 to 4.0, whereas sweetness and umami taste were influenced just slightly and the other taste modalities were not affected (**Figure 1**). As these data clearly demonstrated that the taste modulators are present already in the nonprocessed beans, the aqueous extract isolated from the dried beans was used for sensory-guided fractionation to sort out the tasteless, but taste-modulating, compounds from the bulk of inactive substances.

Sensory-Guided Fractionation. To gain first insight into the kokumi molecules imparting the taste-modulating activity, the bean extract was separated by means of GPC using Sephadex G-10 as the stationary phase and water as the mobile phase (**Figure 2**). Monitoring the effluent by means of a refraction index (RI) detector, the bean extract was separated into the five GPC fractions I−V, which were individually freeze-dried. Sensory analysis of aliquots of the GPC fractions in bottled water demonstrated that fractions I and V were entirely tasteless and fractions II−IV exhibited just an astringent taste sensation (**Table 1**). In parallel, aliquots of the five GPC fractions were added to chicken broth in their natural concentration ratios and presented to the trained sensory panelists, who were asked to evaluate the mouthfulness and complexity compared to the blank chicken broth (control). The data, given in **Table 1**, show that fractions III and IV showed the highest activity for mouthfulness enhancement (2.0→4.0/4.2), followed by fraction II, whereas fractions I and V did not influence the taste profile of the model chicken broth.

To locate the molecules responsible for the enhanced mouthfulness, GPC fractions III and IV should be further separated by preparative HPLC. No chromatographic separation of these GPC fractions could be successfully achieved on RP-18 stationary phases, because the RP material did not allow a sufficient resolution of these highly polar substances (data not shown). Therefore, fractions III and IV were further separated by means of a HILIC using carbamoylated silica gel as the stationary phase. Monitoring the effluent by means of a UV−vis detector (220 nm) as well as an ELSD, GPC fraction III was separated into 25 subfractions (**Figure 3**), which were individually freeze-dried, taken up in the same amount of model chicken broth, and then rated for their activity in mouthfulness enhancement using the model chicken broth as the savory matrix. Strong taste-modulating activity was found for fraction III/9, followed by fraction III/8. All of the other fractions showed only very low activity or were inactive.

To identify the molecules inducing the taste-enhancing effect in fractions III/8 and III/9, these fractions were isolated in a semipreparative scale and, then, analyzed by LC-MS/MS as well as 1D- and 2D-NMR experiments. In the ESI^+ mode the compound isolated from fraction III/9 showed a pseudo-molecular ion of *m*/*z* 261 ($[M + 1]^+$) as well as sodium and potassium adducts with *m*/*z* 283 ($[M + Na]^+$) and *m*/*z* 299 ($[M + K]^+$), respectively, thus suggesting a molecular mass of 260 Da. Fragmentation of the pseudo-molecular ion *m*/*z* 261 by means of collision-activated dissociation (CAD) in the second quadrupole of the triple-quadrupole instrument resulted in a complex fragmentation pattern as expected for peptides. As shown in **Figure 4**, the fragment series of *m*/*z* 130, 102, and 84 is a characteristic indication of a glutamic acid residue; for example, the b_1 fragment ion *m*/*z* 130 is due to the most common cleavage at the peptide bond, and subsequent decarboxylation gives *m*/*z* 84, whereas the corresponding immonium ion was detectable with *m*/*z* 102. Besides glutamic acid, the second amino acid in the peptide needs to be an isoleucine or a leucine residue, fitting well with the immonium ion *m*/*z* 86. The fragment *m*/*z* 132 is 19 amu above the standard fragment mass for leucine or isoleucine, respectively; therefore, one of these amino acids has to be the C-terminal part of the dipeptide.

To differentiate between both hydrophobic amino acids, 1D- and 2D-NMR spectroscopic experiments were performed. The ^1H NMR spectrum showed seven resonance signals, three integrated for three protons, two signals for two protons, and two signals for one proton. The signals resonating at 4.26 and 3.76 ppm are within the typical range for the α-protons of amino acid residues. Analysis of the spectrum obtained by means of homonuclear H,H-correlation spectroscopy (COSY) revealed a coupling between the methylene group with a chemical shift of 2.08 ppm and the methylene group at 2.42 ppm as well as the proton at 3.76 ppm, as expected for a glutamic acid moiety. The two signals with chemical shifts of 0.82 and 0.86 ppm were assigned as the methyl groups of either a leucine or an isoleucine moiety. The differentiation between leucine and isoleucine as part of the isolated peptide could be achieved after comparison of the ^{13}C chemical shifts with those obtained from the single amino acids. The most significant difference was found for the resonance signals of the two methyl groups. Typical ranges for the ^{13}C-chemical shifts of the methyl groups of leucine are between 20.0 and 23.3 ppm, whereas the corresponding resonance signals for isoleucine show a high-field shift to a range from 11.9 to 15.9 ppm. ^{13}C NMR spectroscopy of the isolated peptide shows 11 resonance signals, among which the 3 carbon atoms resonating at 173.5, 174.4, and 177.2 ppm corresponded to the 3 carbonyl carbon atoms. As two signals appeared at 20.6 and 22.2 ppm and no signals were observed at a higher field, the second amino acid of the dipeptide has to be leucine.

Table 1. Taste Quality of GPC Fractions I–V in Water and Their Influence on the Sensory Profile of Model Chicken Broth

fraction[a]	taste quality in water	influence on the sensory profile of chicken broth[b]
I	tasteless	no difference
II	slightly astringent	enhanced mouthfulness and complexity (2.0→3.0); more rounded taste
III	astringent, slightly bitter	enhanced mouthfulness and complexity (2.0→4.0); increased richness and palate length
IV	astringent	enhanced mouthfulness and complexity (2.0→4.2); increased richness and palate length
V	tasteless	no difference

[a] Number of GPC fractions refers to **Figure 2**. [b] The influence of the individual GPC fractions on the mouthfullness/complexity (2.0) of the model chicken broth was evaluated on a scale from 0 to 5.

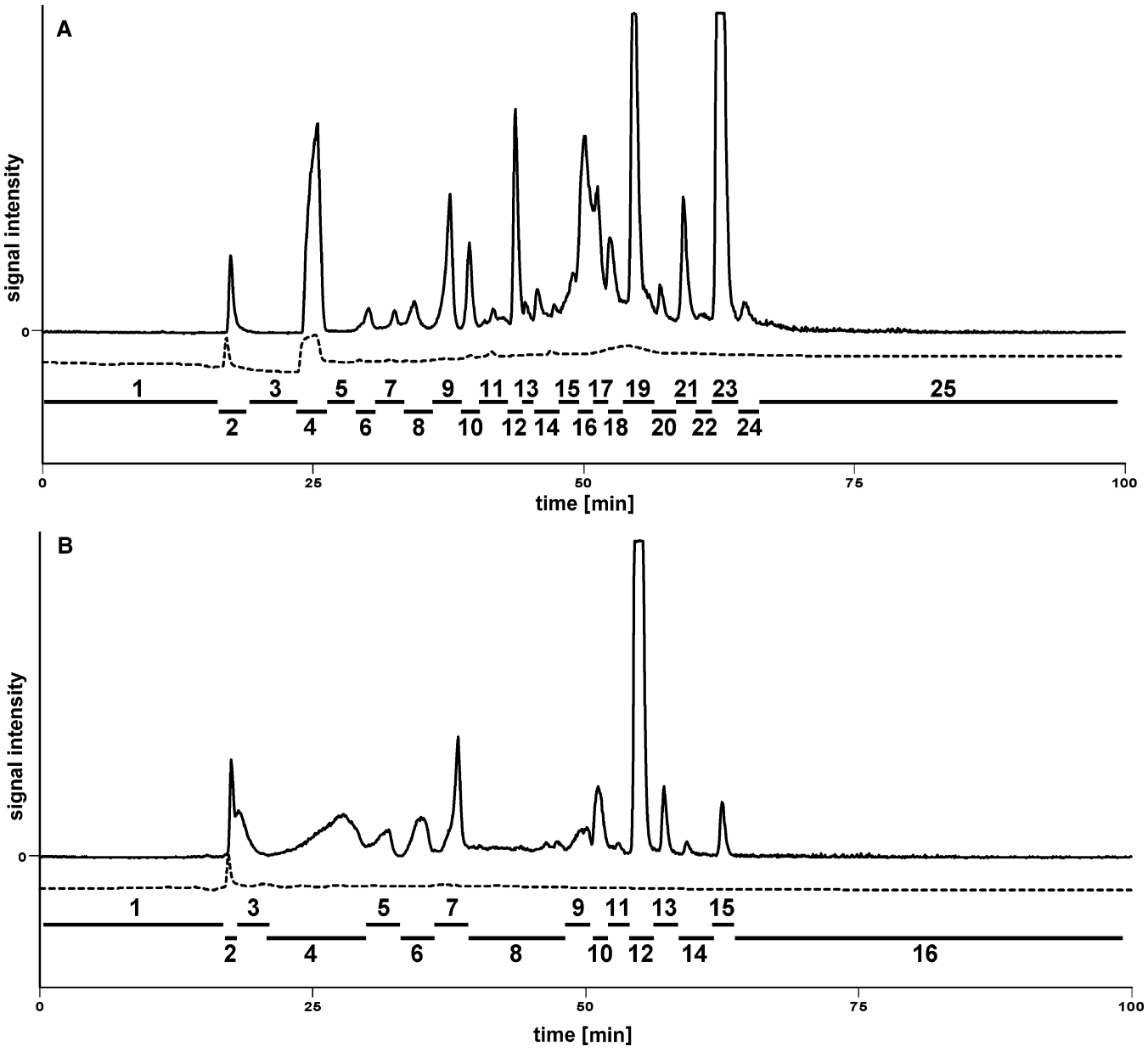

Figure 3. HILIC chromatogram of GPC fractions III (**A**) and IV (**B**), both isolated from an aqueous bean extract; ELSD trace as solid line, UV trace (220 nm) as dashed line.

For the unambiguous characterization of the isolated compound, it was necessary to distinguish between the two possible positions of the peptide bond at the C-terminal group or at the side chain of the glutamic acid residue by means of heteronuclear multiple bond correlation spectroscopy (HMBC) optimized for $^{2}J_{C,H}$ and $^{3}J_{C,H}$ coupling constants. Analysis of the long-range correlations between the proton H−C(6) resonating at 4.26 ppm and neighboring carbon atoms permitted the connection of the quartenary carbon C(5) with a ^{13}C signal at 174.4 ppm and the α-proton of the leucine residue. In addition, no correlation between this proton and the C-terminal carbonyl carbon atom of the glutamic acid, resonating at 173.5 ppm, was observed. On the basis of these findings, the presence of the isopeptide bond could be successfully confirmed and the structure of the taste-modulating compound in HILIC fraction III/9 was determined as γ-L-glutamyl-L-leucine (**Figure 4**).

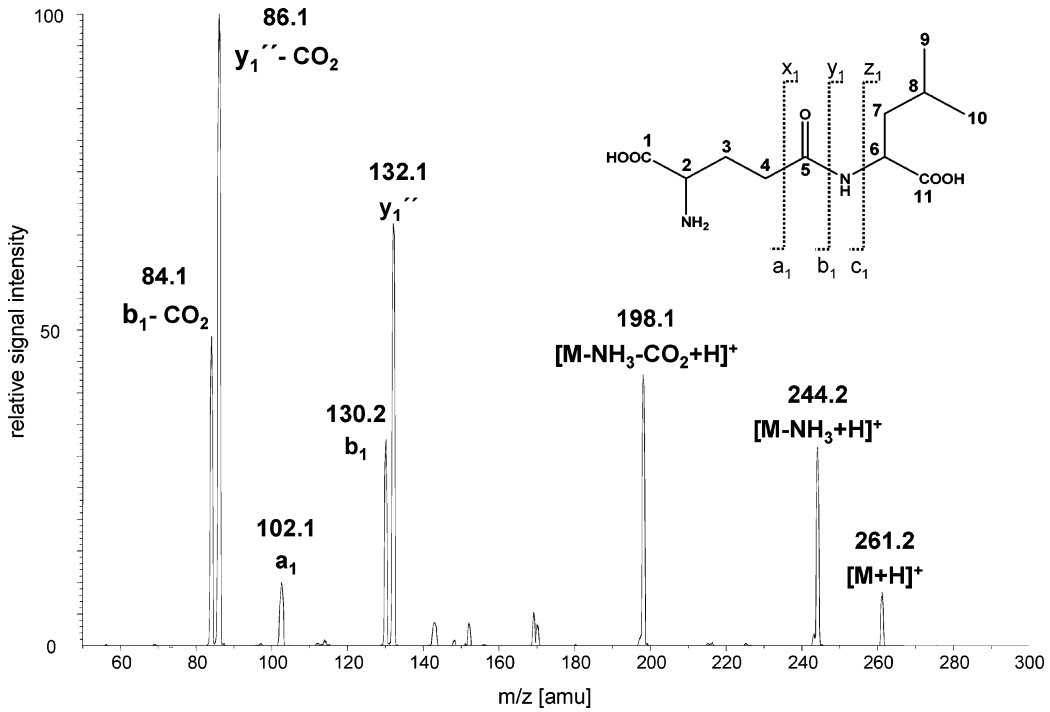

Figure 4. LC-MS/MS spectrum (ESI+) of γ-L-glutamyl-L-leucine isolated from fraction III/9.

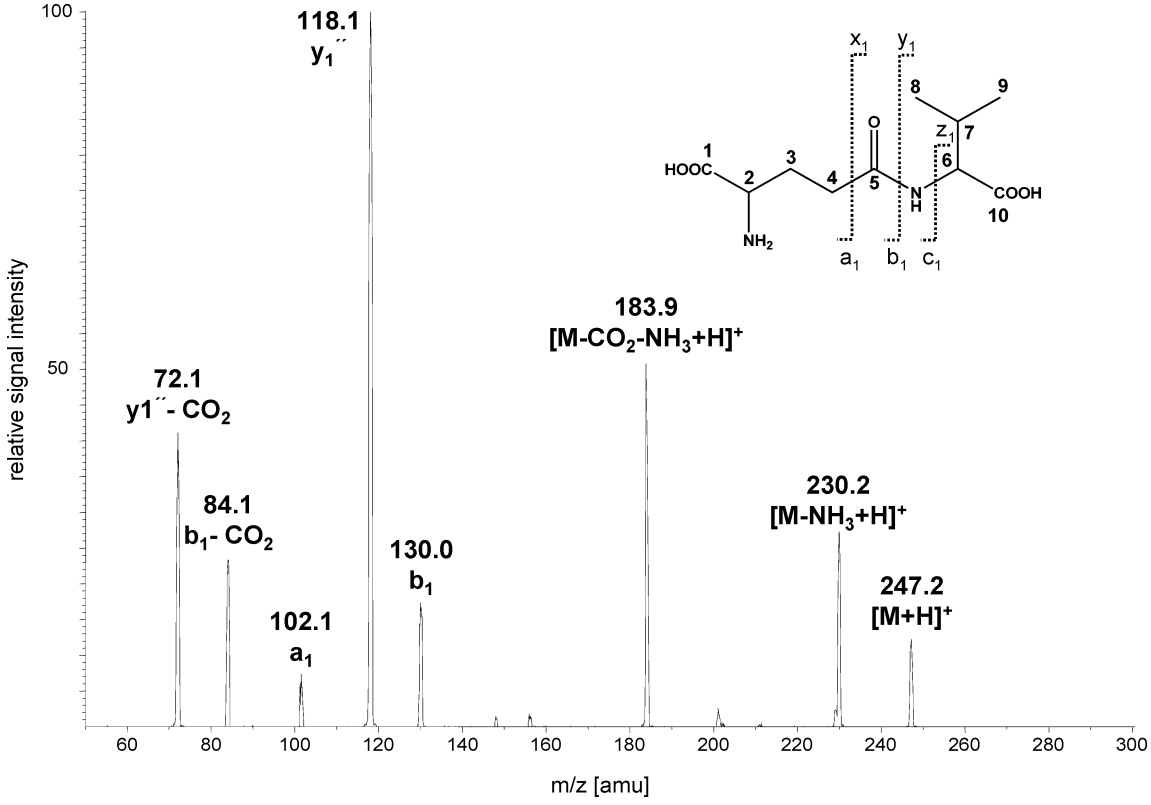

Figure 5. LC-MS/MS spectrum (ESI+) of γ-L-glutamyl-L-valine isolated from fraction III/8.

Mass spectrometry experiments of fraction III/8 showed a molecular mass of 246 Da and a fragmentation pattern similar to that observed for γ-L-glutamyl-L-leucine (**Figure 5**). The fragment series with *m*/*z* 130, 102, and 84 once more pinpointed the presence of a glutamic acid residue, whereas the fragments with *m*/*z* of 118 and 72 indicated the presence of a valine moiety. Comparison of spectroscopic (LC-MS/MS, NMR), chromatographic data (retention time), and sensory data with those of a reference compound led to the unequivocal identification of γ-L-glutamyl-L-valine as the taste-modulating compound in HILIC fraction III/8.

Application of the HILIC separation on the taste-modulating fraction GPC fraction IV (**Figure 3**) revealed only subfraction IV/10 to induce a taste-enhancing effect to the model chicken broth. LC-MS/MS analysis gave a molecular weight of 321 Da, and mass spectrometric fragmentation indicated a tripeptide consisting of the amino acids glutamic acid, cysteine, and alanine (**Figure 6**). Unequivocal structure elucidation was achieved by

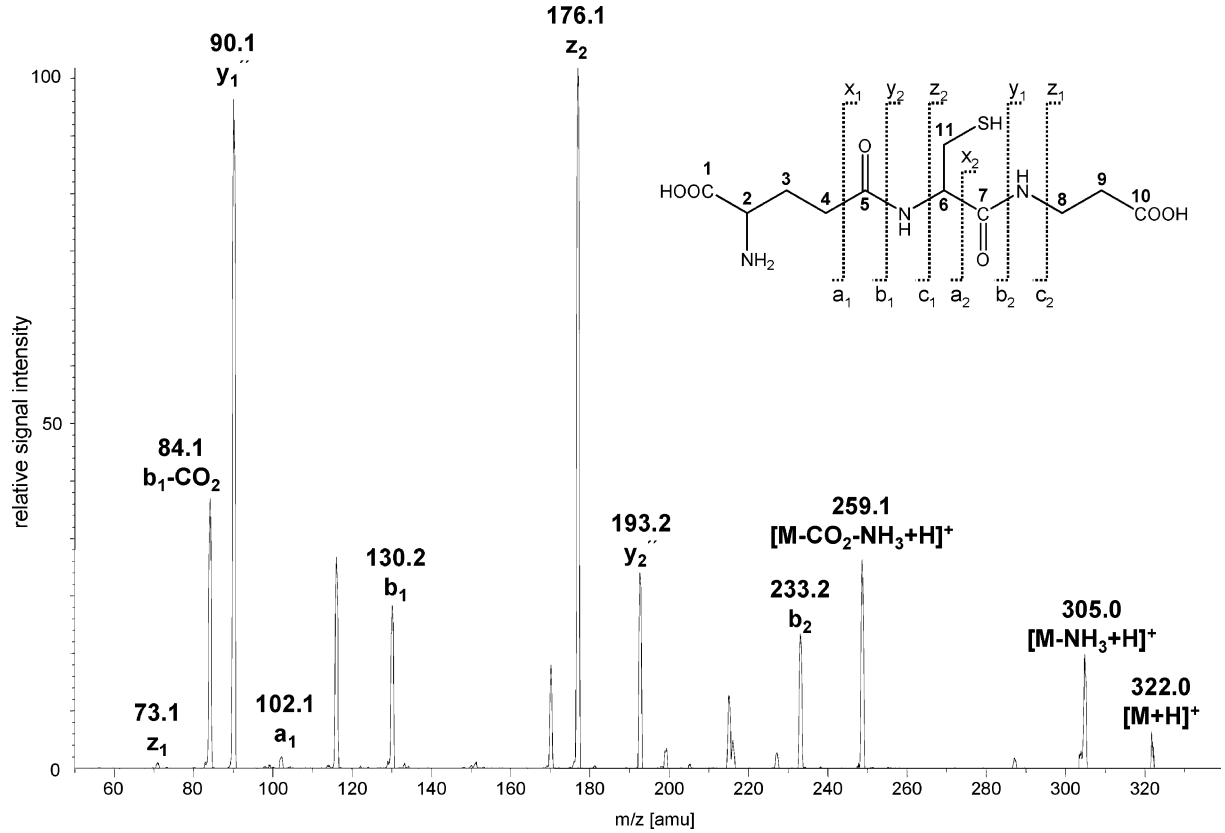

Figure 6. LC-MS/MS spectrum (ESI+) of γ-L-glutamyl-L-cysteinyl-β-alanine isolated from fraction IV/10.

means of ^{1}H and ^{13}C NMR spectroscopy including ^{1}H,^{1}H-COSY, HMQC, and HMBC experiments, which allowed the full assignment of all proton and carbon resonance signals in the target molecule. The absence of the protons of a methyl group in the ^{1}H NMR spectrum and the presence of the four protons of two methylene groups, which do not belong to the glutamoyl moiety, led to the identification of a β-alanyl residue. On the basis of the long-range couplings between the α-proton of cysteine H−C(6) resonating at 4.37 ppm and the terminal carbon atoms C(5) and C(8) obtained from heteronuclear NMR experiments, the cysteine moiety was readily identified to be linked to the γ-carboxylic group of glutamic acid as well as the amino group of the β-alanine. By taking all of these spectroscopic data into consideration, the structure of the taste modulator in HILIC fraction IV/10 was identified as γ-L-glutamyl-L-cysteinyl-β-alanine, known as homoglutathione. This proposal was further confirmed by cochromatography with the corresponding reference compound. Although the occurrence of γ-L-glutamyl-L-leucine and homoglutathione in legumes was reported earlier (*20*, *21*), to the best of our knowledge γ-L-glutamyl-L-valine has previously been identified only from other plant families such as Alliaceae (*22*) and Liliaceae (*23*).

Sensory Activity of γ-L-Glutamyl Peptides. Prior to sensory analysis, the purity and identity of the γ-L-glutamyl peptides were checked by HPLC-MS as well as ^{1}H NMR spectroscopy. To evaluate the sensory activity of the peptides, first, human threshold concentrations for the intrinsic taste of these peptides were determined in water (pH 6.5) by means of an ascending three-alternative forced-choice test (*19*). The γ-glutamyl dipeptides were found to exhibit a nonspecific, slightly astringent oral sensation with threshold concentrations of 3.3 and 9.4 mmol/L for γ-L-glutamyl-L-valine (γ-Glu-Val) and γ-L-glutamyl-L-leucine (γ-Glu-Leu), respectively (**Table 2**). This is in contrast to the sour taste reported for these peptides in the literature (*24*, *25*), but all previous studies were performed at much lower pH values as in the present investigation (pH 6.5).

Table 2. Human Taste Recognition Threshold Concentrations of γ-L-Glutamyl Peptides in Water and Various Savory Systems, Respectively

	taste threshold (mmol/L) in				
peptide	water[a]	NaCl[b]	L-Glu[b]	NaCl/L-Glu[b]	chicken broth[b]
γ-Glu-Leu	9.4	3.2	2.3	0.8	0.8 (0.4–1.6)
γ-Glu-Val	3.3	1.6	0.8	0.4	0.4 (0.2–0.8)
γ-Glu-Cys-β-Ala	3.8	1.9	0.9	0.1	0.2 (0.1–0.4)
γ-Glu-Cys-Gly	3.1	1.6	1.6	0.4	0.2 (0.1–0.4)

[a] Sensory quality was described as a faint astringent sensation. [b] Sensory quality of the savory systems was described as "more complex", "increased mouthfulness", and "more long-lasting" when evaluated in the presence of the peptide.

In a second set of experiments, the influence of the γ-glutamyl peptides on different savory systems was investigated to gain a more detailed insight into their mouthfulness-enhancing effect. To achieve this, recognition threshold concentrations for the individual peptides in sodium chloride as well as L-glutamic acid solution were determined. The sensory analysis showed that in all cases the panelists were able to differentiate between the blank savory base (control) and the corresponding solutions containing one of these peptides. It is interesting to note that the threshold concentration of the peptides in these savory bases was significantly lower than that in water, for example, the threshold concentration of γ-L-glutamyl-L-valine dropped by a factor of 4 to 0.8 mmol/L when added to the L-glutamic acid solution (**Table 2**). In addition, the samples including one of the peptides were evaluated with increased mouthfulness and were more long-lasting.

In a third set of experiments, the thresholds of the γ-glutamyl peptides were determined in a binary solution of L-glutamic acid

(10 mmol/L) and sodium chloride (30 mmol). As given in **Table 2**, the thresholds of the peptides were significantly decreased when compared to the aqueous solutions lacking any other taste compound or containing either L-glutamic acid or sodium chloride. For example, γ-L-glutamyl-L-valine, exhibiting a high intrinsic taste threshold of 3.3 mmol/L, showed a significantly lower threshold value of 0.4 mmol/L in the presence of L-glutamic acid and sodium chloride. Compared to the aqueous solution of the γ-glutamyl peptides, an equimolar concentration of these peptides in binary glutamic acid/NaCl mixtures was described by the panelists to evoke a strongly enhanced mouthfulness and rich complexity as well an increase in the palate length. It is interesting to note that threshold concentrations similar to those found for the peptides in the glutamic acid/sodium chloride mixture were determined when the peptides were evaluated in the model chicken broth (**Table 2**).

Comparative studies on glutathione, γ-Glu-Cys-Gly, revealed the same mouthfulness-enhancing (kokumi) activity for that sulfur-containing tripeptide, thus confirming earlier findings that the taste threshold value of that peptide decreased in umami solutions containing monosodium glutamate and the disodium salt of 5′-inosine monophosphate when compared to the blank aqueous solution (*18*). The first kokumi compounds were isolated from water extracts of garlic and onion and were characterized as sulfur-containing peptide derivatives such as, for example, *S*-allyl-L-cysteine sulfoxide, γ-glutamyl-*trans*-*S*-propenyl-L-cysteine sulfoxide, and 3-(*S*)-methyl-1,4-thiazane-5-(*R*)-carboxylic acid (*S*)-oxide (*17*, *18*).

In conclusion, mouthfulness/complexity-enhancing compounds, so-called kokumi compounds, were identified in common beans by combining analytical natural product chemistry and human sensory analysis. The γ-glutamyl peptides identified were found to possess only a rather unspecific, slightly astringent instrinsic taste perceived at high taste threshold concentrations, whereas in the presence of savory compounds such as sodium chloride and L-glutamic acid, hypo-threshold amounts of these compounds were able to increase the mouthfulness, complexity, and palate length of these systems. Quantitative studies as well as investigations on the structure−activity relationships of these peptides are currently under investigation and will be published elsewhere.

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