JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Series of Kokumi Peptides Impart the Long-Lasting Mouthfulness of Matured Gouda Cheese

SIMONE TOELSTEDE,[†] ANDREAS DUNKEL,[#] AND THOMAS HOFMANN*[,#]

Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany, and Lehrstuhl für Lebensmittelchemie and Molekulare Sensorik, Technische Universität München, Lise-Meitner-Strasse 34, D-85350 Freising-Weihenstephan, Germany

Comparative sensory analysis revealed that a 44-week-matured Gouda cheese (GC44) exhibited a much more pronounced mouthfulness and long-lasting taste complexity when compared to a young Gouda cheese ripened for only 4 weeks (GC4). To identify the molecules underlying that so-called kokumi sensation, a sensomics approach was applied on the water-soluble extract (WSE44) of GC44 by combining gel permeation chromatography (GPC) with analytical sensory tools. HPLC-MS/MS experiments on GPC fractions inducing a kokumi sensation when tasted in an aqueous biomimetic taste recombinant solution (rWSE44) enabled the identification of 8 α-L-glutamyl and 10 γ-L-glutamyl dipeptides as candidate kokumi-enhancing molecules. Among those, only the γ-L-glutamyl dipeptides were found to impart an enhanced kokumi sensation to the matured cheese, whereas none of the α-glutamyl peptides were found to be active. Among the γ-L-glutamyl peptides, the candidates γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, present in GC44 in concentrations between 4.11 and 17.66 μmol/kg, were identified for the first time as the key kokumi molecules enhancing mouthfulness and complex taste continuity of the matured cheese.

KEYWORDS: Cheese; taste; kokumi; mouthfulness; glutamyl peptides

INTRODUCTION

Although multiple studies were performed in the past 30 years to characterize the key taste compounds of Gouda cheese, a highly desirable dairy product appreciated by consumers all over the world, the data available in the literature are rather contradictory (*1*−*8*).

Using a sensometabolomics approach, a total of 49 putative taste-active metabolites and mineral salts were quantitatively determined in the water-soluble extracts prepared from 4-week- (WSE4) and 44-week-matured Gouda cheese (WSE44), respectively, and then ranked in their sensory impact based on dose-over-threshold (DoT) factors (*9*). The bitterness of the cheese matured for 44 weeks was found to be induced by $CaCl_2$ and $MgCl_2$ as previously found for goat cheese (*7*). In addition, various bitter-tasting amino acids were identified to contribute the bitter taste of Gouda cheese, whereas bitter peptides were found to influence more the bitterness quality, rather than the bitter intensity of the cheese. Among the bitter peptides identified, the decapeptide YPFPGPIHNS and the nonapeptides YPFPGPIPN and YPFPGPIHN, assigned to the casein sequences β-CN(60−69) and β-CN(60−68), respectively, as well as the tetrapeptide LPQE released from α_{s1}-CN(11−14) were found with the highest bitterness impacts (*9, 10*). In addition, lactic acid and hydrogen phosphate were identified as the key contributors to the sourness of the cheese, whereas the characteristic umami taste was found to be induced by monosodium L-glutamate and sodium lactate. Moreover, the perceived saltiness was found to be due to sodium chloride and sodium phosphate and was demonstrated for the first time to be significantly enhanced by the basic amino acid L-arginine (*9*). The sensory relevance of the taste compound identified so far was confirmed by carefully designed taste re-engineering and omission experiments. The taste profile of the basic taste recombinant (rWSE4), consisting of a blend of 27 compounds in their "natural" concentration, matched very well that of the authentic WSE4 (*9*). Also, the taste recombinant (rWSE44) and the authentic water-soluble extract of the 44-week-ripened cheese (WSE44) were evaluated with very similar ratings for the basic taste qualities, but the sensory panelists described a significant difference in the perceived complexity, body, and mouthfulness, as well as in the continuity of the orosensation. On the basis of these observations, it was concluded that the molecules imparting the typical mouthfulness and complex body of the matured cheese are still unknown (*9*).

The objectives of the present investigation were, therefore, to screen the water-soluble extract WSE44 for the molecules

* Author to whom correspondence should be addressed (telephone +49-8161/71-2902; fax +49-8161 /71-2949; e-mail thomas.hofmann@wzw.tum.de).
[†] Universität Münster.
[#] Technische Universität München.

10.1021/jf803376d CCC: $40.75
Published on Web 01/26/2009

imparting the mouthfulness of the 44-week-matured Gouda cheese by means of a sensomics approach, to identify the most active compounds, and to determine their human threshold concentrations in water as well as in a biomimetic taste recombinant.

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased from Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany), respectively. Solvents were of HPLC grade (Merck). Deionized water used for chromatography was purified by means of a MilliQ-water Gradient A 10 system (Millipore, Billerica, MA). Reference compounds of the α-L-glutamyl and γ-L-glutamyl dipeptides were purchased from Bachem (Weil am Rhein, Germany). Synthetic bitter peptides were purchased from EZBiolab (Westfield, IN) and purified as described recently (*10*). Gouda cheese samples ripened under controlled standard conditions for 4 weeks (GC4) and 44 weeks (GC44), respectively, were selected by a professional cheese sensory panel in the Dutch food industry and were confirmed to represent the characteristic flavor profiles of a young and a matured Gouda cheese, respectively. Both cheese samples were freshly cut from cheese wheels, sealed in rindless portions (100 g) in nitrogen-flushed packages, and stored at −20 °C until use. Formic acid, which is GRAS listed as a flavoring agent for food and feed applications, was used to adjust the pH value of solutions to be sensorially evaluated; trace amounts (>0.002%) of this acid do not influence the sensory profile of the test solution.

Preparation of the Water-Soluble Extract (WSE). Samples (60 g) of GC4 and GC44, respectively, cut into small pieces and deionized water (240 mL) were placed into a centrifuge beaker, homogenized for 5 min by means of an Ultra-Turrax T 25 basic (Ika Labortechnik, Germany), and then centrifuged at 10000 rpm for 20 min at 4 °C by means of a Varifuge 20 RS (Kendro, Langenselbold, Germany). The upper solid fat layer as well as the protein pellet formed was removed to afford the liquid layer including the cheese water solubles (pH 5.7). The protein pellet as well as the fat layer was re-extracted with deionized water (240 mL) as detailed above, the aqueous layers were pooled, and soluble casein was precipitated upon adjustment of the pH value to 4.6 by the addition of formic acid (1%, v/v; in water). After centrifugation at 10000 rpm at 4 °C for 20 min, followed by paper filtration (Macherey-Nagel, 615-1/4) and freeze-drying (GAMMA 1/2-16LSC, Christ, Germany), a water-soluble extract (WSE4, WSE44) was obtained, which was stored at −20 °C until further analysis.

Analytical Sensory Experiments. *General Conditions, Panel Training.* To familiarize the subjects with the taste language used by our sensory group and to get them trained in recognizing and distinguishing different qualities of oral sensations in analytical sensory experiments, 11 assessors (4 women and 7 men, ages 23−39 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 2 years in weekly training sessions. For example, the subjects were trained to evaluate the taste of aqueous solutions (2 mL; pH 5.7) of the following standard taste compounds in bottled water (Evian; Danone, Wiesbaden, Germany) using the sip-and-spit method: NaCl (20 mmol/L) for salty taste, lactose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, and monosodium L-glutamate (3 mmol/L) for umami taste. For training of bitter taste, solutions of $MgSO_4$ (166 mmol/L) representing a short-lasting, metallic bitter taste quality perceived mainly at the anterior part of the tongue, salicin (1.4 mmol/L), imparting a long-lasting bitter taste sensation perceived mainly in the back of the tongue, and caffeine (8.0 mmol/L), providing a long-lasting bitterness perceived throughout the oral cavity, were used as references. 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, the panel was asked to compare the gustatory impact of a blank chicken broth (3.0 g/100 mL, pH 6.5; Gourmet Bouillon Huhn, Maggi, Singen, Germany) as the control with a solution of reduced glutathione (5 mmol/L) in the same chicken broth (*11*). The sensory sessions were performed at 21 °C in three independent sessions. To prevent cross-modal interactions with odorants, the panelists used nose clips.

Precautions Taken for Sensory Analysis of Food Fractions and Taste Compounds. Prior to sensory analysis buffer compounds and solvent traces were removed from the freeze-dried fractions isolated from cheese. To achieve this, the individual fractions were dissolved in water, and remaining volatiles and solvent traces were removed in high vacuum (<5 mPa, 35 °C) and then again taken up in water and freeze-dried twice. HRGC-MS and ion chromatographic analysis revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used. To minimize the uptake of any toxic compound to the best of our knowledge, all of the sensory analyses were performed by using the sip-and-spit method, which means the test materials were not swallowed but expectorated again.

Preparation of Taste Recombinants rWSE4 and rWSE44. To reconstitute the taste of WSE4 and WSE44, respectively, the taste compounds were dissolved in their "natural" concentrations (*9*) in bottled water to obtain the recombinants rWSE4 and rWSE44, respectively. In detail, for the preparation of the rWSE4 solution, the mineral salts sodium chloride (172.87 mg), disodiumhydrogen phosphate monohydrate (97.89 mg), potassium chloride (26.09 mg), magnesium chloride hexahydrate (48.79 mg), calcium hydroxide (32.30 mg), and calcium chloride (30.63 mg) and the amino acids L-aspartic acid (4.91 mg), L-leucine (42.33 mg), L-alanine (6.47 mg), L-arginine (23.19 mg), L-asparagine (32.35 mg), L-glutamine (26.84), glycine (2.70 mg), L-histidine (4.62 mg), L-isoleucine (13.94 mg), L-lysine (55.92 mg), L-methionine (6.79 mg), L-phenylalanine (21.37 mg), L-proline (5.65 mg), L-serine (5.82 mg), L-threonine (5.15 mg), L-tryptophan (3.21 mg), L-tyrosine (15.14 mg), L-valine (11.13 mg), and monosodium L-glutamate monohydrate (47.91 mg), as well as calcium lactate pentahydrate (416.22 mg) and casein (238.00 mg), were solubilized in bottled water (100 mL); the pH value of that solution was adjusted to 5.6 by the addition of trace amounts of aqueous formic acid (1% in water) and was diluted 1:4 prior to sensory analysis.

To prepare rWSE44, the mineral salts sodium chloride (218.33 mg), disodiumhydrogen phosphate monohydrate (86.15 mg), potassium chloride (29.67 mg), magnesium chloride hexahydrate (49.61 mg), and calcium chloride (252.17 mg), the amino acids L-isoleucine (2.80 mg), L-leucine (151.27 mg), L-tryptophan (37.18 mg), L-tyrosine (50.15 mg), L-arginine (57.97 mg), L-histidine (10.26 mg), L-lysine (201.51 mg), L-phenylalanine (62.21 mg), L-valine (49.58 mg), sodium glutamate monohydrate (188.63 mg), L-aspartic acid (15.19 mg), L-asparagine (72.31 mg), L-glutamine (49.78 mg), L-alanine (26.70 mg), L-methionine (38.10 mg), glycine (13.28 mg), L-proline (20.38 mg), L-serine (27.18 mg), and L-threonine (26.66 mg), the peptides LPQE (23.19 mg), EIVPN (7.00 mg), VRGPFP (4.32 mg), GPVRGPFP (8.89 mg), YPFPGPIHNS (7.53 mg), YPFPGPIHN (7.90 mg), YPFPGPIPNS (0.22 mg), LVYPFPGPIHN (0.52 mg), SLVYPFPGPIHNS (0.30 mg), YPFPGPIPN (24.92 mg), and VYPFPGPIPN (0.50 mg), and sodium lactate (353.21 mg), as well as hydrochloric acid (1 mol/L, 240 μL), were dissolved in bottled water (100 mL); the pH value of that solution was adjusted to 5.7 by the addition of trace amounts of aqueous formic acid (1% in water) and was diluted 1:4 prior to sensory analysis.

The overall taste profile of the basic taste recombinants, rWSE4 and rWSE44, was evaluated by means of taste profile analysis using nose clips. In addition, the extended taste recombinant α/γ-WSE44 was prepared by adding the "natural" amounts of α-L-glutamyl and γ-L-glutamyl dipeptides to the basic taste recombinant rWSE44.

Taste Recognition Threshold Concentrations. The recognition threshold concentrations of the L-glutamyl dipeptides were determined in bottled water (for intrinsic taste) as well as in the aqueous rWSE44 solution (for kokumi activity). By means of a triangle test, aqueous solutions with increasing amounts of L-glutamyl peptides (0.5−20.0 mmol/kg) and rWSE44 solutions with increasing amounts of L-glutamyl peptides (1.0−30.0 μmol/kg), respectively, were presented to the sensory panel, which was asked to identify the correct sample besides two water samples or two rWSE44 solutions lacking the glutamyl peptides (blank solutions) 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

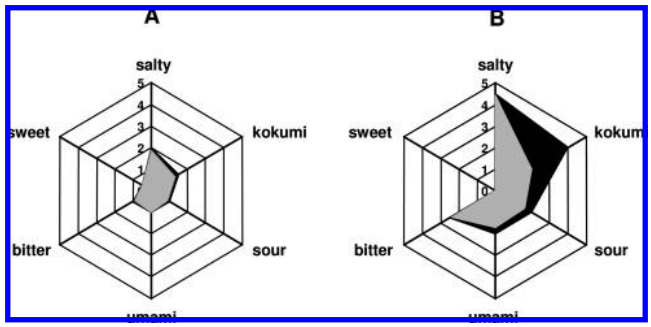

Figure 1. Taste profile analysis of (**A**) the aqueous extract (WSE4, black) prepared from the 4-week-matured Gouda cheese and the basic taste recombinant rWSE4 (gray) and (**B**) the aqueous extract (WSE44, black) prepared from the 44-week-matured Gouda cheese and the corresponding basic taste recombinant rWSE44 (gray), respectively.

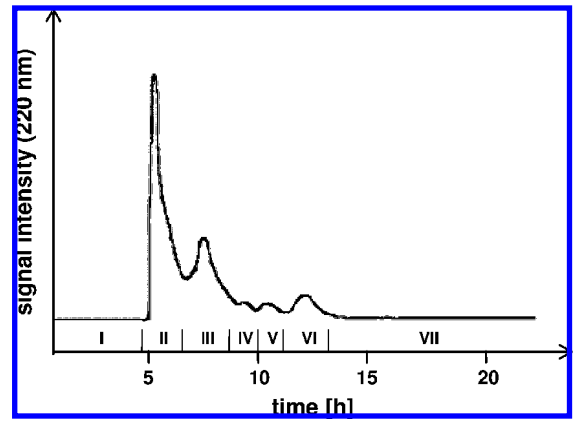

Figure 2. GPC chromatogram (λ = 220 nm) of the WSE44 prepared from the 44-week-matured Gouda cheese.

fatigue, tasting began at a concentration level two 1:2 dilution steps below the individual threshold concentration that had been determined in a preliminary sensory experiment. The geometric mean of the last and the second to 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. Values between individuals and separate sessions differed by not more than two dilution steps.

Taste Profile Analysis. For taste profile analysis of the water-soluble cheese extract, the WSE lyophilisate was dissolved in bottled water in "natural" cheese concentration, and the pH value was adjusted to that of the cheese matured for 4 (pH 5.6) and 44 weeks (pH 5.7), respectively, by adding trace amounts of a 1% aqueous solution of formic acid. An aqueous 1:4 dilution of this stock solution was then presented to the sensory panelists, who were asked to rate the intensity of the individual taste qualities given in **Figure 1** on a scale from 0 (not detectable) to 5 (strongly detectable).

Tastant Spiking of Cheese Sample GC4. Cheese sample GC4 was spiked with the taste compounds in amounts required to reach the concentrations determined in the matured cheese sample, GC44. To achieve this, a tastant cocktail containing the difference in the concentration of each compound between samples GC4 and GC44 (*9*) was suspended with water (1.0 mL) and thoroughly mixed with a sample (20 g) of GC4 in a mortar to give the spiked sample GC4$^+$. After homogenization in a mortar, the resulting solid cheese mixture was wrapped in cling film, pressed into shape, and physically matured overnight at 6 °C. In addition, an aliquot of the sample GC4$^+$ was spiked with a cocktail of the α-L-glutamyl and γ-L-glutamyl dipeptides, containing the difference in the concentration of each peptide between samples GC4 and GC44, to afford sample α/γ-rGC4$^+$. The spiked cheese samples, GC4$^+$ and α/γ-rGC4$^+$, and, in addition, the 44-week-matured cheese sample, GC44, all treated in the same way as described above without the addition of any other tastants, were then evaluated by means of the taste profile analysis.

Taste Omission Experiments. To evaluate the individual taste contribution of the α-L-glutamyl and γ-L-glutamyl dipeptides, partial taste recombinants were prepared by omitting either individual tastant groups or single taste compounds from the total taste recombinant, rWSE44. Each of the partial recombinants was presented to the panelists in comparison with two samples of the total taste recombinant using a triangle test. Panelists were asked to evaluate whether the solutions were identical in the overall taste or not. Those panelists, who detected the odd sample correctly, were asked to rate the intensity of the given taste descriptors of that sample on a scale from 0 (not detectable) to 5 (strongly detectable).

Gel Permeation Chromatography (GPC) and Localization of Kokumi Compounds. An aliquot (1.0 g) of the WSE44 lyophilisate was dissolved in water (10 mL); the pH was adjusted to 4.0 with aqueous formic acid (1% in water) and, after filtration, was placed on top of an XK 50/100 glass column (Pharmacia, Uppsala, Sweden) filled with a slurry of Sephadex G15 (Amersham Bioscience, Uppsala, Sweden) in water (pH 4.0). Monitoring the effluent (2.5 mL/min) at 220 nm by means of an L-7420-type UV−vis detector (Merck Hitachi, Darmstadt, Germany) allowed the collection of seven fractions (I−VII) as given in **Figure 2**, which, after freeze-drying, were stored at −20 °C until use. To locate fractions inducing a kokumi activity, aliquots of the individual GPC fractions were dissolved in water (for intrinsic taste) and in the aqueous taste recombinant solution rWSE44, respectively, in the same ratio as these have been isolated from the cheese sample GC44 (calculated by the yield). Thereafter, the solutions of the individual GPC fractions in water or the recombinant rWSE44 were sensorially evaluated against water or the rWSE44 solution lacking any additive (control). Panelists were asked to evaluate the taste of the GPC fractions in water and to describe the taste difference between the rWSE44 solutions containing and lacking the GPC fractions, respectively. As the rWSE44 solutions spiked with GPC fractions II and III, respectively, exhibited a strong kokumi sensation when compared to the control, these GPC fractions were used for a HPLC-MS/MS profiling. As γ-L-glutamyl-L-leucine and γ-L-glutamyl-L-valine have been recently reported as kokusmi compounds in beans (*11*), the MS/MS-based screening was focused primarily on glutamyl peptides. To achieve this, aliquots of the GPC fractions II and III were dissolved in water, membrane filtered, and injected into the HPLC-MS/MS system equipped with a 250 × 2.0 mm, 5 μm, MonoChrom MS column (Varian, Darmstadt, Germany). The solvent system consisted of acetonitrile (solvent A) and water (solvent B), both containing 1% formic acid. Operating at a flow rate of 0.2 mL/min, chromatography was started with 100% solvent B for 10 min; the content of solvent A was increased within another 10 min from 0 to 10% and then to 100% within another 25 min. By comparison of MS data and retention times with that of the corresponding reference substance, followed by cochromatography, 10 γ-glutamyl peptides, namely, γ-Glu-Glu, γ-Glu-Met, γ-Glu-His, γ-Glu-Gln, γ-Glu-Ala, γ-Glu-Gly, γ-Glu-Leu, γ-Glu-Val, γ-Glu-Tyr, γ-Glu-Phe, and 8 α-glutamyl peptides, namely, α-Glu-Gly, α-Glu-Thr, α-Glu-Val, α-Glu-Glu, α-Glu-Ala, α-Glu-Asp, α-Glu-Tyr, and α-Glu-Trp, were identified in the kokumi-active GPC fractions II and III, respectively.

Quantitative Analysis of Glutamyl Peptides. Lyophilized WSE4 and WSE44, respectively, were dissolved in deionized water (approximately 25 mg/mL), membrane filtered, and aliquots (5 μL) were injected into the HPLC-MS/MS system using the identical parameters given above. As shown in **Figure 3**, the following peptides were analyzed using the mass transitions given in parentheses: the γ-glutamyl dipeptides γ-Glu-Glu (*m/z* 277.4→84.0), γ-Glu-Met (*m/z* 279.4→150.0), γ-Glu-His (*m/z* 285.4→156.2), γ-Glu-Gln (*m/z* 276.4→147.2), γ-Glu-Ala (*m/z* 219.3→90.0), γ-Glu-Gly (*m/z* 205.3→84.0), γ-Glu-Leu (*m/z* 261.4→86.1), γ-Glu-Val (*m/z* 247.4→72.0), γ-Glu-Tyr (*m/z* 311.4→136.1), and γ-Glu-Phe (*m/z* 295.4→166.1), and the α-glutamyl peptides α-Glu-Gly (*m/z* 205.3→84.0), α-Glu-Thr (*m/z* 249.2→120.1), α-Glu-Val (*m/z* 247.4→72.0), α-Glu-Glu (*m/z* 276.4→147.2), α-Glu-Ala (*m/z* 219.3→90.0), α-Glu-Asp (*m/z* 263.2→134.0), α-Glu-Tyr (*m/z* 311.4→136.1), and α-Glu-Trp (*m/z* 334.2→188.1), respectively. Quantitative analysis was performed in triplicates by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions of each reference peptide.

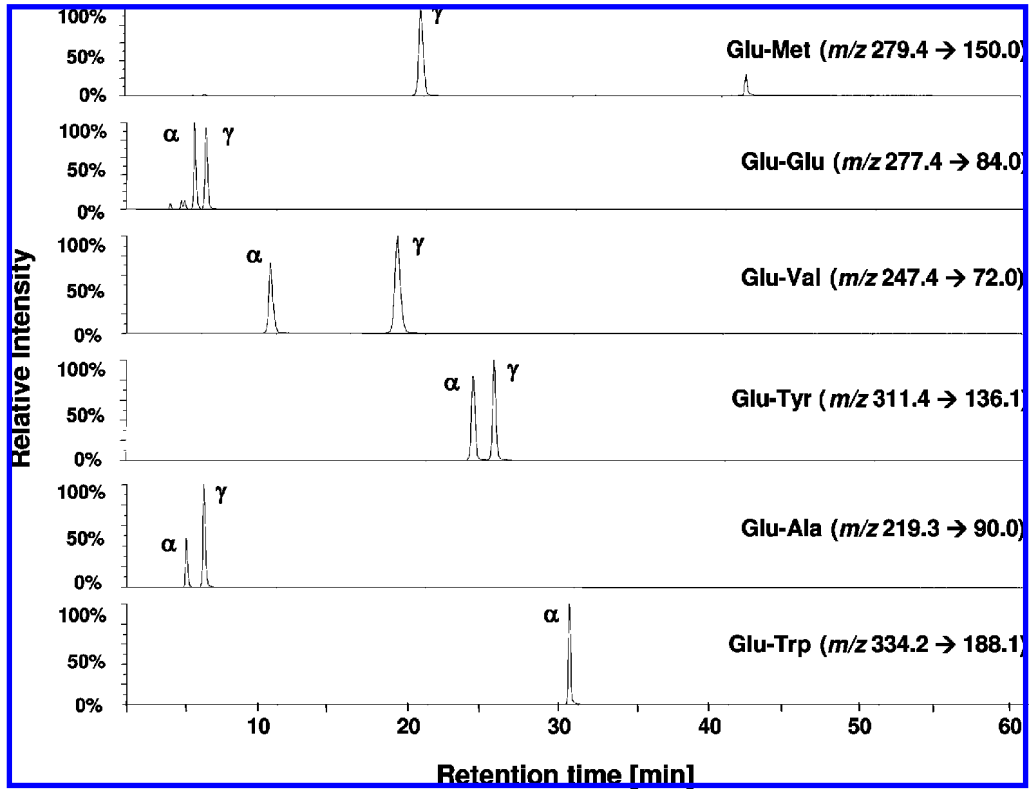

Figure 3. LC-MS/MS (MRM) analysis of selected α- and γ-glutamyl dipeptides in WSE44 prepared from the 44-week-matured Gouda cheese (GC44).

Dry Matter Content. The dry matter content was determined in a vacuum drying oven kept at 65 °C as reported in the literature (*12*).

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). LC-MS/MS determinations of L-glutamyl peptides were acquired on an API 4000 Q-Trap LC-MS/MS system (Applied Biosystems Sciex Instruments, Darmstadt, Germany) connected to an 1100 series HPLC system from Agilent (Waldbronn, Germany) operating in the positive electrospray ionization mode. Detection of α-glutamyl and γ-glutamyl dipeptides was performed in the multiple-reaction monitoring (MRM) mode using the transitions described above. Ion spray voltage was set at 5500 eV, and nitrogen served as curtain gas (1.38 bar).

RESULTS AND DISCUSSION

Previously performed taste reconstitution experiments revealed that the intensity and quality of the five basic taste modalities of the biomimetic water-soluble extract (rWSE44) were almost identical to that of the water-soluble extract (WSE44) obtained from an authentic 44-week-matured Gouda cheese (*9*). However, the sensory panel observed a much more pronounced mouthfulness, thickness, and complex body, as well as a more long-lasting taste sensation of WSE44 when compared to the artificial recombinant. Molecules inducing that type of mouthfulness and thickness and increasing continuity of food taste perception were coined by the Japanese as "kokumi" compounds (*13*, *14*). To identify the molecules imparting the kokumi activity in the matured cheese, first, the taste profiles of the water-soluble extract of 4-week- (WSE4) and 44-week-matured cheeses (WSE44) as well as those of the corresponding taste recombinants (rWSE4, rWSE44) were re-evaluated.

Sensory Re-evaluation of the Taste Profiles of WSE4/44 and rWSE4/44. On the basis of the quantitative data reported recently (*9*), the aqueous basic taste recombinants rWSE4 and rWSE44 of the water-soluble extract of both cheeses (WSE4/44) were prepared, and the taste profiles of these taste recombinants were compared with those of the WSEs. The sensory data revealed the highest ratings for the kokumi sensation in the WSE44 sample; for example, the kokumi sensation was judged with an intensity of 4.0 in WSE44 and with a value of only 2.0 for its recombinant solution rWSE44 (**Figure 1**). These data confirmed the lack of some kokumi compounds in the biomimetic rWSE44 when compared to the authentic WSE44. In comparison, WSE4 showed only a minor kokumi activity judged with an intensity of 1.5 and was very close to the kokumi intensity (1.2) found for the corresponding recombinant solution rWSE4. On the basis of these data, it can be concluded that the kokumi intensity of the 44-week-matured cheese is higher than that of the young, 4-week-ripened cheese and that the molecules imparting the kokumi sensation are generated during the ripening process.

Screening and Identification of Kokumi Compounds in WSE44. To comprehensively map and locate potential candidates imparting the kokumi sensation of the matured cheese, WSE44 was fractionated by GPC using Sephadex G-15 as the stationary and water (pH 4.0) as the mobile phase (**Figure 2**). The seven GPC fractions I−VII were collected and freeze-dried, and an aliquot of each individual fraction was dissolved in water to evaluate the intrinsic taste of the fractions. Whereas GPC fractions I, VI, and VII were entirely tasteless and GPC fractions IV and V exhibited slight saltiness, the aqueous solution of GPC fraction II was described as slightly sour and somewhat bitter, and GPC fraction III imparted strong saltiness and induced a comfortable umami sensation (**Table 1**).

To locate potential kokumi compounds in the matured cheese, in addition, aliquots of the seven GPC fractions were dissolved in the aqueous taste recombinant rWSE44 in their "natural" concentration, and these solutions were then sensorially compared to the aqueous rWSE44 solution lacking any additive (control). Among the seven GPC fractions, only the addition of fraction II and, to a somewhat lower extent, fraction III

Table 1. Sensory Evaluation of Single GPC Fractions in Water and in an Aqueous Solution of the Basic Taste Recombinant rWSE44, Respectively

	sensory evaluation of the	
fraction added	basic taste (in water)[a]	kokumi sensation (in rWSE44)[b]
I	tasteless	no kokumi sensation
II	slightly sour slightly bitter	more rich and complex strong kokumi sensation
III	salty, umami slightly bitter	slightly richer, strongly salty slight kokumi sensation
IV	slightly salty	no kokumi sensation
V	slightly salty	no kokumi sensation
VI	tasteless	no kokumi sensation
VII	tasteless	no kokumi sensation

[a] The GPC fractions were dissolved in bottled water and adjusted to pH 5.7 with trace amounts of formic acid (1% in water). [b] The GPC fractions were dissolved in the basic taste recombinant solution (rWSE44) and adjusted to pH 5.7; a rWSE44 solution lacking any additive was used as the control.

induced a kokumi sensation when added to the rWSE44 solution (**Table 1**). LC-MS/MS profiling of all the GPC fractions and comparison with reference compounds indicated the presence of major amounts of L-glutamyl peptides, namely, the 10 γ-L-glutamyl peptides γ-Glu-Glu, γ-Glu-Met, γ-Glu-His, γ-Glu-Gln, γ-Glu-Ala, γ-Glu-Gly, γ-Glu-Leu, γ-Glu-Val, γ-Glu-Tyr, and γ-Glu-Phe and the 8 α-glutamyl peptides α-Glu-Gly, α-Glu-Thr, α-Glu-Val, α-Glu-Glu, α-Glu-Ala, α-Glu-Asp, α-Glu-Tyr, and α-Glu-Trp, exclusively in the kokumi-active GPC fractions II and III. The other GPC fractions were either free of these dipeptides or contained them in trace amounts only. As γ-L-glutamyl-L-leucine and γ-L-glutamyl-L-valine (*11*) have been recently reported for their kokumi properties, the γ-L-glutamyl peptides detected in GPC fractions II and III were suggested as the kokumi compounds in matured cheese. Although some α-L-glutamyl peptides have been found in Grana Padano (*15*) and γ-Glu-Phe, γ-Glu-Leu, and γ-Glu-Tyr have been reported in Comté cheese (*4*), the potential role of the latter peptides as taste contributors is unclear. As the concentrations of γ-Glu-Phe, γ-Glu-Leu, and γ-Glu-Tyr in Comté cheese were found to be far below their taste detection thresholds, the authors assumed that these peptides do not contribute alone to the cheese taste but might contribute in an interaction with other components to the "complex flavor of cheese" (*4*). To investigate the potential contribution of glutamyl peptides to the mouthfulness and kokumi sensation imparted by a matured cheese, these compounds were quantitatively determined in the cheese samples GC4 and GC44.

Quantitative Analysis of L-Glutamyl Dipeptides in Gouda Cheese. Prior to quantitative analysis, the intensities of the detected pseudo-molecular ions $[M + H]^+$ and the respective fragments were optimized for reference compounds for the 10 γ-glutamyl peptides and the 8 α-glutamyl peptides identified above by software-assisted ramping of the ion path and collision cell settings. Full chromatographic separation of the α-glutamyl and γ-glutamyl peptides failed on an RP18 column, but was successfully achieved by using pentafluorophenylpropyl material as the stationary phase (**Figure 3**).

Quantitative analysis of the glutamyl peptides in the cheese samples revealed that all 18 peptides were present in the 44-week-matured Gouda cheese (GC44), whereas only 13 peptides were detectable at significantly lower amounts in the cheese ripened for only 4 weeks (GC4) (**Table 2**). In the GC44 sample, α-Glu-Glu and α-Glu-Asp were the quantitatively predominating

Table 2. Concentration of α- and γ-Glutamyl Dipeptides in 4-Week-Matured (GC4) and 44-Week-Matured Gouda Cheese (GC44) and Intrinsic Orosensory Threshold Concentrations

	concn (μmol/kg of dw)[a] in			
peptide	GC4	GC44	ratio GC44/GC4	TC (μmol/kg)
γ-Glu-Glu	0.19 (0.11[b])	27.57 (17.66[b])	145.1	5000[c] 10000[d]
γ-Glu-Met	0.71 (0.41[b])	20.27 (12.99[b])	28.5	2500[c]
γ-Glu-His	0.26 (0.15[b])	6.41 (4.11[b])	24.7	2500[c]
γ-Glu-Gln	0.60 (0.34[b])	13.85 (8.87[b])	23.1	2500[c]
γ-Glu-Ala	0.17 (0.10[b])	2.97 (1.90[b])	17.5	900[c]
γ-Glu-Gly	3.32 (1.90[b])	6.26 (4.01[b])	1.9	1250[c]
γ-Glu-Leu	nd	7.10 (4.55[b])		9400[c]
γ-Glu-Val	nd	2.62 (1.68[b])		3300[c]
γ-Glu-Phe	nd	2.00 (1.28[b])		2500[c]
γ-Glu-Tyr	nd	0.31 (0.20[b])		2500[c] 5000[e]
α-Glu-Gly	0.37 (0.21[b])	2.92 (1.87[b])	7.9	2500[c]
α-Glu-Thr	0.49 (0.28[b])	3.22 (2.06[b])	6.6	2500[c]
α-Glu-Val	0.72 (0.41[b])	4.42 (2.82[b])	6.1	5000[c]
α-Glu-Glu	10.93 (6.27[b])	60.65 (38.85[b])	5.5	2500[d]
α-Glu-Ala	0.64 (0.37[b])	2.56 (1.64[b])	4.0	10000[c]
α-Glu-Asp	4.86 (2.79[b])	18.38 (11.77[b])	3.8	1250[c]
α-Glu-Tyr	0.82 (0.47[b])	1.22 (0.78[b])	1.5	5000[c,e]
α-Glu-Trp	nd	0.12 (0.07[b])		5000[c,e]

[a] Concentration is given on a dry weight basis. nd, not detectable. [b] Concentration is given on a wet weight basis. [c] Threshold concentration for an unspecific, slightly astringent mouth coating. [d] Threshold concentration for umami-like taste;. [e] Threshold concentration for bitter taste.

α-glutamyl dipeptides found in concentrations of 60.65 and 18.38 μmol/kg (dm), whereas γ-Glu-Glu, γ-Glu-Met, and γ-Glu-Gln with concentrations of 27.57, 20.27, and 13.85 μmol/kg (dm) were the most abundant representatives among the γ-glutamyl dipeptides. A comparison of the concentrations of these peptides in the 4-week- and 44-week-matured cheese samples indicated that the ripening of the cheese favored the generation of the γ-glutamyl dipeptides more than that of the α-glutamyl peptides; for example, the concentration of γ-Glu-Glu was increased by a factor of 145.1, whereas only 5.5 times higher amounts were found for the corresponding α-Glu-Glu (**Table 2**).

These data indicate that different mechanisms are involved in the generation of α- and γ-L-glutamyl dipeptides. As the caseins contain high amounts (23.4 g/100 g) of L-glutamic acid (*16*) and the amino acid sequence α-L-glutamyl-L-glutamic acid is represented multifold in the milk proteins, particularly in α_{s2}-casein, the α-glutamyl dipeptides are expected to be formed by proteolytic breakdown of caseins. In contrast, the γ-glutamyl dipeptides might be enzymatically generated by a γ-glutamyl transpeptidase or γ-glutamyl transferase (GGT) from amino acids released upon protein hydrolysis. The high concentrations of γ-Glu-Glu are well in line with the finding that L-glutamic acid is one of the major free amino acids in semihard cheeses (*17*). Although the presence of GGT in cheese was suggested earlier (*18*), there is only one study experimentally confirming the GGT activity in cheese (*19*).

Sensory Studies on L-Glutamyl Dipeptides. Prior to sensory analysis, the purity of all compounds was confirmed by HPLC-MS as well as ^{1}H NMR spectroscopy to be >98%. To evaluate the sensory activity of the peptides, first, human threshold concentrations for the intrinsic taste of these peptides were determined in water by means of an ascending triangle test. With the exception of α-Glu-Glu and γ-Glu-Glu, imparting a umami-like sensation with threshold concentrations of 2.5 and 10.0 mmol/kg, respectively, the α- as well as the γ-glutamyl peptides were found to exhibit an unspecific, slightly astringent oral sensation with relatively high threshold concentrations

Table 3. Orosensory Profile of the Water Extract (WSE44), the Basic Taste Recombinant (rWSE44), the Recombinant Including 18 Glutamyl Peptides (α/γ-rWSE44), the 44-Week-Matured Gouda Cheese (GC44), the 4-Week-Matured Gouda Cheese Spiked with the Difference in the Concentration of Each Taste Compound between GC44 and GC4 ($GC4^+$), and $GC4^+$ Spiked with the Difference in the Concentration of the 18 Glutamyl Peptides between GC44 and GC4 (α/γ-$GC4^+$)

	intensities for individual taste qualities[a] in					
	aqueous matrix			cheese matrix		
taste quality	WSE44	rWSE44	α/γ-rWSE44	GC44	$GC4^+$	α/γ-r$GC4^+$
sourness	2.0	1.6	1.6	2.0	1.8	1.8
bitterness	2.5	2.5	2.5[b]	3.0	2.9	2.9[b]
umami	2.0	1.7	2.0	2.0	2.0	2.0
saltiness	4.5	4.4	4.4	4.0	3.7	3.8
sweetness	0	0	0	0.5	0.5	0.5
mouthfulness	4.0	2.0	3.8	4.0	2.3	3.9

[a] Intensities were judged on a scale from 0 (not detectable) to 5 (strongly detectable) and are given as the mean of triplicates. The values of the individual panelists determined in three independent sessions differed by not more than ±0.3 unit. [b] The bitter quality was perceived as less harsh and more pleasant when compared to rWSE44.

between 0.9 (γ-Glu-Ala) and 10.0 mmol/kg (α-Glu-Ala) (**Table 2**). In addition, the peptides γ-Glu-Tyr, α-Glu-Tyr, and α-Glu-Trp exhibited some bitter taste at concentrations above 5.0 mmol/kg. These findings are in contrast to the sour taste reported by other groups (*20, 21*), but all previous studies were performed at much lower pH values (pH <4.0) than in the present investigation (pH 5.7). The concentrations of the α- as well as the γ-glutamyl peptides in both cheese samples were found to be between 100 and 1000 times below the determined intrinsic taste threshold concentrations, thus excluding a direct contribution of these peptides to the taste profile of the cheese samples.

As γ-glutamyl peptides were expected to be kokumi-active in subthreshold concentrations, reconstitution experiments were performed to investigate their impact on the taste profile of the cheese. To achieve this, first, the 18 α/γ-glutamyl dipeptides were added in their "natural" concentrations to the aqueous basic taste recombinant rWSE44 and, using a triangle test, the sensory panel was asked to pick up and evaluate the difference between this total recombinant (α/γ-rWSE44) and the rWSE44 solution lacking these peptides (**Table 3**). As all of the panelists were able to detect the odd sample, the sensory panel was asked to judge the intensity of the basic taste modalities as well as the kokumi sensation on a scale from 0 (not detectable) to 5 (strongly detectable). The addition of "natural" amounts of the glutamyl peptide mixture to the basic taste recombinant rWSE44 affected positively the kokumi sensation; for example, the kokumi intensity was increased from 2.0 to 3.8 units and reached almost the kokumi intensity of the authentic aqueous cheese extract WSE44 (**Table 3**). In addition, the panelists reported that the bitterness quality was modified and was perceived as less harsh and more pleasant in the α/γ-rWSE44 solution when compared to rWSE44 lacking these peptides.

To further confirm the importance of the identified glutamyl peptides for the typical mouthfulness and continuity of the 44-week-matured cheese and to investigate the influence of the cheese matrix in taste perception, the aim of the following experiment was to perform a taste re-engineering experiment using the 4-week-matured cheese (GC4) as a suitable matrix (*9*). The difference in the concentration of each basic tastant between the GC4 and GC44 samples was calculated, and a cocktail of these compounds was added to a defined portion of GC4 to give the sample $GC4^+$ following the protocol reported recently (*9*). In addition, an aliquot of the $GC4^+$ sample was spiked with the difference in the concentration of the 18 glutamyl peptides between GC44 and GC4 to give the total taste recombinant α/γ-$GC4^+$. After homogenization and physical maturation, this sample was sensorially evaluated in comparison to GC44, which was treated in the same way but without the addition of the tastant cocktail (**Table 3**). The basic taste modalities of the spiked Gouda sample $GC4^+$ matched rather well with the authentic cheese matured for 44 weeks (GC44), but the sensory panel picked up a difference in the kokumi impact. The presence of the glutamyl peptide mixture in the sample α/γ-$GC4^+$ was detectable by all panelists and was described with an increase of the kokumi sensation (+1.6) (**Table 3**). Fitting well with the aqueous recombination experiments, the kokumi sensation of the peptide-containing sample α/γ-r$GC4^+$ evaluated with an intensity of 3.9 units was comparable with that the authentic cheese sample GC44 (4.0 units), thus confirming the key contribution of the α/γ-glutamyl peptides to the orosensory perception of a matured cheese. In addition, the bitterness of α/γ-r$GC4^+$ was evaluated by the sensory panelists as more pleasant and was described to fit very well with the bitter taste profile of the matured Gouda cheese sample GC44. Moreover, the panelists reported that the taste of the sample α/γ-r$GC4^+$ was perceived as more long-lasting and lingering when compared to that of r$GC4^+$ and matched very well the continuity of the orosensory perception of the ripened cheese GC44.

Omission Experiments with L-Glutamyl Peptides. To gain more insight into the flavor contribution of α- and γ-glutamyl peptides as well as the congruent basic taste compounds required to induce the kokumi sensation, omission experiments were performed in aqueous solution of the total taste recombinant α/γ-rWSE44. Omission of both the α- and γ-glutamyl peptides induced a highly significant decrease in kokumi impact (3.8→2.0) as well as some reduction of the umami intensity (**Table 4**), thus confirming the key function of glutamyl peptides for the mouthfulness of the α/γ-rWSE44.

In a second experiment, a taste recombinant was prepared by omitting the group of the 8 α-glutamyl peptides. Only a slight decrease in kokumi and umami intensity (−0.2) was observed (**Table 4**). Omission of the group of 10 γ-glutamyl peptides induced a significant decrease of the kokumi sensation (−1.5 units), which unambiguously identified them as the key drivers of this oral sensation. Moreover, the umami intensity was slightly negatively affected (−0.2). To answer the question as to whether the quantitatively predominant γ-glutamyl peptides are suitable to induce the kokumi sensation, a partial recombinant was prepared lacking the minor γ-glutamyl peptides found in cheese in concentrations below 4.0 μmol/kg (**Table 4**). As the sensory panel was not able to distinguish between this partial recombinant and α/γ-rWSE44 containing all 18 glutamyl peptides, the number of kokumi-active key peptides was narrowed to a total of 6 γ-L-glutamyl dipeptides, namely, γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, respectively.

To identify the congruent basic taste compounds required for the kokumi activity of the γ-L-glutamyl peptides, individual tastant groups, namely, a bitter-tasting group I containing the bitter L-amino acids and peptides, as well as $CaCl_2$ and $MgCl_2$, the umami-tasting group II containing L-glutamic acid, L-aspartic acid, L-glutamine, and L-asparagine, and the salty/sour tasting group III containing NaCl, KCl, lactate, and phosphate as well as group IV containing the sweet-tasting L-amino acids were, respectively, omitted from the taste recombinant rWSE44, and the partial recombinants obtained were tasted with and without

Table 4. Influence of the Omission of α- and γ-Glutamyl Peptides on the Taste Profile of the Total Taste Recombinant α/γ-rWSE44

group omitted	S[e]	description of taste difference[f] (change in intensity)
α/γ-glutamyl peptides[a]	***	decrease in kokumi/mouthfulness (3.8→2.0) and umami (2.0→1.7)
α-glutamyl peptides[b]	**	decrease in kokumi/mouthfulness (3.8→3.6) and umami (2.0→1.8)
γ-glutamyl peptides[c]	***	decrease in kokumi/mouthfulness (3.8→2.3) and umami (2.0→1.8)
γ-glutamyl peptides[d] (<4.0 μmol/kg)	nd	

[a] The 18 α-/γ-glutamyl peptides given in **Table 2** were omitted from α/γ-rWSE44. [b] The 8 α-glutamyl peptides given in **Table 2** were omitted from α/γ-rWSE44. [c] The 10 γ-glutamyl peptides given in **Table 2** were omitted from α/γ-rWSE44. [d] All of the minor glutamyl peptides with concentrations below 4.0 μmol/kg (**Table 2**) were omitted from α/γ-rWSE44. [e] Significance of triangle test (participation of six panelists): ***, very highly significant ($p < 0.001$); **, very significant ($p < 0.05$); *, significant ($p < 0.01$). [f] Partial recombinants lacking certain tastants were presented to the panel by means of a triangle test. If sample was correctly chosen, differences in taste intensities should be evaluated on a scale from 0 (not detectable) to 5 (strongly detectable). The values of the individual panelists determined in three independent sessions differed by not more than ±0.3 unit.

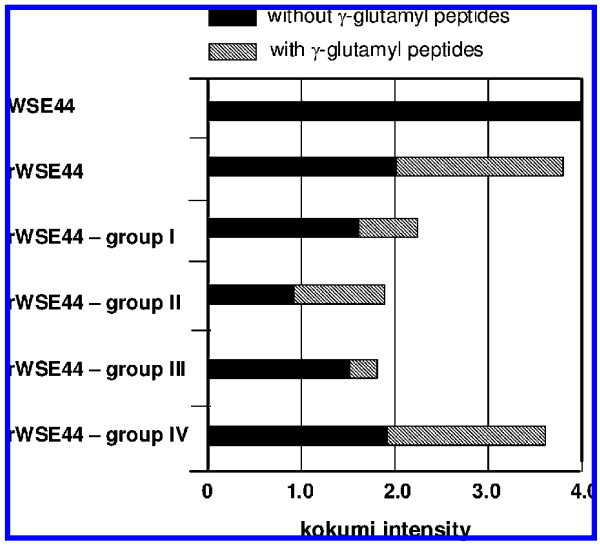

Figure 4. Influence of the "natural" concentration of the γ-glutamyl peptides γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His on the kokumi intensity of the taste recombinant rWSE44 (pH 5.7) as well as partial recombinants (pH 5.7) lacking tastant group I (bitter compounds), group II (umami compounds), group III (salty/sour compounds), and group IV (sweet compounds), respectively.

the addition of the 6 kokumi-active γ-glutamyl peptides γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, respectively (**Figure 4**). As the omission of the tastant group I (bitter) and group III (sour/salty), and to a smaller extent also group II (umami), resulted in only a minor increase of the kokumi intensity by addition of the γ-glutamyl peptides when compared to the total recombinant, this sensory experiment revealed the bitter and the sour/salty as well as the umami components as important congruent molecules required for the kokumi effect. The kokumi intensity of the partial recombinant lacking the bitter group I and the umami group II was judged to be increased by only 0.5 and 0.9 point, respectively, when the γ-glutamyl peptides were added. Even lower (+0.2 unit) was the effect of the γ-glutamyl peptides when the sour/salty group III was lacking. Interestingly, the sweet compounds in group IV increased by almost 2 points, indicating that they show a comparatively negligible effect for the kokumi sensation. Consequently, the congruent basic tastants for the kokumi activity of the γ-glutamyl peptides were identified to be among the bitter and the umami as well as the sour/salty components located in groups I−III, respectively.

To further narrow the number of congruent basic tastants, a salty-tasting biomimetic mixture of the chlorides of magnesium, calcium, and potassium (mix A) and a sour/salty tasting mixture of the sodium salts of phosphate and lactate (mix B) as well as

Table 5. Kokumi Intensity of Tastant Mixtures (pH 5.7) in the Absence and Presence of the Six Major γ-Glutamyl Peptides

	kokumi intensity[g]	
tastant mix	without glutamyl peptides	with glutamyl peptides[h]
mixture A[a]	0	0
mixture B[b]	0	0
mixture C[c]	0.3	0.5
mixture C + NaOH[d]	1.5	2.5
mixture C + KOH[e]	0.3	0.5
mixture C + NaCl[f]	1.9	3.4

[a] Tastant mixture A contained the chlorides of magnesium, calcium, and potassium in the same concentrations as in rWSE44. [b] Tastant mixture B contained the sodium salts of phosphate and lactate in the same concentrations as in rWSE44. [c] Tastant mixture C contained all determined amino acids in the same concentrations as in rWSE44. [d] NaOH was added to mixture C, and pH value was adjusted to pH 5.7 by using 1% aqueous formic acid. [e] KOH was added to mixture C, and pH value was adjusted to pH 5.7 by using 1% aqueous formic acid. [f] NaCl was added to mixture C, and pH value was adjusted to pH 5.7 by using 1% aqueous formic acid. [g] The values of the individual panelists determined in three independent sessions differed by not more than ±0.3 unit. [h] A cocktail of the peptides γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His was added to the tastant mixtures in the same concentrations as used in α/γ-rWSE44.

a mixture of all the amino acids (mix C) were prepared and sensorially evaluated against the corresponding tastant mixtures containing the γ-glutamyl peptides γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His. The panelists were asked to evaluate the kokumi sensation on a scale from 0 (not detectable) to 5 (strongly detectable) with the total recombinant as reference (**Table 5**). Whereas mixtures A and B did not show any kokumi activity at all, the amino acid containing tastant mixture C exhibited a slight kokumi sensation, which was not significantly influenced when the γ-glutamyl peptides were added (**Table 5**). To investigate the influence of additional minerals on the kokumi sensation, sodium ions were introduced to mixture C in the same concentrations as used in rWSE44 in the absence or presence of the γ-glutamyl peptides. The basic tastant mixture was judged with a rather intense kokumi activity (1.5 units), which was impressively increased to an intensity of 2.5 when the γ-glutamyl peptides were added (**Table 5**).

In a comparative experiment, sodium was substituted by an equimolar amount of potassium, but the kokumi activity of this mixture was marginally influenced by the addition of γ-glutamyl peptides only (**Table 5**). Consequently, the sodium ion was identified as a congruent counterpart required for the kokumi activity of the γ-glutamyl peptides.

In a final experiment, sodium chloride was added to mixture C to evaluate the influence of sodium and chloride ions on the kokumi activity in the absence and presence of γ-glutamyl peptides. Mixture C spiked with NaCl exhibited a kokumi

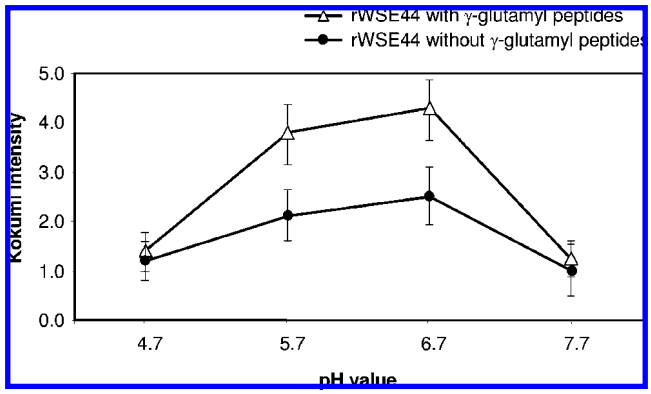

Figure 5. Influence of the pH value on the kokumi intensity of the basic taste recombinant rWSE44 in the absence and presence of natural amounts of the γ-glutamyl peptides γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, respectively. The standard deviation was determined from triplicates.

intensity of 1.9 units, which was only slightly higher that that detected for the NaOH-containing mixture C lacking any chloride ions. Upon addition of the γ-glutamyl peptides, the kokumi intensity was increased from 1.9 to 3.4 units and was almost the same as the kokumi activity of α/γ-rWSE44 (3.8 units) (**Table 5**). As a consequence, NaCl and amino acids are the congruent basic tastants required for the kokumi-enhancing activity of the γ-glutamyl peptides, thus confirming recent findings on the mouthfulness-enhancing activity of γ-glutamyl peptides on sodium chloride/L-glutamate mixtures in beans (*11*).

Influence of the pH Value on the Kokumi Activity of Glutamyl Peptides. To investigate the influence of the pH value on the kokumi activity of the γ-glutamyl peptides, the basic taste recombinant rWSE44 was spiked with the γ-glutamyl peptides and the pH values of the solutions were adjusted to 4.7, 5.7, 6.7, and 7.7, respectively, using trace amounts of formic acid (1% in water) or aqueous KOH (1 mol/L). In addition, the rWSE44 solution lacking the γ-glutamyl peptides was adjusted to exactly the same pH value and used as control. The panelists were asked to evaluate the kokumi sensation of these solutions on a scale from 0 (not detectable) to 5 (strongly detectable) with the total recombinant as reference (**Figure 5**). At pH 5.7, the pH value of the WSE44 solution, the kokumi sensation of rWSE44 was evaluated with intensities of 2.0 and 3.8 in the absence and presence of the peptides, respectively, thus corresponding to an increase of 1.8 units induced by the peptides. A similar increase in kokumi intensity of 1.9 units was found when the experiment was repeated at pH 6.7. In contrast, no significant kokumi activity of the γ-glutamyl peptides was observed at a lower pH value of 4.7 as well as at a higher pH value of 7.7, thus demonstrating that the orosensory activity of these peptides is strongly dependent on the pH value.

Kokumi Threshold Concentrations of Glutamyl Peptides in rWSE44. To determine the minimum concentrations required for the kokumi activity of γ-glutamyl peptides in cheese, recognition threshold concentrations were determined for single γ-glutamyl peptides, namely, γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, by means of a triangle test using rWSE44 as the matrix (**Table 6**). The sensory studies revealed that γ-Glu-Met and γ-Glu-Leu exhibited the lowest recognition threshold of 5 μmol/kg in the presence of the basic cheese taste compounds, whereas the other four γ-glutamyl peptides showed threshold concentrations ranging between 7.5 and 17.5 μmol/kg, respectively (**Table 6**). As the concentration of the six key kokumi peptides γ-Glu-Glu (17.66 μmol/kg), γ-Glu-Gly (1.90 μmol/kg), γ-Glu-Gln (8.87 μmol/kg), γ-Glu-Met (12.99 μmol/kg), γ-Glu-Leu (4.55 μmol/kg), and γ-Glu-His (4.11 μmol/kg) in cheese sample GC44 (**Table 2**) is about 50 μmol/kg (fresh weight) in total, the mean threshold concentration of the γ-glutamyl peptides is significantly exceeded in the WSE44, thus confirming for the first time the orosensory contribution of these peptides to the kokumi sensation of the 44-week-matured cheese. As these γ-glutamyl peptides might have important implications also to the taste profile of other dairy products, studies on the biogeneration of these kokumi peptides during cheese ripening are currently in progress.

Table 6. Kokumi Threshold Concentrations for Selected Glutamyl Peptides in rWSE44

glutamyl peptide	kokumi threshold concn[a] (μmol/kg)
γ-Glu-Glu	17.5
γ-Glu-Gly	17.5
γ-Glu-His	10.0
γ-Glu-Gln	7.5
γ-Glu-Met	5.0
γ-Glu-Leu	5.0

[a] The recognition threshold concentrations, determined in rWSE44 by means of a triangle test, are given as the mean of triplicates.

ACKNOWLEDGMENT

We thank Friesland Foods for providing cheese samples.

LITERATURE CITED

(1) Fox, P. F.; McSweeney, P. L.; Cogan, T. M.; Guinee, T. P. Sensory Character of cheese and its evaluation. In *Cheese—Chemistry, Physics and Microbiology—General Aspects*, 3rd ed.;Elsevier Academic Press: London, U.K., 2004; pp 455−487.

(2) Biede, S. L.; Hammond, E. G. Swiss cheese flavor: II. Organoleptic analysis. *J. Dairy Sci.* **1979**, *62*, 238–248.

(3) Aston, J. W.; Creamer, L. K. Contribution of the components of the water-soluble fraction to the flavor of Cheddar cheese. *N.Z. J. Dairy Sci. Technol.* **1986**, *21*, 229–248.

(4) Roudot-Algaron, F.; Kerhoas, l.; Le Bars, D.; Einhorn, J.; Gripon, J. C. Isolation of γ-glutamyl peptides from Comté cheese. *J. Dairy Sci.* **1994**, *77*, 1161–1166.

(5) Sommerer, N.; Garem, A.; Molle, D.; Septier, C.; Le Quere, J. L.; Salles, C. Isolation of a peptidic fraction from the goat cheese water-soluble extract by nanofiltration for sensory evaluation studies. *Dev. Food Sci.* **1998**, *40*, 207–217.

(6) Molina, E.; Ramos, M.; Alonso, L.; Lopez-Fandino, R. Contribution of low molecular weight water soluble compounds to the taste of cheeses made of cows', ewes' and goats' milk. *Int. Dairy J.* **1999**, *9*, 613–621.

(7) Engel, E.; Nicklaus, S.; Septier, C.; Salles, C.; Le Quere, J. L. Taste active compounds in a goat cheese water-soluble extract. 2. Determination of the relative impact of water-soluble extract components on its taste using omission tests. *J. Agric. Food Chem.* **2000**, *48*, 4260–4267.

(8) Visser, S.; Slangen, K. J.; Hup, G.; Stadhouders, J. Bitter flavor in cheese. 3. Comparative gel-chromatographic analysis of hydrophobic peptide fractions from twelve Gouda-type cheeses and identification of bitter peptides isolated from a cheese made with *Streptococcus cremoris* strain HP. *Neth. Milk Dairy J.* **1983**, *37*, 181–92.

(9) Toelstede, S.; Hofmann, T. Quantitative studies and taste re-engineering experiments towards the decoding of the nonvolatile sensometabolome of Gouda cheese. *J. Agric. Food Chem.* **2008**, *56*, 5299–5307.

(10) Toelstede, S.; Hofmann, T. Sensomics mapping and identification of the key bitter metabolites in Gouda cheese. *J. Agric. Food Chem*. **2008**, *56*, 2795–2804.
(11) Dunkel, A.; Koester, J.; Hofmann, T. Molecular and sensory characterization of γ-glutamyl peptides as key contributors to the kokumi taste of edible beans (*Phaseolus vulgaris* L.). *J. Agric. Food Chem*. **2007**, *55*, 6712–6719.
(12) Analysis of foods—Determination of the dry weight of cheese (reference method). Official collection of analysis methods § 64 LFGB (in German), L 03.00-9, 1984.
(13) Ueda, Y.; Yonemitsu, T.; Tsubuku, T.; Sakaguchi, M.; Miyajima, R. Flavor characteristics of glutathione in raw and cooked foodstuffs. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1978–1980.
(14) Ueda, Y.; Tsubuku, T.; Miyajima, R. Composition of sulfur-containing components in onion and their flavor characters. *Biosci., Biotechmol., Biochem*. **1994**, *58*, 108–110.
(15) Sforza, S.; Ferroni, L.; Galaverna, G.; Dossena, A.; Marchelli, R. Extraction, semi-quantification, and fast on-line identification of oligopeptides in grana padano cheese by HPLC-MS. *J. Agric. Food Chem*. **2003**, *51*, 2130–2135.
(16) Belitz, H. D. *Lehrbuch der Lebensmittelchemie*, 5th ed.; Springer-Verlag: Berlin, Germany, 2001.
(17) Lavanchy, P.; Sieber, R. Proteolysis in different hard and semi-hard cheeses: 1. Amino acids (in German). *Schweiz. Milchw. Forsch*. **1993**, *22*, 59–64.
(18) Fox, P. F.; Kelly, A. L. Indigenous enzymes in milk: overview and historical aspects —Part 2. *Int. Dairy J*. **2006**, *16*, 517–532.
(19) Blel, M.; Guingamp, M.-F.; Gaillard, J.-L.; Humbert, G. Studies on the thermal sensitivity of γ-glutamyl transpeptidase measured with a modified test procedure and compared with that of alkaline phosphatase and lactoperoxidase in milk. *Lait* **2002**, *82*, 555–566.
(20) Kirimura, J.; Shimizu, A.; Kimizuka, A.; Ninomiya, T.; Katsuya, N. The contribution of peptides and amino acids to the taste of foodstuffs. *J. Agric. Food Chem*. **1969**, *17*, 689–695.
(21) Suzuki, H.; Kajimoto, Y.; Kumagai, H. Improvement of the bitter taste of amino acids through the transpeptidation reaction of bacterial γ-glutamyltranspeptidase. *J. Agric. Food Chem*. **2002**, *50*, 313–318.

Received for review October 29, 2008. Revised manuscript received December 22, 2008. Accepted December 23, 2008. We thank Friesland Foods for financial support.

JF803376D