Amino Acids (2014) 46:441–447
DOI 10.1007/s00726-013-1637-3

ORIGINAL ARTICLE

Enzymatic production and degradation of cheese-derived non-proteolytic aminoacyl derivatives

Chiara Bottesini · Tullia Tedeschi · Arnaldo Dossena · Stefano Sforza

Received: 31 July 2013 / Accepted: 5 December 2013 / Published online: 13 December 2013

Abstract Gamma-glutamyl-amino acids, lactoyl-amino acids and pyroglutamyl-amino acids, collectively named Non-Proteolytic Aminoacyl Derivatives (NPADs) are unusual aminoacyl derivatives of non-proteolytic origins found in consistent amount in several cheeses. Although their enzymatic origin arising from lactic acid bacteria has been demonstrated, the exact enzymes originating them, the ones eventually degrading them and also their resistance to digestive enzymes in the human gastrointestinal tract and in the blood serum after eventual absorption are still unknown. In this paper, pure enzymes and biological media were tested on NPAD and their aminoacidic precursors, for identifying the conditions favoring bioproduction and biodegradation of these compounds. Pure gamma-glutamyl-phenylalanine and its precursor (glutamic acid and phenylalanine), also in the isotopically labeled forms, were tested with Parmigiano-Reggiano extracts, blood serum and different pure enzymes, including typical digestion enzymes (pepsin, trypsin and chymotrypsin), gamma-glutamyl transpeptidase and carboxypeptidase. The data suggested that their production in cheese, and also their partial degradation, might be due to the action of peptidases and gamma-glutamyl transpeptidase. Anyway, under simulated gastrointestinal digestion and in blood serum these compounds turned out to be perfectly stable, suggesting a potential to be absorbed as such and possibly being transported to the body tissues.

C. Bottesini · T. Tedeschi · A. Dossena · S. Sforza (✉)
Department of Food Science, University of Parma, Parco Area delle Scienze, 17/a, 43124 Parma, Italy
e-mail: stefano.sforza@unipr.it

Keywords γ-Glutamyl-phenylalanine · Lactoyl-phenylalanine · Parmigiano-Reggiano cheese · Non-proteolytic aminoacyl derivatives

Introduction

Cheese is a complex mixture of many different compounds, originating from the proteins, the sugars and the fats present in milk modified by several biochemical reactions. One of the most important of these processes is the breakdown of the casein matrix, a phenomenon known as proteolysis. Parmigiano-Reggiano (PR-RE) is a hard cheese cooked and long ripened. During the ripening, an intense proteolytic process takes place in this cheese, due to the enzymes present in the milk, calf rennet and starter/non-starter lactic acid bacteria (SLAB/NSLAB) (Forde and Fitzgerald 2000; Fallico et al. 2005).

As a consequence, the nitrogen fraction of PR-RE cheese is constantly evolving during ageing time. As recently demonstrated, peptides in PR-RE cheese can be classified into four groups, according to their origin and to the ageing period in which they are most present: peptides deriving by action of chymosin (mostly present at the beginning of cheese production), peptides deriving from SLAB (mostly abundant in the first 1–3 months of ageing), peptides deriving from NSLAB (mostly abundant at 6–10 months) and Non-Proteolytic aminoacyl derivatives (NPAD), characterizing aged cheeses (Sforza et al. 2012). NPADs, dipeptide-like molecules including γ-glutamyl-, lactoyl- and pyroglutamyl-amino acids, have been identified as characterizing the aged cheeses, not only in PR-RE, but also in Grana Padano, Asiago, and in Comté cheese, in amounts included between few mg and more than 50 mg per 100 g of cheese. (Sforza et al. 2009; Roundot-Algaron et al. 1994)

Although NPAD properties are still largely unknown, their most studied characteristic is their influence on cheese taste In particular, γ-glutamyl peptides have been shown to have a strong kokumi taste (Toelstede and Hofmann 2009). Recently Kuroda et al. (2012a, b) discovered a new γ-glutamyl peptide (γ-glutamyl–valyl–glycine) in vietnamese fish sauce, a potent kokumi compound present in some samples in amounts over 10 mg/l. Other biofunctional or technological properties of these compounds have not been investigated yet, but their abundance in cheese certainly suggests a closer look to their potential.

The origin of these compounds, and particularly the gamma-glutamyl amino acids, the most studied, is still object of a debate. Several papers hinted at their production by γ-glutamyl transpeptidase (GGT) (Hu et al. 2012). GGT is an enzyme present in all the organism mammals, plants and bacteria. The role attributed to this ubiquitous enzyme is transfer of the γ-glutamyl group, using a γ-glutamyl donor substrate (such as glutamine) to an amino acid, through a transpeptidation reaction.

Recently, we have been able to demonstrate that lactic acid bacteria enzymes produce γ-glutamyl-phenylalanine, in vitro condition, starting from glutamic acid, and not from glutamine. This suggest that the enzymatic activity seems to be different by GGT activity, and also that this particular enzymatic activity is strictly related to lactic acid bacteria (Sgarbi et al. 2013).

Beside the enzymes providing their formation, it would also be interesting to assess if these molecules are susceptible to degradation, both in cheese and, mostly, in the human body, during gastrointestinal digestion and/or in blood serum. This prior knowledge would be essential before starting to study any potential biological activity in vivo.

In this work we performed a screening study, to deeply investigate the enzymatic origin of this class of molecules, and also their resistance to enzymatic degradation once formed. In particular, several pure enzymes and biological media, including blood serum, Parmigiano-Reggiano cheese extracts and simulated gastrointestinal digestion mixtures, were tested on pure NPADs (γ-glutamyl-phenylalanine and lactoyl-phenylalanine) and on their precursors (phenylalanine, glutamic acid, lactic acid and glutamine), aimed at identifying the conditions favoring bioproduction and biodegradation of these compounds. These enzymatic activities were investigated also with the help of the isotopically labeled pure compounds.

Materials and methods

Chemicals

Potassium dihydrogen phosphate monohydrate, γ-glutamyl transpeptidase, Carboxypeptidase Y, glutamic acid, D–L lactic acid, L-lactic acid type VIII A R-amylase from barley malt, uric acid, type III mucin from porcine stomach, urea 98 %, D-(þ)-glucose 99.5 %, D-glucuronic acid, D-(þ)-glucosamine hydrochloride 99 %, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancreas, and bovine and ovine bile were purchased. Also *m*-cresol, thioanisole, diisopropylethylamine (DIPEA), methylene chloride were purchased from Sigma (Stuttgart, Germany).

The heavy labeled phenylalanine (D_5) was purchased from Spectra 2000, Rome–Italy (from Cambridge Isotope, Cambridge, UK).

Potassium hydroxide, potassium chloride, sodium chloride, ammonium chloride, 37 % hydrochloric acid, potassium dihydrogen phosphate, sodium hydrogen carbonate, and dried calcium chloride were obtained from Carlo Erba (Milan, Italy), potassium thiocyanate, potassium sulfate and sodium sulfate were purchased from Riedel–de–Haen (Hannover, Germany), magnesium chloride hexahydrate, N^{α}-Boc-L-glutamic acid-α-^{t}butyl ester and *O*-Benzotriazole-*N*,*N*,*N*',*N*'-tetramethyl-uronium-hexafluoro-phosphate were obtained from Merck (Darmstadt, Germany).

THF dry was obtained by distillation and not stabilized by quinones. Formic acid and trifluoroacetic acids were purchased by Acros Organics (Geel, Belgium).

Parmigiano-Reggiano cheese extract (12 months of ripening)

The Parmigiano-Reggiano extract was obtained by dilution and homogenization of 20 g of grated cheese in 90 ml of phosphate buffer (50 mM at 7 pH). After centrifugation at 3,500 rpm at 4 °C (Eppendorf, Hamburg, Germany), the extract was filtered on paper filter and on membrane with 0.45 μm cut-off (Millipore, Cork, Ireland), then incubated with deuterated precursors for 24 h at 37 °C. The presence of γ-glutamyl-phenylalanine was determined by LC/ESI–MS analysis.

Chemical synthesis of γ-glutamyl-phenylalanine

The standard γ-Glu-Phe was chemically synthesized using solution methodologies. The compounds were purified by RP-HPLC and characterized by LC/ESI–MS.

The deuterated γ-Glu-Phe was also synthesized by solution methodologies following the same procedure.

In brief, N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester (0.20 mmols) was dissolved in methylene chloride together with HBTU, *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro phosphate, (0.19 mmol) and the mixture was stirred at room temperature for 30 min. Phenylalanine-methyl ester (0.23 mmols) and DIPEA (0.41 mmol) were

added and the reaction was left under magnetic stirring for 24 h at room temperature. To make the remaining phenylalanine react, 0.10 mmols of N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester were added and, after 30 min of magnetic stirring, also 0.205 mmols of DIPEA. The reaction goes to completion in 16 h. The reaction was monitored by TLC using ethyl acetate as eluent. The organic solution was washed with a saturated solution of $KHSO_4$, $NaHCO_3$ and NaCl, dried with Na_2SO_4 and filtered.

The solvent was evaporated under reduced pressure and the residue was dissolved in 4 ml of a trifluoroacetic acid/methylene chloride 1:1 solution, containing also 4 % (v/v) of *m*-cresol and thioanisole as scavengers. The mixture was stirred at room temperature for 1 h and then the solvent was evaporated under reduced pressure. Ethyl ether was added to the residue to precipitate the free dipeptide. The precipitate was washed several times with ethyl ether and product dried at rotavapor. The product was diluted in a solution made by water, plus 0.2 % of acetonitrile and 0.1 % of formic acid. The reaction was analyzed by means of LC/ESI–MS, the experimental procedure described in "LC/ESI–MS analysis of NPADs".

The purification was performed with semipreparative HPLC–UV (Water, Milford, MA, USA). The eluents used were: (A) water acidified with 0.1 % trifluoroacetic acid and (B) acetonitrile acidified with 0.1 % trifluoroacetic acid. The separation gradient began at the 100 % of eluent A for 5 min, then until 80 % of eluent A in 15 min, subsequently the gradient followed a cycle of washing with eluent B, and reconditioning. The flow rate was set at 4 ml/min and the column used was Jupiter 5μ C18 300 Å 250 × 10 mm. The purified fraction was dried through rotavapor. The product was diluted in a solution made by water, plus 0.2 % of acetonitrile and 0.1 % of formic acid. The experimental procedure of LC/ESI–MS analysis of the purified fraction is described in "LC/ESI–MS analysis of NPADs".

LC/ESI–MS analysis of NPADs

Chromatographic analyses were performed with an UPLC chromatographic system (Waters, Milford, MA, USA) coupled to a SQD detector.

A C_{18} BEH Acquity Waters column (2.1 × 150 mm, 1.7 μm particles) was used. The eluents were two: water, 0.2 % acetonitrile, acidified with 0.1 % of formic acid (A) and acetonitrile with 0.1 % of formic acid (B). A flow rate was set at 0.2 ml/min. The injection volume was 2 μl. The chromatographic gradient was performed according to the following steps: 0–7 min isocratic 100 % A, 7–50 min linear gradient from 100 % A to 50 % A, 50–52.6 min isocratic 50 % A, 53–58 min from 50 % A to 0 % A and reconditioning.

The electrospray parameters in the positive ion mode are the following: the capillary and the cone voltages were 3.2 kV and 30 V, respectively; the source and the desolvation temperatures were 150 and 300 °C, respectively. Cone gas flow (N_2) 100 l/h, desolvation gas (N_2) 650 l/h.

Characterization MH^+ (ESI–MS): 295.1 γ-Glu-Phe, 300.1 deuterated-γ-Glu-Phe, 243.1 deuterated Lac-Phe, and 171.2 for deuterated Phenylalanine.

Formation and degradation in cheese extracts at different pH values

The extracts were obtained with the same procedure previously described but using phosphate buffer at different pH (4.4; 6; 7; 8). For the experiments aimed at studying formation, a defined amount of extract (900 μl) was incubated with 3.2 mg of heavy labeled Phenylalanine for 24 h at 37 °C. The presence of γ-glutamyl-phenylalanine was determined by LC/ESI–MS by evaluating the ratio between deuterated γ-Glu-Phe/undeuterated γ-Glu-Phe (methods described above).

A similar experiment was set up to evaluate the hydrolysis rate of γ-Glu-Phe at different pH: 7 ml of cheese extract was adjusted at pH 4–6–7–8 values and equilibrated to 10 ml for all samples. 900 μl of previous extracts was added of deuterated γ-Glu-Phe (1 mM) and incubated for 24 h at 37 °C, evaluating the appearance of deuterated Phe by LC/MS. Again, the ratio between deuterated γ-Glu-Phe/undeuterated γ-Glu-Phe was evaluated; the instrumental analysis was performed as previously reported.

Kinetic of bond formation

To evaluate the kinetic of NPAD formation, the soluble fraction of Parmigiano-Reggiano cheese was placed in contact with deuterated phenylalanine (4.14 mg) and the sample was analyzed by LC/ESI–MS every 72 min for 13 h to evaluate the kinetics of γ-Glu-Phe formation. In addition the extract was incubated 5 h with different amounts of deuterated phenylalanine (1.6, 3.2, 8 mg) and the enzymatic reaction was stopped with 20 % of acetonitrile. The samples were analyzed by LC/ESI–MS and the ratio between deuterated γ-Glu-Phe/undeuterated γ-Glu-Phe was evaluated.

Evaluation of the potential ability of pure enzymes to produce and degrade NPADs

Carboxypeptidase Y (CPY) and γ-glutamyl transpeptidase (GGT), were utilized to test their ability to form and/or or to cleave the deuterated γ-Glu-Phe. The reaction solvent was a phosphate buffer at pH = 6 for CPY and at pH 8 for

GGT, the reaction was kept overnight at 37 °C with a enzyme:substrate ratio of 1:50. The substrates for the enzymatic reaction were prepared as follows: glutamine + deuterated phenylalanine, glutamic acid + deuterated phenylalanine, lactic acid + deuterated phenylalanine (all in equimolar amounts 1.175 mmols) and deuterated γ-Glu-Phe.

The production or degradation of γ-Glu-Phe was determined by LC/ESI–MS analysis.

Simulated in vitro gastrointestinal digestion

Two different experiments of simulated gastrointestinal digestion, differing for the degree of complexity of the simulation, were set up to test the behavior of NPADs under these conditions, and also to eventually test the ability of the proteolytic enzymes to form NPADs in presence of the appropriate precursors.

Pepsin, chymotrypsin, trypsin were reacted individually and pooled together with the precursors (solution 2 mg/ml of phenylalanine + glutamic acid) and directly added in the optimal buffer used for the enzyme reactions (HCl 10 mM for pepsin, Phosphate Buffer 100 mM at pH 7 for trypsin and chymotrypsin), with an enzyme: substrate ratio of 1:100 at 37 °C. The samples were analyzed by LC/ESI–MS (Prandi et al. 2012)

The complete gastrointestinal digestion and the preparation of the artificial digestive juices (saliva, gastric juice, duodenal juice, and bile) were performed according to the protocol of Versantwoort et al. (2005).

Either L-glutamic acid and L-phenylalanine-D5 (1:1 stoichiometric ratio 1.175 mmols) or 1 mM of previously synthesized deuterated γ-Glu-Phe, were placed in contact with 300 µl saliva and incubated for 5 min at 37 °C (pH 6.8). After the first step, 600 µl gastric juice at the proper pH (pH 2–3) was added, and reacted for 2 h, later bicarbonate (100 µl 1 M) and duodenal juice-bile, 300 µl, were added and left to react for others 2 h (pH 6.5–7). The reaction was incubated at 37 °C and maintained under magnetic stirring. The enzyme reaction was stopped warming at 95 °C for 10 min and centrifuged at 3,500 rpm for 15 min. The supernatant was directly injected in LC/ESI–MS.

Stability in blood serum

The γ-Glu-Phe, also, was kept in contact with human blood serum for 1 h and samples were taken at 2–5–15–30–60 min of incubation at 37 °C.

The reaction was stopped by adding a solution containing 50 % of acetonitrile and after centrifugation (10,000 rpm at 4 °C) the outcome was monitored by LC/ESI–MS analysis. A control sample was performed with pure γ-Glu-Phe (without serum) and another control sample was done by replacing the γ-Glu-Phe with a generic peptide (sequence: LQLQPFPQPQLPY) incubated with the serum in the same amounts (0.476 mmols) and conditions.

All the samples were analyzed by LC/ESI–MS.

A similar test aimed at detecting a possible γ-Glu-Phe formation in blood serum was performed with precursors of γ-Glu-Phe: glutamine or glutamic acid or lactic acid and deuterated phenylalanine in ratio 1:1 (1.175 mmols). The samples were monitored at 0–15–60 min, then the reaction was stopped with acetonitrile and finally analyzed by LC/ESI–MS.

Results

Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe in cheese aqueous extracts

Using isotopically labeled precursors (to discriminate the newly produced γ-Glu-Phe from that already present in the extracts), the presence in cheese aqueous extracts, obtained at pH = 7, of an enzymatic activity able to produce γ-glutamyl-phenylalanine was demonstrated. Deuterated phenylalanine was added to the cheese extracts and the mixture was incubated at 37 °C for 24 h, by monitoring through LC/ESI–MS the deuterated γ-Glu-Phe/undeuterated γ-Glu-Phe ratio. To evaluate the kinetic of formation, the production of new γ-Glu-Phe was monitored for 13 h. As shown in Fig. 1, the amount of produced γ-Glu-Phe increased during the time.

The extract pretreated at 95 °C for 30 min before incubation did not yield the compound after incubation, demonstrating thus the enzymatic origin of γ-Glu-Phe.

In a subsequent experiment, the correlation between the amount of γ-Glu-Phe newly formed and the amount of deuterated Phe added was studied, stopping the reaction

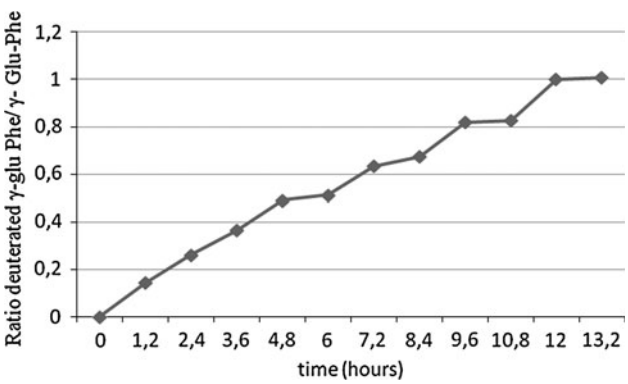

Fig. 1 Relative amount of deuterated γ-Glu-Phe formed by incubating deuterated Phe with cheese extract for 13 h

after a predefined time (5 h) with addition of acetonitrile up to 20 % (Fig. 2). Again, the amount of deuterated γ-Glu-Phe increased according to the increased amount of the precursor, demonstrating that this molecule is actually produced by using phenylalanine as precursor.

Subsequently, experiments aimed at evaluating the efficiency of formation of γ-Glu-Phe at different pH values were performed. An increased amount of deuterated γ-Glu-Phe was obtained by increasing the pH, whereas acidic pH values seemed to inhibit its formation (Fig. 3).

The same experiment was then performed for studying Lac-Phe formation. Quite interestingly, when adding deuterated Phe and monitoring deuterated Lac-Phe formation, a different behavior was observed: no product formation at acidic pH, (Fig. 4) and then a decreasing trend going from pH = 6–8, differently as seen for γ-Glu-Phe, suggesting that the responsible enzymes in the extracts might be different (Fig. 4). Also the efficiency seemed to be lower, as indicated by the lower ratios to the undeuterated compounds measured.

The samples analyzed were complex non-homogeneous mixtures, so it is reasonable to expect that the amount of enzyme extracted was not the same in every repetition of the experiment. Thus, the high errors observed are likely due to the natural variability of the biological system. The bars bearing different letters resulted significantly different by Tukey's test ($p < 0.05$).

To evaluate the condition of γ-Glu-Phe degradation in cheese aqueous extracts, deuterated γ-glutamyl-phenylalanine was added to the extract, followed by incubation at 37 °C for 24 h, and monitoring again the ratio of deuterated γ-Glu-Phe/undeuterated γ-Glu-Phe. The experiment showed a clear decrease of the added deuterated γ-glutamyl-phenylalanine, as compared to the amount of γ-Glu-Phe naturally present in the samples, indicating that in cheese extracts an enzymatic activity able to degrade γ-Glu-Phe is also present.

The degradation of the target molecule at different pH values indicated an increased degradation rate when

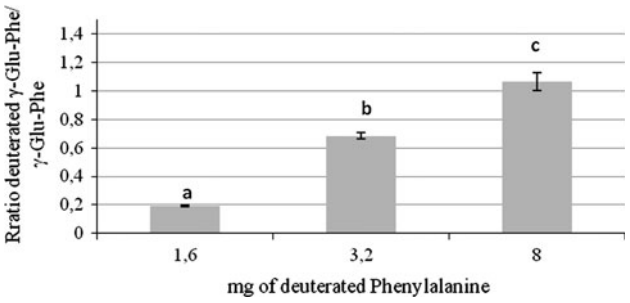

Fig. 2 Relative amount of deuterated γ-Glu-Phe formed after 5 h by incubating increasing amounts of deuterated Phe with cheese extract showing a growth when increasing the amount of precursors

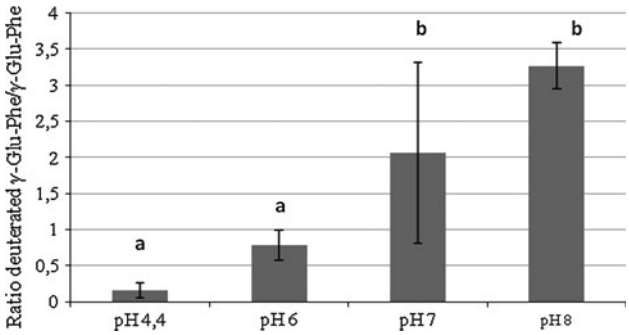

Fig. 3 Formation of deuterated γ-Glu-Phe at different pH values, acidic pH values seemed to inhibit its formation

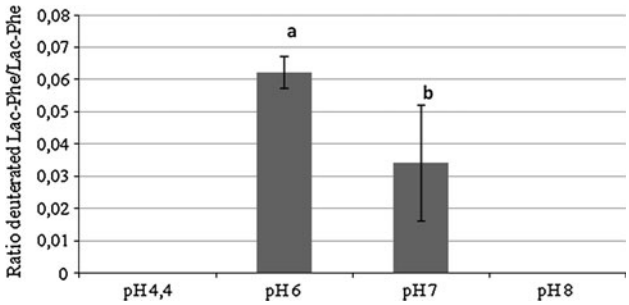

Fig. 4 Formation of deuterated Lac-Phe at different pH values. The efficiency seemed to be lower, as indicated by the lower ratios to the undeuterated compounds measured

increasing the pH, suggesting that the producing and the degrading enzyme have the same optimal pH and might possibly be the same enzyme (Fig. 5).

The bars bearing different letters are significantly different by Tukey's test ($p < 0.05$) all the data represent means and standard deviation.

Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe by pure enzymes

To assess the ability of pure enzymes to produce and degrade NPADs, different enzymes were considered, with different precursor (Gln, Glu, Lac, Phe) or full γ-Glu-Phe (when studying degradation). The results of these experiments are briefly summarized in Table 1.

First, common endoproteolytic enzymes common in the human digestive tract (trypsin, chymotrypsin and pepsin) were tested, both singularly and in combination. However, γ-Glu-Phe demonstrated to be totally resistant to their action. Even a simulated complete gastrointestinal digestion in more physiological conditions (details in the experimental section) resulted to be totally ineffective in degrading the compounds, demonstrating their extreme resistance in the human digestive tract. The same enzymes were also incubated with the precursors of the above molecules, to possibly test the ability of these digestive

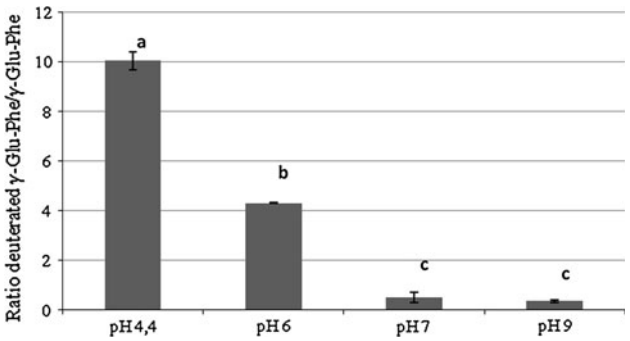

Fig. 5 Degradation of deuterated γ-Glu-Phe at different pH values. The degradation of the target molecule at different pH values indicated an increased degradation rate when increasing the pH

Table 1 Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe by pure enzymes

Precursors	γ-Glutamyl transpeptidase	Carboxypeptidase Y
Bond formation GLN + PHE(D_5)	Yes	No
Bond formation GLU + PHE-(D_5)	Yes	No
Bond formation LAC + PHE-(D_5)	No	Yes
Bond degradation γ-Glutamyl-Phenylalanine-(Ds)	Yes	No

enzymes to produce them, but no γ-Glu-Phe and Lac-Phe were formed, demonstrating that these enzymes are uneffective also for production, beside degradation, of NPADs.

Carboxypeptidase Y, a common exopeptidase, was then tested both with γ-Glu-Phe and Lac-Phe. Again, no degradation of both molecules was outlined. On the other hand, when incubated with the suitable precursors, a production of Lac-Phe by carboxypeptidase Y was observed, but not of γ-Glu-Phe. This Lac-Phe production was totally inhibited when carrying the experiment in presence of protease inhibitors (Sigma FAST Protease Inhibitor Tablets). On the other side, when incubating cheese extracts with protease inhibitors, deuterated γ-Glu-Phe was anyway formed, suggesting that only the enzyme responsible of Lactoyl-Phenylalanine production is a protease.

γ-Glutamyl transpeptidase (GGT) was then tested for γ-Glu-Phe production, being usually indicated as the enzyme able to originate γ-Glu-Phe in foods. As expected, when using glutamine as precursor, which is a known substrate for GGT (Suzuki et al. 2005), γ-Glu-Phe was produced in high yield, but even when using glutamic acid, small amounts of γ-Glu-Phe could be detected. As expected, GGT was also found to be able to cleave the bond of γ-Glu-Phe degrading it.

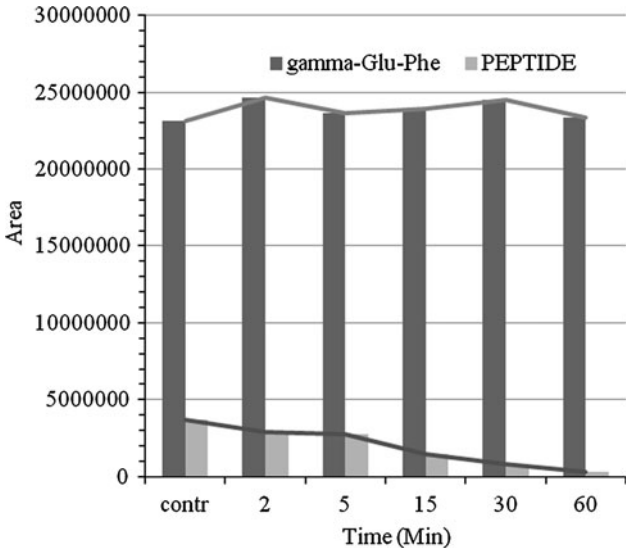

Fig. 6 Degradation of γ-Glu-Phe and a generic peptide in blood serum, γ-Glu-Phe, unlike the control peptide, was not degraded by the proteolytic activities present in blood serum, showing a perfect resistance over 1 h time

Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe in blood serum

To test the possibility of enzymatic production of γ-Glu-Phe and Lac-Phe in blood serum, the aminoacidic precursors (L-phenylalanine and L-glutamic acid or glutamine or lactic acid) were placed in contact with a blood serum for 1 h at 37 °C. In all cases, no formation of the target molecules was outlined by LC/ESI–MS.

To test the resistance at degradation in blood serum, γ-Glu-Phe was placed in contact with blood serum and monitored for 1 h. As control, the degradation of a standard peptide in serum was monitored over the same time.

The Fig. 6 shows that, γ-Glu-Phe, unlike the control peptide, was not degraded by the proteolytic activities present in blood serum, showing a perfect resistance over 1 h time.

Discussion

γ-Glu-Phe and Lac-Phe are produced by yet unknown one or more enzymes, already demonstrated to be of bacterial origin, present in cheese. A degrading activity of these compounds is also present in cheese, possibly due to the same enzymes, an observation consistent with the hypothesis that these enzymes might be proteases. Anyway, the different efficiency of production at different pH hints for γ-Glu-Phe and Lac-Phe being produced by different enzymes. With the aim of testing the hypothesis that proteolytic enzymes are responsible for the formation of γ-

Glu-Phe and Lac-Phe, common endoproteases (trypsin, pepsin, chymotrypsin) and one exoprotease (carboxypeptidase Y) were tested with the precursors. Only carboxypeptidase Y was found to be able to produce small amounts of Lac-Phe, but not of γ-Glu-Phe. On the other hand, γ-Glu-Phe was produced by GGT, as expected, also, quite surprisingly, when glutamic acid is used as precursor. Therefore, a combined action of GGT and exoproteases might be at the origin of these compounds in cheese.

γ-Glu-Phe and Lac-Phe were also tested for their resistance to gastrointestinal digestion and in blood serum, to define their bioaccessibility, and they were found to be perfectly resistant, indicating their potential absorption.

More studies will be needed to outline their ability to be absorbed in the gastrointestinal tract and their eventual biological and nutritional functionalities.

Acknowledgments Ajinomoto Inc. is gratefully acknowledged for its support to this research through the Ajinomoto Amino Acids Research Program ("Identification of microorganisms and conditions for optimal bioproduction of gamma-glutamyl aminoacids as new flavoring agents").

Conflict of interest The authors declare that they have no conflict of interest.

References

Fallico V, McSweeney PLH, Horne J, Pediliggieri C, Hannon JA, Carpino S, Licitra G (2005) Evaluation of bitterness in Ragusano cheese. J Dairy Sci 88:1288–1300. doi:10.3168/jds.S0022-0302(05)72795-8

Forde A, Fitzgerald GF (2000) Biotechnological approaches to the understanding and improvement of mature cheese flavour. Curr Opin Biotechnol 5:484–489. doi:10.1016/S0958-1669(00)00130-0

Hu X, Legler PM, Khavrutskii I, Scorpio A, Compton JR, Robertson KL, Friedlander AM, Wallqvist A (2012) Probing the donor and acceptor substrate specificity of the γ-glutamyltranspeptidase. Biochemistry 51:1199–1212. doi:10.1021/bi200987b

Kuroda M, Kato Y, Yamazaki J, Kageyama N, Mizukoshi T, Miyano H, Eto Y (2012a) Determination of γ-glutamyl–valyl–glycine in raw scallop and processed scallop products using high pressure liquid chromatography–tandem mass spectrometry. Food Chem 134:1640–1644. doi:10.1016/j.foodchem.2012.03.048

Kuroda M, Kato Y, Yamazaki J, Kai Y, Mizukoshi T, Miyano H, Eto Y (2012b) Determination of γ-glutamyl–valyl–glycine in commercial fish sauces. J Agric Food Chem 60:7291–7296. doi:10.1021/jf3012932

Prandi B, Bencivenni M, Tedeschi T, Marchelli R, Dossena A, Galaverna G, Sforza S (2012) Common wheat determination in durum wheat samples through LC/MS analysis of gluten peptide. Anal Bioanal Chem 403(10):2909–2914. doi:10.1007/s00216-012-5731-2

Roundot-Algaron F, Kerhoas L, Le Bars D, Einhorn J, Gripon JC (1994) Isolation of γ-glutamyl peptides from Comtè cheese. J Dairy Sci 77:1161–1166. doi:10.3168/jds.S0022-0302(94)77053-3

Sforza S, Cavatorta V, Galaverna G, Dossena A, Marchelli R (2009) Accumulation of non-proteolytica aminoacyl derivatives in Parmigiano Reggiano cheese during ripening. Int Dairy J 19:582–587. doi:10.16/j.idairyj.2009.04.009

Sforza S, Cavatorta V, Lambertini F, Galaverna G, Dossena A, Marchelli R (2012) Cheese peptidomics: a detailed study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano cheese from curd to 24 months of aging. J Dairy Sci 95:3514–3526. doi:10.3168/jds.2011-5046

Sgarbi E, Lazzi C, Iacopino L, Bottesini C, Lambertini F, Sforza S, Gatti M (2013) Microbial origin of non proteolytic aminoacyl derivatives in long ripened cheeses. Food Microbiol 35:116–120. doi:10.1016/j.fm.2013.02.013

Suzuki H, Kajimoto Y, Kumagai H (2005) Improvement of the bitter taste of amino acids through the transpeptidation reaction of bacterial γ-glutamyltranspeptidase. J Agric Food Chem 50:313–318. doi:10.1021/jf010726u

Toelstede S, Hofmann T (2009) Kokumi-Active Glutamyl Peptides in Cheeses and their biogeneration by Penicillium roqueforti. J Agric Food Chem 57:3738–3748. doi:10.1021/jf900280j

Versantwoort CHM, Oomen AG, Van de Kamp E, Rompelberg CJM, Sips AJAM (2005) Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem Toxicol 43:31–40. doi:10.1016/j.fct.2004.08.007