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ARTICLE

Kokumi-Active Glutamyl Peptides in Cheeses and Their Biogeneration by *Penicillium roquefortii*

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Recently, a group of γ-glutamyl dipeptides, but not the α-glutamyl dipeptides, were found to induce the attractive kokumi flavor of matured Gouda cheese. In the present investigation, the spatial distribution of α- and γ-glutamyl dipeptides in Gouda cheese wheels and the concentration of these peptides in other cheese types were determined by means of HPLC-MS/MS. Among all cheeses investigated, by far the highest γ-glutamyl peptide concentration (3590 μmol/kg) was found for Blue Shropshire, a blue-veined cheese. To check whether the γ-glutamyl transferase (GGT) from *Penicillium roquefortii* is involved in γ-glutamyl peptide production in this cheese, the GGT activity was measured and γ-glutamyl peptides were analyzed in liquid cultures of mold isolated from Blue Shropshire as well as single *P. roquefortiii* strains incubated with the γ-glutamyl donor L-glutamine and the candidate substrates L-glutamic acid, L-histidine, L-leucine, and L-methionine. Being well in line with the GGT activity found in Blue Shropshire, *P. roquefortii* was found for the first time to produce and secrete γ-glutamyl peptides. Among the amino acids tested, L-methionine was found as a preferred γ-glutamyl acceptor; for example, γ-Glu-Met was produced in yields of about 50 mmol/mol and [$^2H_3$]-γ-Glu-Met was obtained when [$^2H_3$]-L-methionine was used as substrate amino acid.

KEYWORDS: γ-Glutamyl transferase; GGT; γ-glutamyl peptides; kokumi; taste; cheese; mouthfulness; *Penicillium roquefortii*

INTRODUCTION

Due to their attractive taste profiles, multiple studies were focused in the past on the characterization of the key compounds evoking the salty, sour, sweet, bitter, and umami taste of cheese products (*1*−*10*). Besides these basic taste modalities, ripened cheeses are highly appreciated by consumers mainly because of their desirable long-lasting mouthfulness, complexity, and continuity of taste. This sensory sensation, coined kokumi taste (*11*−*14*), was recently confirmed by means of a sensomics approach for a 44-weeks ripened Gouda cheese (*15*). A family of γ-L-glutamyl dipeptides, namely, γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met, γ-Glu-Leu, and γ-Glu-His, were found for the first time to impart an enhanced kokumi sensation perceived for the matured Gouda cheese (*15*). Carefully planned taste reconstitution and omission experiments in aqueous solution as well as in a cheese matrix undoubtedly confirmed that the γ-L-glutamyl dipeptides enhance the mouthfulness, complexity, and continuity of the typical cheese taste, whereas none of the corresponding α-glutamyl dipeptides, present in the cheese as well, were found to exhibit this kokumi activity (*15*). Quantitative analysis of glutamyl dipeptides in Gouda cheese samples matured for 4 and 44 weeks, respectively, revealed that ripening of the cheese facilitates the generation of the kokumi-active γ-glutamyl dipeptides more than that of the α-glutamyl dipeptides.

As the parameters influencing the generation of these glutamyl dipeptides in cheese are not known, the objectives of the present investigation were, first, to investigate the spatial distribution of the γ-L-glutamyl as well as the α-L-glutamyl dipeptides in a young (4 weeks) and a 44-weeks matured Gouda cheese wheel, respectively, and then to determine the concentrations of these sapid peptides in various types of cheeses made from either raw or pasteurized milk. Moreover, the ability of *Penicillium roquefortii* strains, used as ripening cultures in blue-veined cheese, to produce γ-L-glutamyl dipeptides was studied for the first time to gain insight into the parameters governing the formation of these peptides in cheese.

MATERIALS AND METHODS

Chemicals. L-Glutamine, L-glutamic acid, L-histidine, L-methionine, L-leucine, potato dextrose agar, Tris buffer, and the clarifying reagent for dairy products were purchased from Fluka (Buchs, Switzerland), L-glutamic acid γ-(*p*-nitroanilide) was from Sigma-Aldrich (Steinheim, Germany), sterile phosphate-buffered saline (PBS Dulbecco w/o Mg^{2+}, Ca^{2+}) was from Biochrom AG (Berlin, Germany), formic acid was from Grüssing (Filsum, Germany), and [$^2H_3$]-L-methionine (98%) was from Cambridge Isotope Laboratories (Andover, MA). Reference compounds of the α-L-glutamyl and γ-L-glutamyl dipeptides as well as glycyl-glycine were purchased from Bachem (Weil am Rhein, Germany). Solvents were

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of HPLC grade (Merck, Darmstadt, Germany). Deionized water used for chromatography was purified by means of a Milli-Q water Gradient A 10 system (Millipore, Billerica, MA). *P. roquefortii* strains 1079, 1080, 1999, and 1812 were obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen, DSMZ (Braunschweig, Germany). The lyophilized cultures were grown and maintained on potato dextrose agar (Biochemika, for microbiology, Fluka, Neu-Ulm, Germany) at 7 °C.

Cheese and Milk Samples. Cheese samples made from bovine milk, namely, Leerdammer (5-weeks ripened), Camembert Le Rustique (21-days ripened), Swiss Gruyere (8-months ripened), Blue Shropshire (7-weeks ripened), light ripened and extra-ripened Milner (30 weeks), and young and matured goat cheese, as well as Mouton made from ewe's milk, were obtained from a local cheese store. Gouda cheese samples ripened under controlled conditions for 4 (GC4) or 44 weeks (GC44) and 4-weeks matured Gouda cheese manufactured from raw (rGC) and pasteurized milk (pGC) of the same milk batch, respectively, as well as Kernhem, a semihard smear cheese ripened for 4 weeks, were obtained from the Dutch dairy industry. The individual cheese samples were sealed in rindless portions (50−100 g) in nitrogen-flushed packages and stored at −20 °C until use. In addition, half-wheels of 4-weeks matured Gouda cheese (GCW4; 36.4 cm in length, 11.9 cm in height, 16.5 cm in depth) and 44-weeks matured Gouda cheese (GCW44; 34.4 cm in length, 10.5 cm in height, 18.1 cm in depth) as well as raw and pasteurized milk samples were obtained from the Dutch dairy industry.

Preparation of the Water-Soluble Extract (WSE) of Cheese. Following a protocol reported recently (*9*), cheese samples (60 g) were cut into small pieces, deionized water (240 mL) was added, and the mixture was homogenized for 5 min by means of an Ultra-Turrax T 25 basic (Ika Labortechnik, Germany) and, finally, centrifuged at 10000 rpm for 20 min at 4 °C by means of a Varifuge 20 RS (Kendro, Langenselbold, Germany). To prepare the WSE from the cheese samples (15 g each) collected from the half-wheels by means of a drilling knife, the extraction was performed with deionized water (80 mL). The upper solid fat layer as well as the protein pellet formed was removed to afford the liquid layer including the cheese water-solubles. After re-extraction of the protein pellet as well as the fat layer with deionized water (240 mL), 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 WSE was obtained from each cheese sample and was stored at −20 °C until further analysis.

Spatial Distribution of Peptides in Cheese Wheels. By means of a drilling knife, six holes were drilled into the half-wheel of 4-weeks (GC4) and 44-weeks (GC44) matured Gouda cheese, respectively, as depicted in **Figure 1**. The corresponding cheese samples obtained were 6.1 cm in depth and 1.9 cm in diameter and showed a weight of about 15 g. From each sample, a WSE was prepared and used for the quantitative analysis of glutamyl dipeptides by means of HPLC-MS/MS.

Incubation Experiments with Mold Isolated from Blue-Veined Cheese. Mold samples (300 mg) were hand-picked from a sample of Blue Shropshire with a pair of tweezers, frozen in liquid nitrogen, and ground in a mortar. An aliquot (130 mg) of the powdered material was suspended in an aqueous solution (2.0 mL) containing the amino acids L-glutamic acid, L-glutamine, L-histidine, L-leucine, and L-methionine (25 mmol/L each) with or without the addition of D-glucose (5% w/w). As a control, an aqueous suspension of the powdered blue mold (130 mg in 2.0 mL) was incubated in the absence of amino acids and glucose. These mixtures were incubated in sealed glass vials in an incubator at 37 °C for up to 21 days. After 3, 7, and 21 days, aliquots (400 μL) were taken, membrane-filtrated (Spartan, 13/0.45 μm RC, Schleicher & Schuell), diluted 1:10 with water, and injected into the LC-MS/MS system tuned for the analysis of α- and γ-glutamyl dipeptides.

Inoculation Experiments with Single *P. roquefortii* Strains. After detachment of the *P. roquefortii* strains 1080, 1079, 1999, and 1812, respectively, from the potato dextrose agar with PBS buffer (4.0 mL), the mold was maintained overnight at 25 °C in a small glass vessel under agitation. An aliquot (400 μL) of this overnight culture of *P. roquefortii* and sterile-filtered test (SFT) solutions (14.0 mL) varying in amino acid composition, in pH value, and in the absence or presence of further additives were placed into autoclaved Sovirel glasses (50 mL) coated with aluminum foil. The individual sample glasses were agitated under light exclusion either at room temperature or at 37 °C in a laboratory incubator. After 5, 10, and 20 days, aliquots (1 mL) were taken, membrane-filtrated (Spartan, 13/0.45 μm RC, Schleicher & Schuell), and then analyzed for α- and γ-glutamyl dipeptides by means of LC-MS/MS.

In a first set of experiments, the SFT solution contained a mixture of the donor amino acid L-glutamine (25 mmol/L) as well as the acceptor amino acids L-glutamic acid, L-histidine, L-methionine, and L-leucine (25 mmol/L each) and was sterile-filtered (0.2 μm, 25 mm, cellulose acetate, VWR, Darmstadt, Germany) prior to use. In a second set of experiments, various concentrations of glucose or sucrose (5% each) were added to the SFT solution prior to incubation. In a third set of experiments, the amino acid mixture was used in concentrations of 25.0, 6.25, 5.0, and 1.0 mmol/L per amino acid. In a fourth set of experiments, the SFT solution containing the aforementioned amino acids in a concentration of 5 mmol/L was adjusted to pH 10.0 by the addition of sodium hydroxide or was spiked with glutathione (5 mmol/L) prior to incubation. In addition, the influence of anaerobic and aerobic conditions was studied by using airtight sealed (anaerobic) and cotton plugged vessels (aerobic), respectively, and the influence of the absence or presence of UV light was studied by using a UV lamp or by keeping the incubated sample solutions in the dark.

Biogeneration of [$^2H_3$]-γ-Glutamyl-L-methionine ([$^2H_3$]-γ-Glu-Met). Equal portions of an aqueous sterile-filtered solution (60 mL) containing [$^2H_3$]-L-methionine (0.74 mmol) and L-glutamine (0.74 mmol) were placed in five Sovirel glasses (50 mL), and an aliquot (400 μL) of an overnight culture of *P. roquefortii* strain 1812 was added to each preparation. After agitation for 20 days at 25 °C in the dark, the mold was separated from the solution by filtration (Macherey-Nagel, 615-1/4), the solution was freeze-dried, and the target compound [$^2H_3$]-γ-glutamyl-L-methionine was purified by means of preparative HPLC using an ELSD detector. Comparison of the mass spectrometric data obtained from the isolated target compound with those recorded for the nondeuterated reference compound γ-Glu-Met confirmed the incorporation of three deuterium atoms in the methionine moiety of [$^2H_3$]-γ-Glu-Met.

Determination of the γ-Glutamyl Transferase (GGT) Activity. Following a previously published protocol (*16*), the GGT activity in milk and cheese samples was determined by the photometric measurement of *p*-nitroaniline (λ = 410 nm) liberated at pH 8.5 and 37 °C from the donor γ-glutamyl-*p*-nitroanilide, from which the glutamyl moiety is transferred to the acceptor substrate glycylglycine upon action of the GGT. The photometer used was an UV−vis spectrometer Lambda 40 from Perkin-Elmer (Boston, MA). Data handling was performed with the software UV WinLab 2.0.

For the analysis of the GGT activity in *P. roquefortii*, strain 1080 was proliferated in a submerse medium containing 10% sucrose and 2% yeast extract (powdered, for bacteriology, Roth, Karslruhe, Germany). After 5 days, the mold was washed with water, freeze-dried, and ground in a mortar, and a defined amount (30 mg) of the powdered material was extracted for 30 min with an aqueous Tris buffer (0.1 mol/L, pH 8.8; 1.0 mL) in an ice bath with agitation. After filtration (Spartan, 13/0.45 μm RC, Schleicher & Schuell, Germany), the aqueous solution was analyzed for its GGT activity following the protocol reported earlier (*16*) with the slight modification that the clarifying and stopping reagent used for the GGT analysis in milk and cheese samples was substituted by acetic acid (2 mol/L). The calibration curve for the determination of the *p*-nitroaniline produced was performed using 2 mol/L acetic acid as solvent.

Quantitative Analysis of α- and γ-Glutamyl Dipeptides. A portion of the lyophilized WSE (25 mg) prepared from each cheese sample was dissolved in deionized water (1.0 mL) and membrane-filtered (Spartan, 13/0.45 μm RC, Schleicher & Schuell), and aliquots (5 μL) were injected into the HPLC-MS/MS system. For the analysis of glutamyl peptides in *P. roquefortii* incubations, samples (1 mL) were taken, filtered (Spartan, 13/0.45 μm RC, Schleicher & Schuell), diluted with water (1:2 dilution), and injected into the HPLC-MS/MS system. Following the protocol reported recently (*15*), the following α- and γ-glutamyl dipeptides were analyzed using the mass transitions given in parentheses: 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), γ-Glu-Phe (*m*/*z* 295.4→166.1), 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 triplicate by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions of each reference peptide ranging from 0.01 to 10 μmol/L (8-point calibration).

Dry Matter Content. The dry matter (dm) content was determined in a vacuum drying oven kept at 65 °C as reported in the literature (*17*).

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). LC-MS/MS analysis of α- and γ-L-glutamyl peptides was 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

Influence of Maturation on the Spatial Distribution of Glutamyl Dipeptides in Gouda Cheese Wheels. Recent studies demonstrated that a group of γ-glutamyl dipeptides, but not the corresponding α-glutamyl dipeptides, induce the attractive kokumi flavor of matured Gouda cheese (*15*). To gain some insight into the factors governing the formation of these peptides during cheese ripening, first, the spatial distribution of both types of glutamyl peptides in 4-weeks (GCW4) and 44-weeks matured Gouda cheese wheel (GCW44) was analyzed quantitatively. To achieve this, six samples were taken from a half-wheel of each cheese by means of a drilling knife as depicted in **Figure 1**. After preparation of a water-soluble extract (WSE) from each drilled sample, the dry matter (dm) content was determined and α-glutamyl and γ-glutamyl dipeptides were quantitatively determined by means of HPLC-MS/MS using a pentafluorophenylpropyl stationary phase (*15*). The results, calculated on the basis of the dry matter content of each drilling hole, revealed the highest concentrations of the major glutamyl peptides γ-Glu-Glu and γ-Glu-Met in the drilling holes A, D, and F; for example, 36.5 and 33.1 μmol/kg (dm) of γ-Glu-Glu and γ-Glu-Met, respectively, were found in drilling hole D, whereas only 26.2 and 24.1 μmol/kg (dm) were present in sample E (**Table 1**). Interestingly, the highest amount of 55.5 μmol/kg (dm) was found for the corresponding α-glutamyl peptide α-Glu-Glu in drilling hole E, whereas the outer parts of the cheese wheel such as the sample taken from drilling hole D contained significantly lower amounts of 20.1 μmol/kg (dm). Surprisingly, the α-Glu-Met was not detectable in any sample, thus indicating that the generation of α-glutamyl peptides is not related to the formation of γ-glutamyl peptides. When compared to the concentrations of the individual peptides in the drilling holes taken from GCW44, the young GCW4 sample contained only very low amounts of these peptides, thus confirming previous results (*15*).

To gain a clearer picture on the spatial distribution of the α-glutamyl and γ-glutamyl dipeptides in the cheese wheels, the average concentrations of γ- as well as α-glutamyl peptides within the drilled samples taken from GCW4 and GCW44 were calculated (**Figure 2**). The average content of α-glutamyl peptides in the samples taken from GCW4 ranged between 0.7 and 1.9 μmol/kg (dm), and the γ-glutamyl peptides were almost equally distributed, centering around an average concentration of about 0.2 μmol/kg (dm). In contrast, samples taken from the 44-weeks matured cheese wheel (GCW44) revealed by far

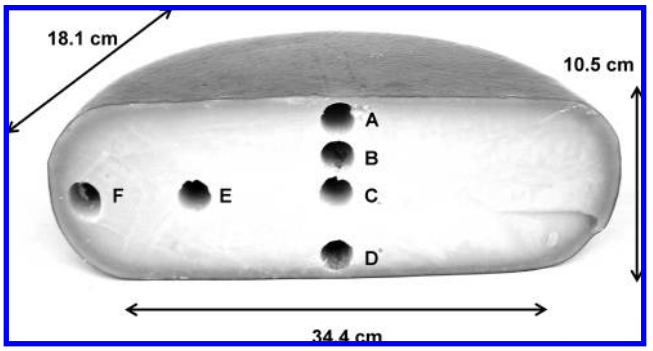

Figure 1. Location and coding of drilling holes A−F taken for the analysis of glutamyl peptides in half-wheels of Gouda cheese. For each drilled sample taken from the cheese wheels, the dry weight (GCW4/GCW44; g/100 g) was determined as follows: A (59.09, 68.88), B (58.88, 65.18), C (57.46, 64.62), D (59.16, 70.35), E (57.26, 64.50), and F (64.06, 72.37).

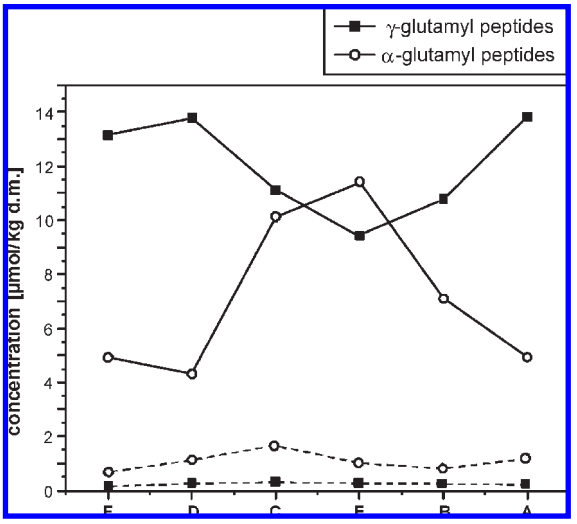

Figure 2. Average concentration of α- and γ-glutamyl peptides in the drilling holes of a 4-weeks (dotted lines) and 44-weeks ripened Gouda cheese wheel (solid lines), respectively. Location and coding of the drilling holes refer to **Figure 1**. The data are given as the mean of three measurements.

the highest average concentration of 14.0 μmol/kg (dm) for the γ-glutamyl peptides in the subrind samples A, D, and F, whereas α-glutamyl peptides showed a vice versa distribution and reached a maximum average concentration of about 11.0 μmol/kg (dm) in the center part E (**Figure 2**).

It is interesting to note that not only the concentration but also the ratio of γ- to α-glutamyl dipeptides in the drilled cheese samples were dependent on the ripening time. For example, a γ-/α-glutamyl peptide ratio of 0.1−0.3 was found for all samples A−F taken from the young Gouda wheel GCW4, whereas ratios of 1.2−2.0 were determined for samples B, C, and E and ratios of >3.1 were found for samples A, D, and F taken from the matured cheese GCW44 (**Table 1**).

These data clearly indicate that the spatial distribution of the α- and γ-glutamyl peptides is significantly influenced by the maturation stage of the cheese and that different mechanisms seem to be 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 (*18*) 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

Table 1. Spatial Distribution of α-/γ-Glutamyl Peptides in Half-Wheels of 4- (WGC4) and 44-Weeks Ripened Gouda Cheese (WGC44)

	concentration[a] [μmol/kg of dm] (rel std dev in %) in the drilling hole[b] taken from											
	GCW4						GCW44					
peptide	F	E	C	B	D	A	F	E	C	B	D	A
α-Glu-Gly	nd[c]	nd	<0.1	nd	0.1 (±9.9)	nd	5.4 (±4.9)	3.8 (±1.7)	4.3 (±2.8)	4.2 (±3.5)	4.9 (±5.9)	5.1 (±2.1)
α-Glu-Val	0.2 (±4.3)	0.4 (±5.2)	0.5 (±5.5)	0.4 (±2.4)	0.5 (±7.4)	0.4 (±10.8)	9.0 (±1.2)	9.0 (±9.5)	10.7 (±1.1)	6.5 (±8.4)	5.1 (±3.8)	4.0 (±0.4)
α-Glu-Ala	0.1 (±10.9)	0.1 (±8.9)	0.1 (±5.6)	<0.1 (±12.9)	0.1 (±9.1)	0.1 (±12.3)	0.5 (±10.1)	0.9 (±8.7)	1.0 (±2.3)	0.7 (±4.3)	0.5 (±3.5)	0.6 (±7.7)
α-Glu-Tyr	0.1 (±16.4)	0.5 (±9.2)	0.9 (±4.7)	0.4 (±3.9)	0.4 (±9.2)	0.4 (±9.8)	0.5 (±1.0)	0.8 (±4.4)	0.8 (±12.1)	0.6 (±10.7)	0.4 (±6.0)	0.4 (±11.7)
α-Glu-Glu	2.9 (±5.7)	5.6 (±1.2)	9.5 (±2.5)	4.0 (±5.5)	6.3 (±11.6)	6.8 (±1.2)	21.5 (±1.5)	55.5 (±4.9)	47.9 (±2.3)	33.9 (±9.9)	20.1 (±5.6)	25.4 (±1.2)
α-Glu-Asp	1.6 (±4.6)	1.1 (±1.7)	2.0 (±2.3)	1.1 (±2.7)	1.1 (±4.8)	1.4 (±1.5)	2.6 (±4.1)	10.3 (±6.4)	8.5 (±3.2)	6.3 (±2.5)	2.6 (±1.2)	3.7 (±10.2)
α-Glu-Thr	<0.1	0.1 (±10.1)	<0.1	nd	<0.1	0.1	2.4 (±1.5)	2.2 (±3.9)	2.3 (±2.7)	1.9 (±2.8)	1.7 (±5.1)	2.0 (±1.2)
α-Glu-Trp	0.1 (±2.9)	0.2 (±8.3)	0.2 (±2.5)	0.2 (±2.1)	0.2 (±1.9)	0.1 (±6.4)	nd	nd	nd	nd	nd	nd
Σ α-Glu-X	4.9	8.0	13.2	6.1	8.7	9.3	41.9	82.5	75.5	54.1	35.3	41.2
γ-Glu-Gly	0.4 (±4.3)	1.0 (±1.8)	0.9 (±3.3)	0.8 (±9.9)	0.9 (±3.2)	0.7 (±6.6)	7.1 (±1.5)	5.1 (±5.5)	5.7 (±10.0)	5.8 (±4.4)	7.3 (±2.8)	5.8 (±1.9)
γ-Glu-Val	nd	nd	nd	nd	nd	nd	4.7 (±2.2)	3.7 (±4.7)	4.2 (±2.7)	4.2 (±1.9)	5.8 (±4.0)	6.0 (±1.5)
γ-Glu-Ala	nd	nd	nd	nd	nd	nd	5.5 (±1.7)	4.0 (±7.3)	4.4 (±3.5)	4.2 (±4.4)	5.3 (±2.6)	5.2 (±1.2)
γ-Glu-Tyr	nd	nd	nd	nd	nd	nd	1.3 (±1.4)	1.2 (±1.3)	1.5 (±1.5)	1.7 (±2.3)	1.9 (±4.2)	2.2 (±1.2)
γ-Glu-Glu	<0.1 (±16.2)	0.4 (±3.5)	0.6 (±10.9)	0.4 (±10.4)	0.3 (±2.7)	0.2 (±7.2)	35.8 (±1.5)	26.2 (±2.9)	29.5 (±1.2)	27.6 (±2.6)	36.5 (±3.0)	35.1 (±1.4)
γ-Glu-Lys	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
γ-Glu-Gln	0.1 (±3.4)	0.3 (±5.5)	0.4 (±5.8)	0.3 (±6.8)	0.3 (±3.0)	0.3 (±1.5)	16.4 (±1.9)	11.5 (±7.0)	13.2 (±1.7)	12.4 (±3.4)	15.0 (±2.6)	15.3 (±2.0)
γ-Glu-His	0.2 (±5.8)	0.2 (±2.7)	0.2 (±5.7)	0.2 (±2.7)	0.2 (±2.2)	0.2 (±1.2)	9.6 (±5.2)	5.8 (±3.8)	6.7 (±1.2)	7.0 (±1.7)	9.4 (±3.4)	10.0 (±5.4)
γ-Glu-Leu	nd	nd	nd	nd	nd	nd	15.6 (±4.0)	12.2 (±6.9)	14.9 (±2.7)	14.2 (±1.8)	18.4 (±4.3)	18.7 (±1.7)
γ-Glu-Phe	<0.1	0.2 (±10.3)	0.2 (±4.7)	0.2 (±3.0)	0.2 (±5.5)	0.1 (±9.8)	3.8 (±3.6)	2.9 (±5.7)	3.6 (±1.5)	3.6 (±3.5)	5.2 (±4.0)	5.0 (±1.3)
γ-Glu-Met	<0.1	0.2 (±5.2)	0.2 (±10.1)	0.1 (±15.1)	0.3 (±3.3)	0.2 (±2.9)	31.9 (±1.2)	24.1 (±5.5)	27.7 (±3.7)	27.4 (±1.5)	33.1 (±1.7)	35.5 (±1.3)
Σ γ-Glu-X	0.7	2.3	2.5	2.0	2.2	1.7	131.7	96.50	111.4	108.1	137.9	138.8
Σ γ/Σ α	0.1	0.3	0.2	0.3	0.3	0.2	3.1	1.2	1.5	2.0	3.9	3.4

[a] The concentrations are given as the mean of three measurements. [b] The coding of the drilling wholes is depicted in **Figure 1**. [c] nd, not detectable.

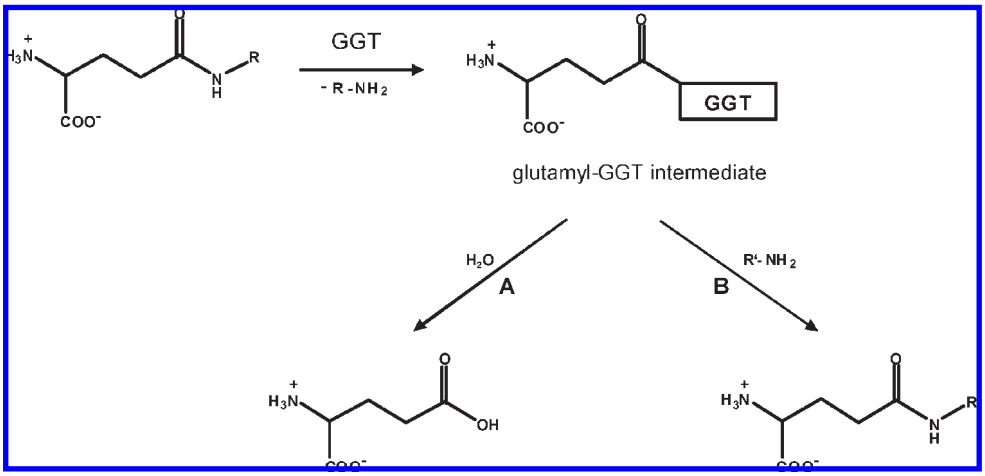

Figure 3. Reaction sequence of the GGT-catalyzed hydrolysis (**A**) and transpeptidation (**B**) of L-glutamine.

enzymatically produced by a γ-glutamyl transferase (GGT) involving free amino acids released upon protein hydrolysis. GGT is known to catalyze the hydrolytic cleavage of the isopeptide bond of γ-glutamyl compounds such as glutathione or L-glutamine as well as the transfer of the γ-glutamyl unit to the amino function of amino acids or peptides (*19*). Both reactions are believed to proceed via a covalent γ-glutamyl−enzyme conjugate as the common key intermediate, followed by hydrolysis to liberate L-glutamate or transpeptidation giving rise to γ-glutamyl peptides (**Figure 3**). As during maturation of the cheese wheel the water content of the outer parts of the cheese is known to be more strongly reduced when compared to the center parts (*20*), the reduced water content in the GCW44 samples A, D, and F might favor GGT-catalyzed transpeptidation reactions to yield the γ-glutamyl peptides. On the other hand, the higher concentrations of α-glutamyl peptides in the center parts might be due to the higher content of water that is required for proteolytic degradation of the caseins.

To gain a some more detailed insight into the occurrence of α- and γ-glutamyl dipeptides in other cheeses and to elucidate factors influencing their concentration in cheese, a selection of commercially available cheese samples was quantitatively screened for α- and γ-glutamyl dipeptides by means of LC-MS/MS.

Concentration of Glutamyl Dipeptides in Cheeses. For the analysis of the α-glutamyl as well as γ-glutamyl dipeptides in cheese, a WSE was prepared from each cheese sample and then analyzed by means of HPLC-MS/MS. As given in **Table 2**, the α-glutamyl dipeptides α-Glu-Ala, α-Glu-Asp, α-Glu-Glu, α-Glu-Gly, α-Glu-Thr, α-Glu-Trp, α-Glu-Tyr, and α-Glu-Val, as well as the γ-glutamyl dipeptides γ-Glu-Ala, γ-Glu-Glu, γ-Glu-Gln, γ-Glu-Gly, γ-Glu-His, γ-Glu-Leu, γ-Glu-Lys, γ-Glu-Met, γ-Glu-Phe, γ-Glu-Tyr, and γ-Glu-Val, were identified and quantitatively determined in each cheese sample. The total amount of α-glutamyl or γ-glutamyl dipeptides ranged between 4.0 and 1005.8 μmol/kg (dm) or between 5.4 and 3590.0 μmol/kg (dm), thus indicating that the concentration of these peptides is strongly dependent on the cheese sample.

By far the highest γ-glutamyl peptide concentrations of 3590.0, 2621.2, and 2007.3 μmol/kg (dm) were found for Blue Shropshire, a blue-veined cheese made from pasteurized bovine milk, followed by the ripened goat cheese and the Swiss Gruyere, both made from raw milk (**Table 2**). In comparison, rather low amounts of γ-glutamyl peptides of 5.4−89.5 μmol/kg (dm) were found in the Gouda cheese samples GC4, GC44, and pGC made from pasteurized milk and in the Gouda sample rGC made from raw milk as well as in the Camembert cheese, respectively.

It is interesting to note that not only the concentration but also the ratio of γ- to α-glutamyl dipeptides was strongly dependent on the cheese sample; for example, γ-/α-glutamyl peptide ratios of 0.3−3.3 were found for the Gouda samples as well as the Camembert and ratios of 17.5 and 19.1 were determined for the Swiss Gruyere and the ripened goat cheese, respectively (**Table 2**).

Among the γ-glutamyl peptides identified, γ-Glu-Glu was the quantitatively predominant derivative in most of the cheese samples with the exception of Camembert (γ-Glu-His), Kernhem (γ-Glu-Leu), Blue Shropshire (γ-Glu-Val), and Gouda cheese sample GC4 (γ-Glu-Gly), pGC as well as rGC (γ-Glu-Lys) containing the peptide given in parentheses as the major γ-glutamyl peptide (**Table 2**). 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 (*21*).

Although the presence of GGT in cheese was suggested earlier (*22*), there is only a single study on the experimental measurement of GGT activity in cheese (*16*). GGT in cheese might originate either from the milk or from the starter and/or ripening cultures used for cheese manufacturing. GGT present in milk was reported to be heat sensitive, and it could be shown that this enzyme is inactivated upon pasteurization (*16*, *23*). If the GGT activity in cheese originates from the milk alone, cheeses manufactured from raw and heat-treated milk should differ significantly in their contents of γ-glutamyl peptides. Most of the cheeses investigated, namely, Leerdammer, Milner, Camembert, Kernhem, Blue Shropshire, and Gouda samples GC4, GC44, and pGC, are made from pasteurized milk, whereas Swiss Gruyere and Gouda sample rGC are made by using raw milk. With the exception of the blue-veined cheese Blue Shropshire, the total γ-glutamyl peptide content of the cheeses prepared from pasteurized milk ranged between 5.4 and 867.8 μmol/kg (dm), whereas the raw milk cheeses Swiss Gruyere and the long-ripened goat cheese contained these peptides in much higher concentrations of >2000 μmol/kg (dm) (**Table 2**). These data imply that raw milk cheeses exhibit higher GGT activities than cheeses made from heat-treated milk.

Interestingly, the highest total concentration of γ-glutamyl peptides (3590.0 μmol/kg dm) was found for Blue Shropshire, a blue-veined cheese (**Table 2**). As this cheese is made from pasteurized milk, another source for the GGT has to be taken into account. Besides the milk, microorganisms as part

Table 2. Concentration α- and γ-Glutamyl Peptides in Selected Cheese Samples

	concentration[a] [μmol/kg of dm] (rel std dev in %) in													
	Gouda cheese				goat cheese		Milner							
peptide	pGC	rGC	GC4	GC44	young	ripened	light	extra	Camembert	Mouton	Kernhem	Leerdammer	Swiss Gruyere	Blue Shropshire
α-Glu-Ala	0.6 (±10.9)	0.3 (±11.8)	0.6 (±10.0)	2.6 (±4.3)	nd[b]	nd	2.1 (±3.9)	3.5 (±4.7)	16.1 (±4.6)	nd	nd	1.6 (±5.7)	nd	nd
α-Glu-Asp	0.8 (±14.0)	0.9 (±9.4)	4.9 (±5.7)	18.4 (±3.1)	5.7 (±7.6)	2.7 (±7.0)	27.4 (±10.7)	17.1 (±2.6)	53.9 (±10.1)	15.4 (±4.8)	6.9 (±4.3)	5.2 (±7.9)	3.3 (±5.8)	177.6 (±2.2)
α-Glu-Glu	1.1 (±11.6)	1.1 (±8.9)	10.9 (±4.3)	60.7 (±2.9)	56.0 (±3.6)	56.5 (±4.6)	90.6 (±6.1)	109.7 (±5.2)	142.0 (±12.2)	64.8 (±5.1)	25.2 (±8.0)	29.4 (±5.5)	52.3 (±4.2)	644.1 (±2.5)
α-Glu-Gly	1.4 (±10.0)	1.3 (±8.5)	0.4 (±15.2)	2.9 (±4.9)	0.8 (±11.9)	25.0 (±5.3)	11.4 (±10.9)	37.1 (±10.9)	3.6 (±8.8)	20.9 (±3.3)	21.6 (±5.5)	1.9 (±10.0)	18.4 (±4.5)	12.1 (±7.8)
α-Glu-Thr	nd	nd	0.5 (±7.4)	3.2 (±10.0)	15.5 (±7.2)	15.3 (±7.7)	1.8 (±9.5)	7.1 (±7.5)	1.5 (±10.6)	18.0 (±4.6)	0.1 (±12.3)	2.7 (±10.7)	13.5 (±3.4)	15.6 (±4.6)
α-Glu-Trp	nd	nd	nd	0.1 (±13.9)	1.3 (±12.4)	15.0 (±3.6)	0.1 (±12.9)	1.5 (±10.1)	0.4 (±12.1)	0.8 (±11.4)	4.5 (±9.4)	0.4 (±13.1)	10.0 (±7.9)	29.4 (±5.9)
α-Glu-Tyr	nd	0.1 (±14.9)	0.8 (±12.6)	1.2 (±8.8)	nd	nd	1.0 (±4.7)	1.1 (±4.5)	1.6 (±6.1)	27.6 (±4.8)	nd	nd	nd	nd
α-Glu-Val	0.1 (±16.5)	0.5 (±3.7)	0.7 (±7.0)	4.4 (±1.9)	5.4 (±10.3)	22.2 (±6.7)	nd	15.5 (±6.7)	127.8 (±7.6)	14.3 (±5.6)	3.2 (±10.9)	6.4 (±3.8)	17.3 (±6.9)	127.0 (±7.7)
Σ α-Glu-X	4.0	4.2	18.8	93.5	84.7	136.7	134.4	192.6	346.9	161.8	61.5	47.3	114.8	1005.8
γ-Glu-Ala	3.8 (±7.9)	3.0 (±8.9)	0.2 (±9.1)	3.0 (±5.3)	8.4 (±4.8)	82.0 (±2.7)	7.4 (±5.0)	31.5 (±6.1)	5.5 (±11.2)	5.63 (±4.3)	16.0 (±8.9)	12.4 (±9.8)	63.8 (±5.2)	54.1 (±5.6)
γ-Glu-Glu	<0.1 (±17.0)	0.6 (±11.0)	0.3 (±9.0)	27.6 (±2.7)	68.0 (±3.6)	768.9 (±2.3)	78.1 (±0.9)	198.2 (±3.0)	26.0 (±3.5)	71.8 (±3.6)	47.2 (±6.8)	134.7 (±4.8)	599.3 (±7.0)	619.5 (±6.0)
γ-Glu-Gln	nd	nd	0.6 (±12.4)	13.9 (±2.4)	48.8 (±2.6)	193.5 (±2.2)	46.5 (±3.0)	107.1 (±2.9)	13.3 (±4.4)	27.2 (±4.5)	32.9 (±8.9)	94.5 (±3.4)	147.3 (±2.5)	234.1 (±3.9)
γ-Glu-Gly	1.4 (±6.1)	1.3 (±9.5)	3.3 (±6.9)	6.3 (±3.2)	nd	nd	6.3 (±2.7)	12.5 (±2.2)	21.4 (±3.6)	2.5 (±4.9)	6.2 (±3.5)	9.3 (±2.8)	nd	108.4 (±7.4)
γ-Glu-His	nd	0.3 (±11.3)	0.3 (±10.9)	6.4 (±4.8)	21.6 (±4.2)	243.1 (±2.6)	31.1 (±4.9)	117.7 (±3.7)	29.7 (±5.9)	71.6 (±5.9)	40.1 (±4.9)	106.6 (±5.5)	201.3 (±6.7)	59.0 (±5.0)
γ-Glu-Leu	nd	0.7 (±8.7)	nd	7.1 (±2.9)	49.3 (±10.9)	351.0 (±4.2)	34.9 (±8.1)	102.6 (±3.1)	11.9 (±7.9)	58.7 (±6.5)	58.2 (±8.4)	58.0 (±6.4)	264.0 (±4.7)	705.1 (±9.0)
γ-Glu-Lys	7.4 (±5.1)	6.0 (±6.7)	nd	nd	16.5 (±3.8)	161.7 (±2.3)	nd	20.1 (±2.3)	9.1 (±3.9)	12.5 (±4.8)	15.9 (±6.7)	4.7 (±5.1)	119.1 (±4.1)	22.7 (±5.2)
γ-Glu-Metd	0.2 (±13.9)	0.4 (±9.5)	0.7 (±12. 2)	20.3 (±4.2)	53.1 (±3.2)	268.8 (±2.3)	43.1 (±4.3)	164.2 (±7.9)	6.5 (±3.0)	36.8 (±5.6)	41.8 (±5.9)	82.9 (±3.8)	206.7 (±4.8)	458.5 (±5.9)
γ-Glu-Phe	0.3 (±11.0)	0.4 (±8.7)	nd	2.0 (±7.7)	23.8 (±4.5)	212.3 (±1.5)	22.5 (±5.3)	55.5 (±5.7)	4.7 (±9.0)	32.3 (±6.1)	44.6 (±6.9)	31.7 (±6.7)	155.3 (±5.9)	366.1 (±4.7)
γ-Glu-Tyr	0.1 (±18.2)	0.1 (±17.1)	nd	0.3 (±15.9)	12.2 (±5.6)	44.6 (±2.9)	5.6 (±2.6)	18.8 (±4.5)	7.7 (±5.1)	0.3 (±8.9)	13.1 (±8.9)	10.2 (±5.6)	32.8 (±3.7)	10.7 (±6.1)
γ-Glu-Val	0.1 (±15.4)	0.5 (±6.8)	nd	2.6 (±1.8)	18.7 (±7.4)	295.3 (±2.1)	nd	39.6 (±2.4)	21.3 (±4.1)	21.3 (±4.3)	50.7 (±5.1)	13.2 (±6.9)	217.7 (±4.9)	951.8 (±3.6)
Σ γ-Glu-X	13.3	13.3	5.4	89.5	320.4	2621.2	275.5	867.8	157.1	340.6	366.7	558.2	2007.3	3590.0
Σ γ/Σ α	3.3	3.2	0.3	1.0	3.8	19.1	2.1	4.5	0.5	2.1	5.9	11.9	17.5	3.6
Σ Glu-X	17.3	17.5	24.2	183.0	405.1	2757.9	409.9	1060.4	504.0	502.4	428.2	605.5	2122.1	4595.8

[a] The concentrations are given as the mean value obtained for three cheese samples. [b] nd, not detectable.

Table 3. γ-Glutamyl Transferase (GGT) Activity of Miscellaneous Samples

sample	GGT activity[a]
P. roquefortii	0.37 U/g of protein
milk, raw	5.18 U/mL
milk, pasteurized	nd
Gouda cheese, 4-weeks matured (GC4)	nd
Gouda cheese, 44-weeks matured (GC44)	nd
Gouda cheese, made from raw milk (rGC)	3.91 U/g (dm)
Gouda cheese, made from pasteurized milk (pGC)	nd
ripened goat cheese	0.12 U/g (dm)
Blue Shropshire	0.54 U/g (dm)
Gruyere Swiss	2.54 U/g (dm)

[a] The GGT activity was determined by means of the photometric assay reported earlier (*7*) and is given in activity units (U) per g or mL as the mean of triplicates. nd, not detectable; dm, dry mass.

of starter and/or ripening cultures might have to be taken into consideration as a candidate source for GGT in cheese products. As the screening of various *Lactococcus* strains has not yet been successful in detecting any major GGT activity (*24*), the high amounts of γ-glutamyl peptides in the blue-veined cheese might be due to the GGT activity of the blue mold *P. roquefortii*. This mold is reported to contain proteinase as well as GGT activity (*25*) and, therefore, this microorganism might be a suitable candidate facilitating the proteolytic liberation of precursor amino acids, which are then used to produce the kokumi-active γ-glutamyl peptides.

GGT Activity in Cheese and Milk Samples. To gain a more detailed insight into the GGT activity of some cheese and milk samples, the GGT activity was determined by a photometric measurement of *p*-nitroaniline liberated from the donor substrate γ-glutamyl-*p*-nitroanilide upon GGT-catalyzed transfer of the γ-glutamyl moiety onto the acceptor substrate glycylglycine, yielding γ-glutamylglycylglycine (*16*).

The photometric assay was applied on extracts made from the GC4 and GC44 samples, the Gouda samples made from pasteurized (pGC) or raw milk (rGC), the Swiss Gruyere made from raw milk, the blue-veined Blue Shropshire, and the ripened goat cheese (**Table 3**). In addition, the GGT activity was measured in the raw as well as the pasteurized milk samples used for the manufacturing of the Gouda cheeses rGC and pGC (**Table 3**). Whereas the raw milk showed the highest GGT activity of 5.18 U/mL, which is even somewhat higher than the activity of 2.09 U/mL reported earlier (*16*), pasteurization induced complete inactivation of the enzyme; for example, the pasteurized milk sample did not exhibit any GGT activity, confirming previous reports (*16*, *23*). Interestingly, the Gouda sample rGC produced from the raw milk showed a GGT activity of 3.91 U/g (dm), whereas no significant GGT activity could be measured in the Gouda sample pGC or in GC4 and GC44, all of which were made from pasteurized milk (**Table 3**). These data clearly demonstrate that the GGT activity in raw milk cheeses such as the Swiss Gruyere might partially originate from the milk used for cheese manufacturing, whereas the GGT activity found in cheeses made from pasteurized milk such as Gouda cheese or Blue Shropshire is likely to originate either from the starter cultures or from the ripening cultures used for each specific cheese product.

Blue Shropshire, a blue-veined cheese made from pasteurized milk, revealed an enzyme activity of 0.54 U/g (dm), which is most likely to originate from mold *P. roquefortii*. To confirm the occurrence of GGT in *P. roquefortii*, samples of the mold were hand-picked and an aqueous extract of the isolated mold was analyzed for its GGT activity (**Table 3**). Although the determined specific activity of 0.37 mU/mg of protein of the extract of

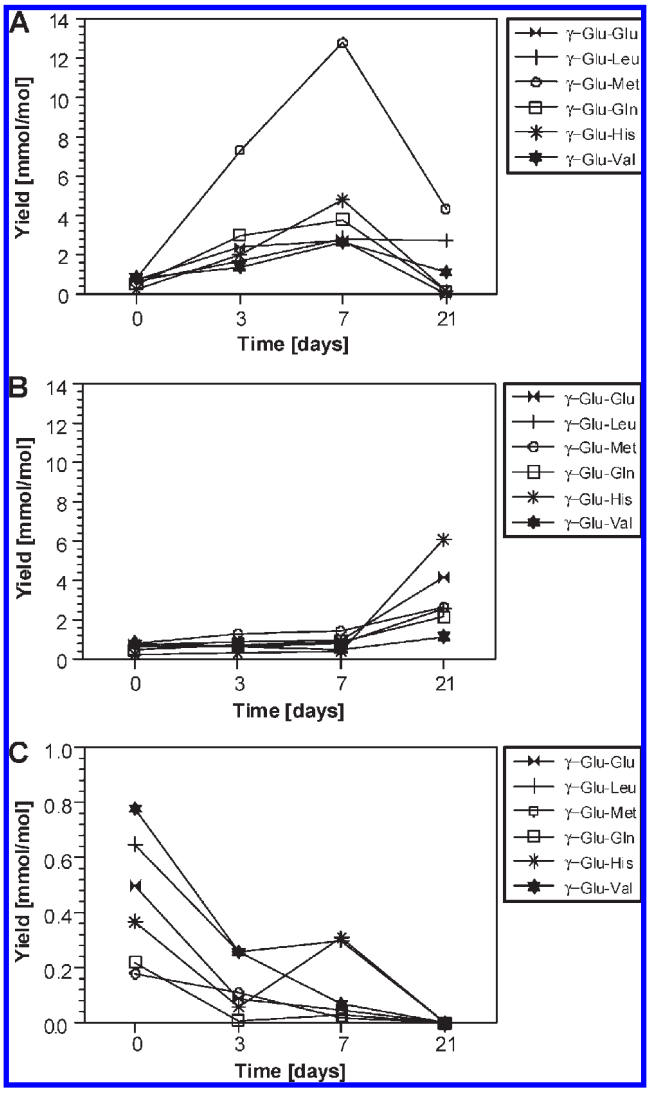

Figure 4. Yield of γ-glutamyl peptides generated upon incubation (37 °C) of mold isolated from Blue Shropshire cheese in the presence of (**A**) L-glutamine, L-glutamic acid, L-histidine, L-leucine, and L-methionine (25 mmol/L each) and (**B**) L-glutamine, L-glutamic acid, L-histidine, L-leucine, L-methionine (25 mmol/L each), and 5% glucose; and (**C**) in the absence of any additives (control). The data are given as the mean of triplicates (rel std dev for each data point < ±10.0%).

P. roquefortii was lower than that reported earlier (3.4 mU/mg of protein) (*25*), this experiment confirmed the GGT activity of *P. roquefortii*.

Incubation Experiments with Mold Isolated from Blue-Veined Cheese. To check whether the GGT in the blue mold *P. roquefortii* from blue-veined cheese is able to produce the γ-glutamyl peptides detected, samples of the mold were hand-picked from the Blue Shropshire cheese and incubated in sealed vessels with the γ-glutamyl donor amino acid L-glutamine and a mixture of the candidate substrate amino acids L-glutamic acid, L-histidine, L-leucine, and L-methionine in the absence or presence of 5% glucose at 37 °C for 21 days. After 3, 7, and 21 days, samples were taken and analyzed by HPLC-MS/MS for the α- and γ-glutamyl peptides. In addition, control experiments were performed by incubating the mold in the absence of the amino acid mixture and glucose.

LC-MS/MS analysis revealed that the sample containing the amino acid mixture showed a maximum in the concentration of γ-glutamyl peptides after 7 days of incubation; for example, a

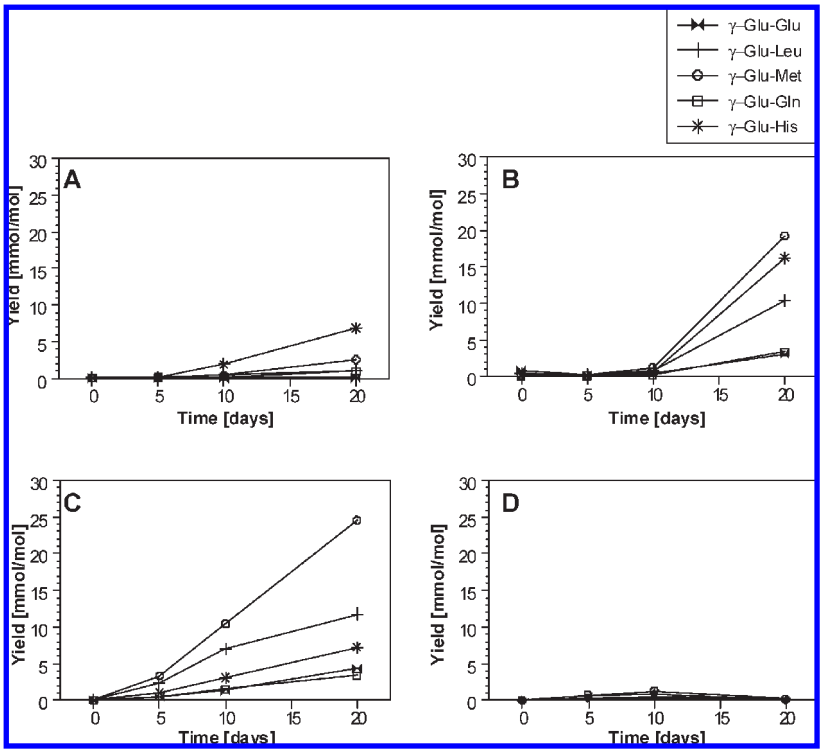

Figure 5. Yield of of γ-glutamyl peptides generated upon incubation (25 °C) of *Penicillium roquefortii* strain 1080 with (**A**) 25.0 mmol/L, (**B**) 6.25 mmol/L, (**C**) 5.0 mmol/L, and (**D**) 1.0 mmol/L of an equimolar mixture of L-glutamine, L-glutamic acid, L-histidine, L-leucine, and L-methionine. The data are given as the mean of triplicates (rel std dev for each data point < ±10.0%).

maximum yield of 12 mmol/mol was found for γ-Glu-Met after 7 days (**Figure 4A**). Interestingly, the generation of the γ-glutamyl peptides was marginal within the first 7 days when 5% glucose was present, but was facilitated when the incubation time was increased to 21 days (**Figure 4B**). In this experiment, γ-Glu-His was the most abundant glutamyl peptide after 21 days with 6.0 mmol/mol. The γ-glutamyl peptide concentration in the control sample was comparatively low (0.2−0.8 mmol/mol) prior to incubation (0 days) and was strongly depleted with increasing incubation time (**Figure 4C**). It is interesting to note that in all of the experiments performed, the corresponding α-glutamyl peptides were either not detectable or present only in trace amounts (< 0.01 mmol/mol). Being well in line with the GGT activity found above (**Table 3**), these data indicate that *P. roquefortii* is apparently able to convert amino acids into γ-glutamyl peptides and to secrete them into solution.

Inoculation Experiments with Single *P. roquefortii* Strains. To gain a more detailed insight into the parameters influencing the γ-glutamyl peptide production, the donor amino acid L-glutamine as well as a mixture of the acceptor amino acids L-glutamic acid, L-leucine, L-methionine, and L-histidine, each in a concentration of 25 mmol/L, was inoculated with the single *P. roquefortii* strain 1080. After the liquid culture had been maintained under agitation for 5, 10, and 20 days at room temperature, samples were taken and analyzed by HPLC-MS/MS for γ-glutamyl peptides. Being well in line with the data found for the mold isolated from Blue Shropshire cheese (**Figure 4**), the incubation with the pure strain of *P. roquefortii* 1080 induced the time-dependent generation of all candidate γ-glutamyl peptides, among which γ-Glu-His and γ-Glu-Met were produced as the major peptides in yields of 7.4 and 2.5 mmol/mol, respectively (**Figure 5A**). In conclusion, incubation of *P. roquefortii* with amino acids clearly showed that this microorganism not only possesses GGT activity but also utilizes the provided substrates to produce and secrete γ-glutamyl dipeptides into solution.

To answer the question as to whether the overall yield of the peptides might be further increased, various parameters were modified in the following. But neither the increase of the incubation temperature to 37 °C nor the addition of 5% glucose and 5% sucrose, respectively, was successful in significantly increasing the yields of γ-glutamyl peptides (data not shown). However, a decrease of the concentration of the amino acids from 25.0 to 6.25 mmol/L resulted in an increase of the yield of γ-glutamyl peptides after 20 days; for example, the highest yields with 20.0 and 16.0 mmol/mol were found for γ-Glu-Met and γ-Glu-His, respectively (**Figure 5B**). A further decrease of the amino acid concentration to 5.0, 2.5, 1.0, and 0.5 mM, respectively, showed just a slight improvement for the 5 mM amino acid mixture (**Figure 5C**) but not for all other tested concentrationsl for example, only marginal amounts of glutamyl peptides were formed in the sample containing 1 mM amino acids (**Figure 5D**). On the other hand, neither the addition of glutathione as another potential γ-glutamyl donor, a change in the pH value (5.0, 7.0, and 10.0), nor a change of the atmosphere from anaerobic to aerobic as well as from dark to light conditions resulted in a significant increase of the yields of γ-glutamyl peptides generated (data not shown).

To investigate whether the ability to produce γ-glutamyl peptides is dependent on the *P. roquefortii* strain, the four strains 1079, 1080, 1812, and 1999 were incubated at room temperature with an amino acid mixture containing L-glutamine, L-glutamic acid, L-leucine, L-methionine, and L-histidine, each in a concentration of 5.0 mmol/L. As depicted in **Figure 6**, only small differences in the kinetics of glutamyl peptide generation were observed between the *P. roquefortii* strains. Independent from the *P. roquefortii* strain, γ-Glu-Met and γ-Glu-His were produced as the predominant glutamyl peptides, but the highest yields of

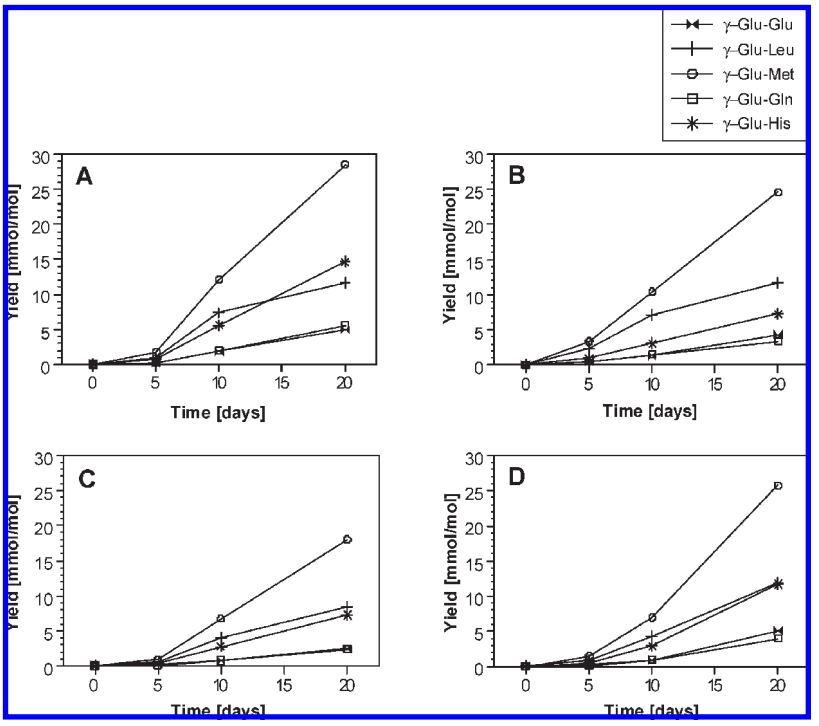

Figure 6. Yield of γ-glutamyl peptides generated upon incubation (25 °C) of *Penicillium roquefortii* strain 1812 (**A**), strain 1080 (**B**), strain 1999 (**C**), and strain 1079 (**D**) in the presence of L-glutamine, L-glutamic acid, L-histidine, L-leucine, and L-methionine (5.0 mmol/L each). The data are given as the mean of triplicates (rel std dev for each data point < ±10.0%).

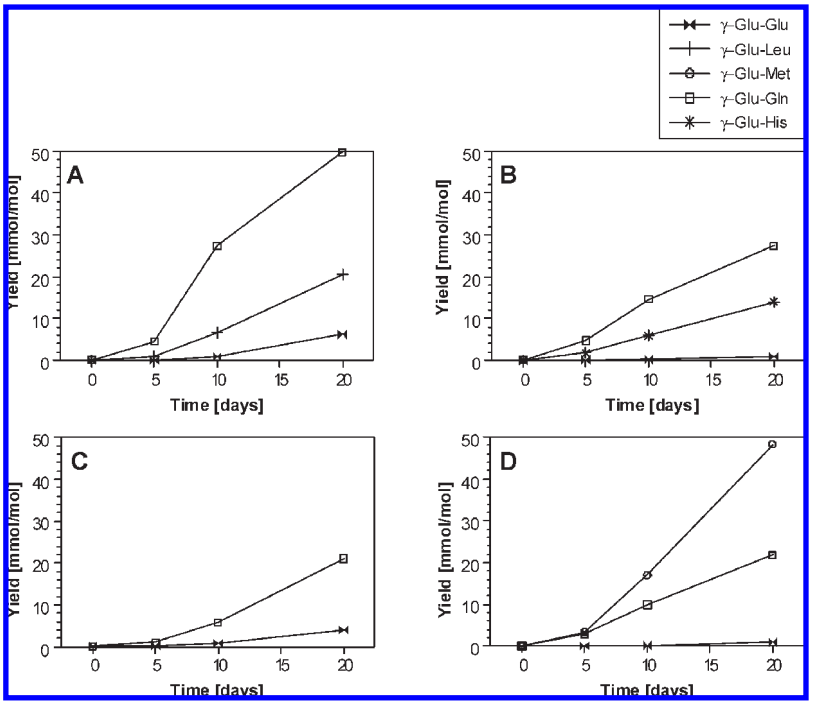

Figure 7. Yield of γ-glutamyl peptides generated upon incubation of *Penicillium roquefortii* strain 1812 at 25 °C in the presence of a binary mixture of L-glutamine (12.5 mmol/L) and (**A**) L-leucine (12.5 mmol/L), (**B**) L-histidine (12.5 mmol/L), (**C**) L-glutamic acid (12.5 mmol/L), or (**D**) L-methionine (12.5 mmol/L), respectively. The data are given as the mean of triplicates (rel std dev for each data point < ±10.0%).

γ-Glu-Met (29.0 mmol/mol) were found when *P. roquefortii* strain 1812 was used for inoculation.

Due to the high efficacy of *P. roquefortii* strain 1812 in glutamyl peptide production, this strain was incubated at pH 8.0 at room temperature with the glutamyl donor L-glutamine (12.5 mmol/L) and single acceptor amino acids (12.5 mmol), namely, L-methionine, L-histidine, L-leucine, and L-glutamic acid, respectively, and the individual samples were analyzed by means of HPLC-MS/MS for the glutamyl peptides formed to disclose the best acceptor amino acid. In the experiments containing either L-leucine, L-histidine, or L-glutamic acid (**Figure 7A−C**), the glutamyl-donor L-glutamine was also the best acceptor amino acid, and γ-Glu-Gln, formed by the transpeptidation of two molecules of L-glutamine, was found as the favored conversion product in yields ranging from 20 to 50 mmol/mol after 20 days. For example, the inoculation

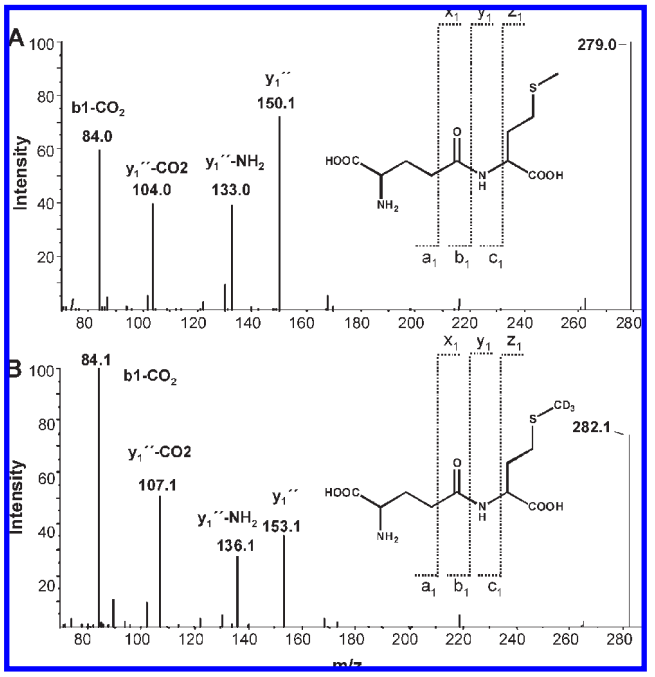

Figure 8. LC-MS/MS spectrum of (**A**) γ-glutamyl-L-methionine and (**B**) [$^2H_3$]-γ-glutamyl-L-methionine biogenerated by *Penicillium roquefortii* from L-glutamine and [$^2H_3$]-L-methionine.

experiment with equimolar amounts of L-glutamine and the hydrophobic amino acid L-leucine revealed the homotranspeptidation product γ-Glu-Gln in rather high yields of 50 mmol/mol, whereas the heterotranspeptidation product γ-Glu-Leu was generated in lower amounts of 20 mmol/mol (**Figure 7A**). The incubation of L-glutamine in the presence of the basic acceptor amino acid L-histidine (**Figure 7B**) or the acidic acceptor substrate L-glutamic acid (**Figure 7C**) resulted in somewhat lower yields of γ-Glu-Gln (29 mmol/mol) and only small yields of the heterotranspeptidation product γ-Glu-His (15 mmol/mol) and γ-Glu-Glu (5 mmol/mol), respectively, thus indicating L-histidine and L-glutamic acid to be poor γ-glutamyl acceptors. Interestingly, the amino acid L-methionine was found as a preferred γ-glutamyl acceptor when compared to L-glutamine; for example, after 20 days, the heterotranspeptidation product γ-Glu-Met was produced in yields of 50 mmol/mol but only 22 mmol/mol of γ-Glu-Gln was found (**Figure 7D**). These data confirm the previous findings that acidic and basic amino acids are poor acceptor amino acids, and neutral amino acids, among which L-methionine is the most preferred, are the most effective amino acid acceptors for the γ-glutamyl transferase (*25*). However, depending on its source, the GGT seems to differ in its substrate specifity; for example, the enzyme isolated from *Escherichia coli* was reported to prefer the basic amino acids L-arginine and L-lysine, whereas mammalian GGT showed high affinities for neutral amino acids (*26*).

In contrast to these findings, γ-glutamyl-L-glutamic acid was found to be preferentially formed in most of the cheese samples (**Table 2**). This might be due to the high natural abundance of L-glutamic acid within the casein sequences and its pronounced proteolytic liberation as reported for matured semihard and hard cheeses (*27*). Although L-glutamic acid was found to be a less active glutamyl acceptor when compared to L-methionine or L-glutamine, the huge concentration of that free amino acid in cheese (*10*) might result in the production of γ-Glu-Glu as the major γ-glutamyl dipeptide.

Generation of [$^2H_3$]-γ-Glutamyl-L-methionine. To confirm that *P. roquefortii* strain 1812 utilizes the acceptor amino acids added to the inoculation experiments and not endogenous amino acids to be converted to the corresponding γ-glutamyl dipeptide by transglutamylation, a mixture of the glutamyl donor L-glutamine and the deuterated acceptor amino acid [$^2H_3$]-L-methionine was incubated in the presence of *P. roquefortii* in a submerse culture for 20 days at room temperature. After removal of the mold by filtration, the aqueous solution was freeze-dried, and the target peptide [$^2H_3$]-γ-Glu-Met was isolated by HPLC on a preparative pentafluorophenyl column. The LC-MS/MS spectrum of [$^2H_3$]-γ-Glu-Met and γ-Glu-Met, displayed in **Figure 8**, clearly revealed the presence of three deuterium atoms in the methionine moiety of [$^2H_3$]-γ-Glu-Met, thus demonstrating that *P. roquefortii* is using the exogenously added amino acid L-methionine as the acceptor amino acid. Furthermore, this experiment demonstrated that the incubation of *P. roquefortii* in the presence of glutamyl donor L-glutamine and an acceptor amino acid is a suitable and conventient method to generate either kokumi-active γ-glutamyl peptides to be used as flavorings or stable isotope labeled γ-glutamyl peptides to be used as internal standards for the development of stable isotope dilution assays.

The data show that γ-glutamyl transferase (GGT), identified as an important enzyme involved in the generation of kokumi-active γ-glutamyl peptides upon cheese maturation, might originate from different sources depending on the type of cheese. Further studies are necessary to clarify and quantify the contribution of the GGT originating from milk, the starter cultures, and/or the various ripening cultures to the overall content of γ-glutamyl peptides in cheese. This information will be essential to tailor the production of kokumi-active γ-glutamyl peptides and to systematically optimize the taste profile of cheeses by technological means. In addition, biotechnological approaches, for example, by using *P. roquefortii* submerse cultures, might be suitable for the ex food large-scale production of flavor-active γ-glutamyl peptides to be used as natural flavorings in food and pet food applications. Investigations of the contribution of different GGT sources to the production of glutamyl peptides in cheese as well as on the role of various starter cultures in glutamyl peptide formation are currently in progress.

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