



Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening

Stefano Sforza*, Valeria Cavatorta, Gianni Galaverna, Arnaldo Dossena, Rosangela Marchelli

Department of Organic and Industrial Chemistry, University of Parma, Viale G.P. Usberti 17a, University Campus, I-43100 Parma, Italy

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ABSTRACT

For the first time, the identification of *N*- γ -glutamyl-, *N*-pyroglutamyl- and *N*-lactoyl-amino acids in Parmigiano-Reggiano cheese is reported. Their structures were assigned by electrospray mass spectrometric analysis and demonstrated for several compounds by the synthesis of authentic specimens and sample spiking. The comparison with the synthesised compounds also allowed the determination of their absolute stereochemistry (L,L), which indicated a common enzymatic origin. All these molecules were shown to accumulate in Parmigiano-Reggiano during ripening, probably because of a resistance to proteolysis due to their unusual structures. Thus, these compounds can be used as markers of ripening time and may be involved in the development of the pleasant flavour of aged cheeses. Their presence in other cheeses was also demonstrated.

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1. Introduction

Free amino acids and small peptides are characteristically formed in cheese during ripening by the proteolytic activities arising from milk, rennet and lactic acid bacteria (LAB) proteases (Sousa, Ardo, & McSweeney, 2001). As a consequence, the free amino acids and, in particular, oligopeptides present in cheeses mainly derive from the proteolysis of caseins (Piraino et al., 2007). Beside their high nutritional value, these low molecular weight nitrogenous compounds play an important role in flavour development (McSweeney & Sousa, 2000) and the oligopeptides are increasingly being studied for their potential functional activities (Contreras, Lopez-Exposito, Hernandez-Ledesma, Ramos, & Recio, 2008).

New aminoacyl derivatives, of non-proteolytic origin but synthesised de novo in cheeses by enzymatic activities, are scarcely known and poorly studied; in particular, compounds derived from glutamic acid have been reported. Isopeptides γ -glutamyl-phenylalanine (γ -Glu-Phe), γ -glutamyl-tyrosine (γ -Glu-Tyr) and γ -glutamyl-leucine (γ -Glu-Leu) have been identified in Comté cheese (Roudot-Algaron, Kerhoas, Le Bars, Einhorn, & Gripon, 1994). These compounds were also synthesised and tasted: γ -Glu-Tyr was sour and salty, whereas γ -Glu-Phe had a more intense and complex taste, which was brothy and slightly sour, salty, and metallic.

Non-dairy foods have also been reported to contain γ -glutamyl-amino acids and peptides: γ -glutamyl-leucine, γ -glutamyl-valine

and γ -glutamyl-cysteinyl- β -alanine were isolated from edible beans and have been characterized as providing the “Kokumi” taste (Dunkel, Köster, & Hofmann, 2007). “Kokumi” is a term used in the flavour industry to describe characteristics such as continuity, mouthfulness, richness and thickness, in contrast to monosodium glutamate, which is usually characterized as having the “Umami” taste (Ueda, Tsubuku, & Miyajima, 1994). *Allium* species have been reported to contain γ -glutamyl compounds, in particular γ -glutamyl-trans-prop(1)enyl cysteine sulfoxide and γ -glutamyl-phenylalanine, probably acting as storage compounds for nitrogen and sulphur (Mutsch-Eckner, Meier, Wright, & Sticher, 1992). A recent patent concerned γ -glutamyl peptides, and the enzymatic methods to prepare them, as compounds which provide a “Kokumi” flavour to consumables and flavour composition (Hofmann & Dunkel, 2007). The presence of γ -Glu-Phe, γ -Glu-Ile and γ -Glu-Leu in Parma ham has been reported: their content was found to increase during the ripening time and to be possibly linked to the pleasant aged flavour (Sforza et al., 2006).

The non-protein amino acid pyroglutamic acid has been reported to be present in Parmigiano-Reggiano and other hard cheeses, derived from an enzymatic reaction that catalyses the internal cyclization of glutamic acid (Mucchetti et al., 2000, 2002). Pyroglutamyl peptides have never been reported in cheeses so far, but they have been reported in wheat (Higaki-Sato et al., 2003) and mushrooms (Altamura, Andreotti, & Longjir, 1970). Derivatives of pyroglutamyl peptides have been patented by Nestlé SA as taste enhancers that impart umami taste to food-stuffs (Schlichtherle-Cerny & Amado, 2003).

* Corresponding author. Tel.: +39 0521 905406; fax: +39 0521 905472.
E-mail address: stefano.sforza@unipr.it (S. Sforza).

In this work the identification of several *N*- γ -glutamyl and *N*-pyroglutamyl-amino acids in Parmigiano-Reggiano cheese is reported. Moreover, some *N*-lactoyl-amino acids, never reported in any food so far, were also identified. Their structures were determined by electrospray mass spectrometry (ESI-MS) and demonstrated for several compounds by the synthesis of authentic specimens, also allowing the determination of their absolute configurations.

2. Materials and methods

2.1. Cheese samples

Parmigiano-Reggiano cheese samples (28) coming from 14 different cheese wheels, aged from 0 (fresh curd) to 24 months, were obtained from the “Consorzio del Parmigiano-Reggiano” (Reggio Emilia, Italy). The 14 cheese wheels were produced in the same factory, on the same day, starting from the same batch of milk, to reduce the variability usually present in Parmigiano-Reggiano cheese. Cheese wheels were then ripened in the factory, following the standard ripening conditions followed in the production of Parmigiano-Reggiano cheese, and sampled at regular intervals during the following 2 years. Additional Parmigiano-Reggiano, Grana Padano and Asiago cheese samples were taken from the Italian market.

2.2. Extraction of the oligopeptide fraction

The oligopeptide fraction was extracted according to the method reported by Sforza et al. (2004). Finely grated cheese (10 g) was suspended in 45 mL of 0.1 M HCl and (L,L)-phenylalanyl-phenylalanine (Phe-Phe) was added as an internal standard (2.5 mL of a 1 mM solution). The suspension was homogenized for 1 min and then centrifuged at $3400 \times g$ for 40 min at 4 °C. The solution was filtered through paper filters and then extracted three times with 40 mL of ethyl ether to eliminate the lipids. The residual aqueous solution was filtered through 0.45 μ m filters. A total of 3 mL of the resulting solution was dried, then dissolved in 900 μ L of a 0.1% formic acid solution (pH 3), ultrafiltered at $2800 \times g$ in an Amicon Micropartition System MPS-1 using Millipore PLGC filters with nominal molecular cut-off 10 kDa (Millipore, Billerica, MA, USA). The filtrate was dried under nitrogen, then dissolved in 500 μ L of 0.1% aqueous HCOOH and analysed by LC/MS.

2.3. Liquid Chromatography/Electrospray Mass Spectrometry (LC/ESI-MS) analysis of the oligopeptide fraction

LC/ESI-MS analyses were performed on an Alliance 2695 separation system (Waters, Milford, MA, USA) according to the following conditions. Eluent A was H₂O (0.2% CH₃CN and 0.1% HCOOH); eluent

B was CH₃CN (0.2% H₂O and 0.1% HCOOH). The elution program was as follows: 100% A for 15 min, a linear gradient from 100% A to 67% A between 15 and 60 min, eluting at 67% A from 60 to 69 min, reducing to 60% A between 69 and 70 min, reducing to 0% A between 70 and 80 min, and eluting at 0% A from 80 to 85 min, followed by reconditioning. The chromatographic column used was a Gemini C18 column (5 μ m, 110 Å, 250 $\times$ 4.6 mm) (Phenomenex, Torrance, CA, USA) and the flow rate was 1 mL min⁻¹. The detection was performed on a single quadrupole ESI mass spectrometer (Acquity SQD, Waters, Milford, MA, USA). Only 20% of the column flow (about 200 μ L min⁻¹) was allowed to enter into the ESI interface by a manually calibrated T-valve. The MS detector was used in the positive ion mode, by setting the capillary voltage at 3.2 kV, the cone voltage at 30 V, the source temperature at 100 °C, the desolvation temperature at 150 °C, the cone gas (N₂) at 100 L h⁻¹ and the desolvation gas (N₂) at 450 L h⁻¹. The acquisition was performed in the total ion current mode (TIC, 100–1900 *m/z*) with a scan time of 2 s. The data were collected and analysed with Mass Lynx 4.0 software.

2.4. Synthesis of *N*- γ -glutamyl, *N*-pyroglutamyl and *N*-lactoyl derivatives of L-phenylalanine and spiking experiments

For each one of the three families of molecules (*N*- γ -glutamyl-amino acids, *N*-pyroglutamyl-amino acids and *N*-lactoyl-amino acids), the corresponding L-phenylalanine derivative was synthesised both with the D- and the L-acyl component. Syntheses were performed by applying standard solid-phase and solution methodologies (see Supplementary Data).

An aliquot of the final solution coming from the extraction of cheese (50 μ L) was spiked with 5 μ L of the synthesised standards at an approximate concentration of 100 μ g (crude weight) per millilitre, then an H₂O:CH₃CN:HCOOH solution (40:60:0.1, by vol) was added up to a final volume of 100 μ L. Spiked samples (25 μ L) were analysed by LC/ESI-MS (same conditions utilised for samples).

2.5. Quantitative determination of the aminoacyl derivatives in cheese samples

For every compound in every sample, extract ion chromatograms (XICs) were obtained from the full-scan chromatograms by extracting the signals corresponding to the ions reported in Table 1. The XIC corresponding to the internal standard Phe-Phe was also obtained, by extracting the characteristic ions 313.1, 166.1, 120.0. The chromatographic area of every compound in the corresponding XIC was normalized to the area of Phe-Phe. The relative response factor for *N*- γ -glutamyl-phenylalanine, *N*-pyroglutamyl-phenylalanine and *N*-lactoyl-phenylalanine was calculated by the injection of the corresponding synthesised standards, together with the

Table 1

Peaks of interest, relative characteristic ions and tentative identification based on Rt, molecular mass and characteristic ions.

Retention time, Rt (min)	Characteristic ions (<i>m/z</i>) and assignments	Molecular mass (Da)	Tentative identification
19.5	279.3 (MH ⁺), 150.1 (MetH ⁺)	278	γ -Glu-Met
21.7	247.2 (MH ⁺), 118.1 (ValH ⁺)	246	γ -Glu-Val
28.4	311.0 (MH ⁺), 182.0 (TyrH ⁺)	310	γ -Glu-Tyr
30.7	261.2 (MH ⁺), 132.1 (IleH ⁺)	260	γ -Glu-Ile
31.7	261.2 (MH ⁺), 132.1 (LeuH ⁺)	260	γ -Glu-Leu
34.7	295.2 (MH ⁺), 166.0 (PheH ⁺), 120.0 (immonium ion Phe)	294	γ -Glu-Phe
35.8	222.1 (MH ⁺), 176.1 (M-COOH) ⁺	221	Lac-Met
36.6	190.2 (MH ⁺), 144.1 (M-COOH) ⁺	189	Lac-Val
37.3	254.2 (MH ⁺), 208.2 (M-COOH) ⁺ , 136.1 (immonium ion Tyr)	253	Lac-Tyr
38.7	243.2 (MH ⁺), 197.1 (M-COOH) ⁺ , 132.1 (IleH ⁺)	242	Pyr-Ile
40.5	243.2 (MH ⁺), 197.1 (M-COOH) ⁺ , 132.1 (LeuH ⁺)	242	Pyr-Leu
44.2	204.2 (MH ⁺), 158.2 (M-COOH) ⁺ , 132.1 (IleH ⁺)	203	Lac-Ile
44.5	204.2 (MH ⁺), 158.2 (M-COOH) ⁺ , 132.1 (LeuH ⁺)	203	Lac-Leu
44.8	277.2 (MH ⁺), 231.1 (M-COOH) ⁺ , 166.0 (PheH ⁺), 120.0 (immonium ion Phe)	276	Pyr-Phe
48.6	238.1 (MH ⁺), 192.1 (M-COOH) ⁺ , 166.0 (PheH ⁺), 120.0 (immonium ion Phe)	237	Lac-Phe

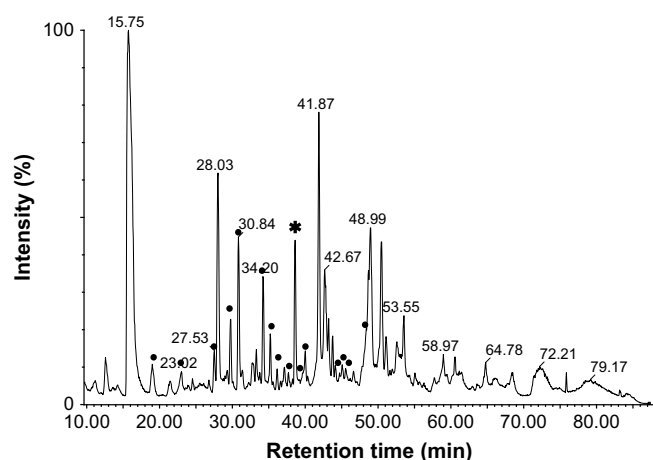


Fig. 1. LC/MS profile (total ion current trace) of a Parmigiano-Reggiano cheese sample ripened for 12 months. Dots indicate the peaks of interests and the asterisk indicates the internal standard.

internal standard Phe-Phe, and used for quantifying the amounts of the above compounds in all the samples analysed. For the quantification of the other *N*- γ -glutamyl-, *N*-pyroglutamyl- and *N*-lactoyl-amino acids, the response factors were approximated to be the same as the corresponding phenylalanyl derivatives. Every sample was analysed in duplicate.

3. Results and discussion

3.1. Identification of non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese

During a study of the oligopeptide fraction of Parmigiano-Reggiano cheese between 0 and 24 months of ripening, some compounds with low molecular mass, mainly present in long-ripened samples, were found to be of particular interest: these molecules attracted attention since, unlike most of the other peptides, they seemed to regularly increase during ripening, apparently unaffected by protease activities. In the LC/MS chromatogram of the oligopeptide fraction of a Parmigiano-Reggiano cheese (aged 12 months) shown in Fig. 1, the dots indicate the peaks of interest at the different retention times (Rt).

Their molecular masses and characteristic ions found in ESI spectra are reported in Table 1. Fragment ions corresponding to free

apolar amino acids were identified in several spectra. For example, ions at *m/z* 132 (corresponding to Leu or Ile) were identified either at Rt 30.7 and 31.7 min (in two molecules both of 260 Da), at Rt 38.7 and 40.5 min (242 Da) and Rt 44.2 and 44.5 min (203 Da). Analogously, ions at *m/z* 166, together with the corresponding immonium ion at *m/z* 120 (diagnostic for Phe), were identified at Rt 34.7 min (294 Da), Rt 44.8 min (276 Da) and Rt 48.6 min (237 Da). The difference between the masses of the full molecules and the free amino acids identified always corresponded to residues of glutamic acid, pyroglutamic acid and lactic acid, respectively. These data suggest structures in which the amino acids are *N*-acylated with the above reported acids, giving rise to glutamyl-amino acids (Glu-aa), pyroglutamyl-amino acids (Pyr-aa) or lactoyl-amino acids (Lac-aa). Glu-aa were assigned as γ -Glu-aa, on the basis of retention times (too high for standard α -Glu-aa), the fact that the accumulation of the molecules indicate non-standard peptide structures, and to the previous identification of the molecules in different foods (Roudot-Algaron, Kerhoas, Le Bars, Einhorn, & Gripon, 1994; Sforza et al., 2006). On the basis of retention times and mass spectra analogies, all the other molecules were assigned as γ -glutamyl-, lactoyl- or pyroglutamyl-amino acid derivatives. According to previously published data (Sforza et al., 2006), and in agreement with the chromatographic behaviour of isoleucine and leucine in reversed-phase LC, isoleucine-containing compounds were assigned as eluting before leucine-containing compounds. Tentative assignments are reported in Table 1 and the hypothetical structures of the compounds are reported in Fig. 2.

3.2. Synthesis of *D*- and *L*- γ -glutamyl, lactoyl and pyroglutamyl derivatives of *L*-phenylalanine and their use as authentic standards

To confirm the hypothetical structures deduced by MS and Rt data and to verify the stereochemistry of the molecules, it seemed necessary to make a comparison with authentic specimens. For the sake of simplicity, it was decided to synthesise all the derivatives corresponding to only one of the apolar amino acids putatively identified, i.e., phenylalanine. Since its origin should be mainly proteolytic, *L*-Phe was used, whereas glutamic acid and lactic acid, which are also of bacterial origin, can be either *D*- or *L*- (Careri, Spagnoli, Panari, Zannoni, & Barbieri, 1996; Marchelli et al., 2007). Pyroglutamic acid derives from *D*- and *L*-glutamic acid by cyclization (Mucchetti et al., 2000). To overcome these uncertainties, all the possible diastereomeric derivatives containing *L*-phenylalanine were synthesized: γ -*L*-Glu-*L*-Phe, γ -*D*-Glu-*L*-Phe, *L*-Pyr-*L*-Phe, *D*-Pyr-*L*-Phe, *L*-Lac-*L*-Phe, *D*-Lac-*L*-Phe. The syntheses were performed

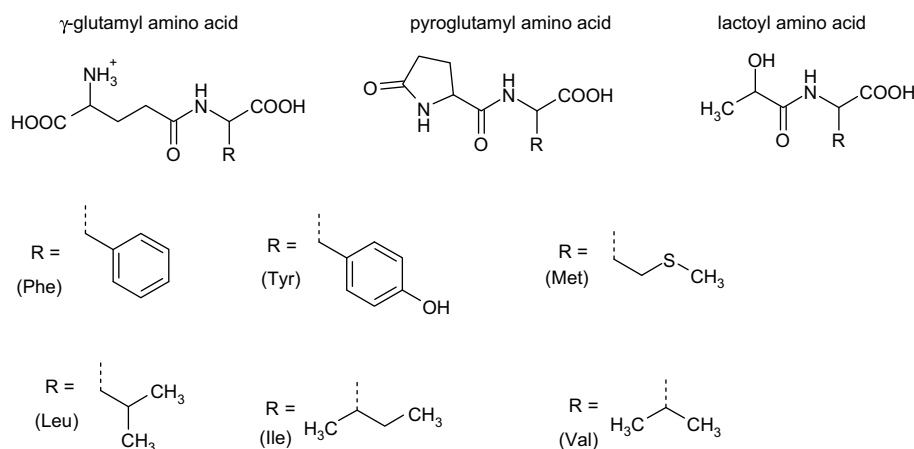


Fig. 2. Proposed structures of the aminoacyl derivatives identified in Parmigiano-Reggiano cheeses.

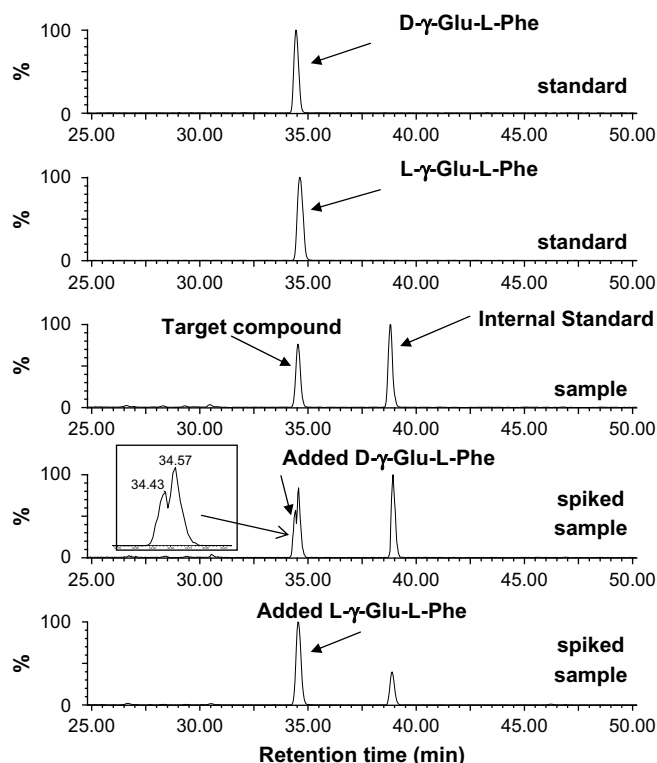


Fig. 3. Extract ion chromatograms (XICs) of D-γ-Glu-L-Phe and L-γ-Glu-L-Phe standards, a Parmigiano-Reggiano cheese sample (the internal standard Phe-Phe is coextracted to assist comparison) and the same sample alternatively spiked with the two synthesised standards. The two γ-Glu derivatives have very close elution times, but can be easily discriminated (insert). The target compound area only increases when L-γ-Glu-L-Phe is added.

by following standard protocols for peptide synthesis either in homogeneous phase or in solid phase (details are reported in [Supplementary Data](#)). The synthesised standards were analysed by LC/MS, following the same method previously used for peptide analysis, confirming that all the diastereomeric compounds eluted at different retention times. In all cases, the retention times and the MS spectra of L,L diastereomers of the synthesised standards perfectly matched with the phenylalanine derivatives identified in the hard cheeses. To get a definitive confirmation, samples of Parmigiano-Reggiano cheeses were also spiked with the standards, definitely confirming the attributions (Figs. 3–5).

As far as the other derivatives are concerned, the similarities in the MS spectra between the phenylalanine and the other derivatives indicate that, for the other compounds, the attributions are also likely to be correct. As far as the stereochemistry is concerned, since the apolar amino acids involved are known to be present in Parmigiano-Reggiano only in the L-form, and having detected only one diastereomer for every single amino acid derivative identified, the same (L,L) configuration could be tentatively hypothesised for all of them, by analogy with the phenylalanine derivatives.

3.3. Levels of γ-glutamyl, lactoyl and pyroglutamyl derivatives in cheeses at different ripening times

The levels of every compound in the Parmigiano-Reggiano samples ranging from 0 to 24 months of age were obtained by calculating the relative response factor for the synthesised phenylalanyl derivatives against the standard phenylalanyl-phenylalanine, which was added to all samples, and assuming for the other N-γ-glutamyl-, N-pyroglutamyl- and N-lactoyl-amino acids, the same response factors of the

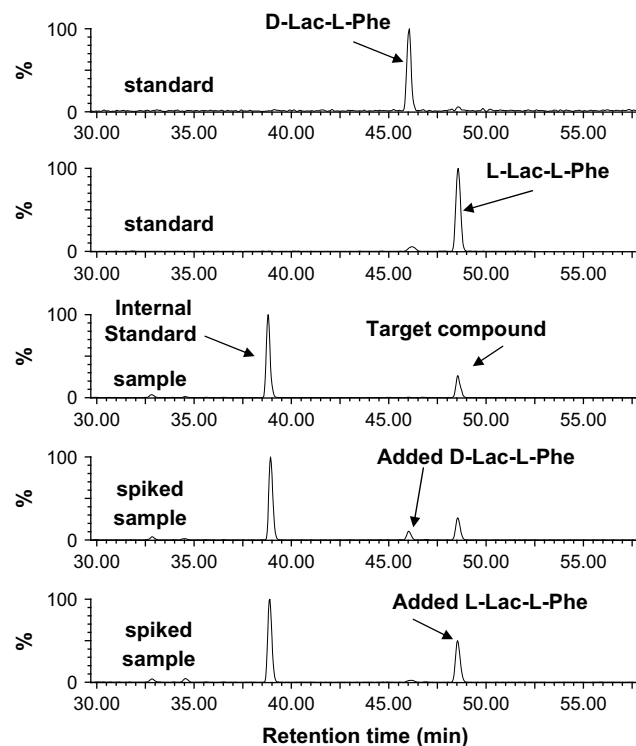


Fig. 4. Extract ion chromatograms (XICs) of D-Lac-L-Phe and L-Lac-L-Phe standards, a Parmigiano-Reggiano cheese sample (the internal standard Phe-Phe is coextracted to assist comparison) and the same sample alternatively spiked with the two synthesised standards. The target compound area only increases when L-Lac-L-Phe is added.

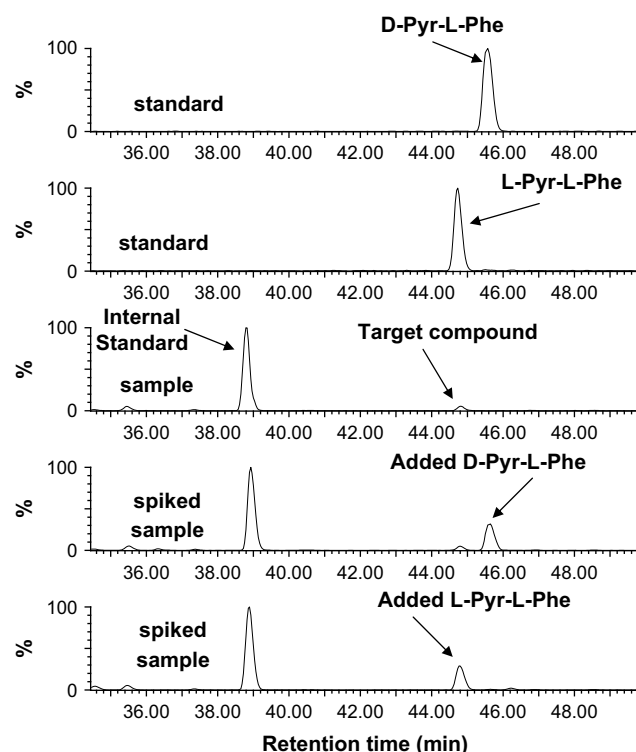


Fig. 5. Extract ion chromatograms (XICs) of D-Pyr-L-Phe and L-Pyr-L-Phe standards, a Parmigiano-Reggiano cheese sample (the internal standard Phe-Phe is coextracted to assist comparison) and the same sample alternatively spiked with the two synthesised standards. The target compound area only increases when L-Pyr-L-Phe is added.

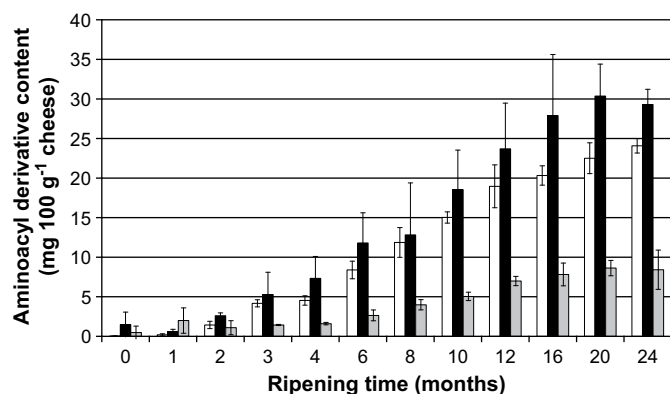


Fig. 6. Quantitative content of different aminoacyl derivatives at different times of ripening: (□), total N - γ -glutamyl-amino acids; (■), total N -lactoyl-amino acids; (▨), total N -pyroglyutamyl-amino acids.

corresponding phenylalanyl derivatives (details in the Section 2). The data are reported in Fig. 6, expressed as total content of γ -glutamyl, pyroglyutamyl and lactoyl derivatives in the samples at different ripening times. It is evident that these compounds, hardly present in the first months, strongly increased during the ripening time. Their accumulation in Parmigiano-Reggiano cheese during ripening could be a consequence of their resistance to proteases due to their unusual structures.

The distribution of the different aminoacyl derivatives in every class of compounds, calculated at month 24 of ripening is shown in Table 2. In every class, the phenylalanine, leucine and isoleucine derivatives are the most prevalent, whereas the methionine and tyrosine derivatives are the least prevalent. These distributions are consistent with the relative abundance of the corresponding free amino acids in aged hard cheeses (Careri et al., 1996). It is quite likely that Pyr-Val, Pyr-Met and Pyr-Tyr could not be detected, even if present, given the general low abundance of the pyroglyutamyl derivatives as compared with the other two classes.

To investigate the presence of such compounds in other aged cheeses, several other samples of aged Parmigiano-Reggiano, Grana Padano and Asiago cheeses were bought in the Italian market and analysed according to the method reported above (Table 3). In all of them, considerable amounts of the N - γ -glutamyl-, N -pyroglyutamyl- and N -lactoyl-amino acids reported above were found, indicating that their presence is a common feature of aged cheeses.

The dipeptides Glu-Ile, Glu-Leu and Glu-Phe, previously reported by the authors as present in Grana Padano cheeses (Sforza, Ferroni, Galaverna, Dossena, & Marchelli, 2003), can now be reliably reassigned as γ -glutamyl derivatives. Beside the already cited γ -glutamyl-amino acids found in Comté (Roudot-Algaron et al., 1994), the presence of γ -glutamyl-amino acids has also very recently been reported in Gouda cheeses (Toelstede, Dunkel, & Hofmann, 2009).

3.4. Origin of γ -glutamyl, lactoyl and pyroglyutamyl derivatives

Several indications point to a common origin, likely enzymatic, of the identified compounds: (a) all the aminoacyl derivatives

Table 2

Percentage distribution of the different aminoacyl derivatives in every class (calculated at the 24th month of ageing), calculated by the relative area ratios in the corresponding XICs.

	Phe	Tyr	Met	Ile	Leu	Val
γ -Glu-AA	25%	4%	4%	17%	38%	12%
Lac-AA	30%	8%	11%	14%	19%	18%
Pyr-AA	38%	n.d.	n.d.	35%	27%	n.d.

Table 3

Levels^a of γ -glutamyl-, lactoyl-, pyroglyutamyl-amino acids found in commercial cheeses at different times of ripening.

N-acyl-amino acids ^b	Grana Padano cheese			Parmigiano-Reggiano cheese			Asiago cheese		
	7	10	33	12	18	18	6	12	18
Months of ageing									
γ -Glu-AA	34.3 ±3.8	81.0 ±8.9	58.0 ±6.4	22.0 ±2.4	43.6 ±4.8	35.8 ±3.9	1.6 ±0.2	6.6 ±0.7	13.1 ±1.4
Lac-AA	9.0 ±1.0	22.8 ±2.5	39.5 ±4.3	14.1 ±1.6	27.6 ±3.0	23.4 ±2.6	4.0 ±0.4	10.8 ±1.2	6.3 ±0.7
Pyr-AA	8.8 ±1.0	22.8 ±2.5	26.1 ±2.9	16.3 ±1.8	31.0 ±3.4	24.8 ±2.7	15.5 ±1.7	2.8 ±0.3	0.6 ±0.1

^a Values given are total levels ($\pm$ standard deviation) of N -acyl-amino acids in mg 100 g⁻¹ cheese.

^b Abbreviations are: γ -Glu-AA, Lac-AA, Pyr-AA: γ -glutamyl-, lactoyl-, pyroglyutamyl-amino acids, respectively.

identified contain an apolar amino acid (Phe, Val, Met, Ile, Leu, Tyr), suggesting a common specificity in the substrate choice; (b) these amino acids are all acylated at the α nitrogen with carboxylic acids commonly found in cheeses (glutamic, pyroglylamic or lactic acid), indicating a common reaction and quite interestingly, according to Careri et al. (1996), by considering all the amino acids and the other organic acids, these three compounds are among the most abundant in aged cheeses; and (c) the reaction appears to be highly stereoselective, since only the L -configuration has been found in the acyl moieties, although the corresponding D -acids are known to be present in hard cheeses. Although one can only speculate on the origin of such enzymes, they might derive from the lactic acid bacteria, either starter or non-starter, or from other bacteria. Further investigations are needed to better elucidate the origin of these unusual amino acid derivatives.

4. Conclusions

The occurrence and the accumulation of non-proteolytic peptide-like molecules in Parmigiano-Reggiano cheeses, γ -glutamyl-, pyroglyutamyl- and lactoyl-amino acids, was demonstrated. These molecules could be generated by an enzyme-catalysed coupling of some apolar free amino acids with L -pyroglylamic acid, L -lactic acid or with the γ -carboxylic group of L -glutamic acid. To the best of our knowledge, lactoyl-amino acids are here reported for the first time to be naturally present in foods. The accumulation of γ -glutamyl-, pyroglyutamyl- and lactoyl-amino acids in Parmigiano-Reggiano cheeses could be usefully exploited to estimate the actual age of Parmigiano-Reggiano samples, a characteristic strongly linked to their quality and to their prices in the market. Moreover, their formation sequesters bitter amino acids (Phe, Leu, Ile, Val) from the amino acidic pool, transforming them into derivatives which have been demonstrated to have a more “umami” taste. The possibility to control such a reaction in cheeses and other foods, by identifying the enzymes involved, could be a way to induce the development of a pleasant flavour.

Acknowledgements

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Appendix. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.idairyj.2009.04.009.

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Supplementary Data

1. Synthesis of γ -D-glutamyl-L-phenylalanine and γ -L-glutamyl-L-phenylalanine

γ -D-glutamyl-L-phenylalanine (γ -D-Glu-L-Phe) was synthesized starting from 0.1 g of Fmoc-Phenylalanine immobilized on a Wang resin (Fmoc-Phe-Wang, Calbiochem-Novabiochem, L  ufelfingen, Switzerland, loading 0.65 mmol g^{-1} , 0.065 mmol). The resin was placed in a reaction vessel for solid-phase peptide synthesis, stirred for 30 min in CH_2Cl_2 for optimal swelling, and washed twice with dimethylformamide (DMF). The Fmoc protective group was then removed with a solution of 20 % piperidine in DMF ($2 \times 5 \text{ min}$) and the resin was washed twice with DMF. A mixture of 0.11 g (0.325 mmol) of Boc-D-Glu-OBzl, 0.12 g (0.310 mmol) of hexafluorophosphate benzotriazolyltetrametiluronium (HBTU), and 0.11 mL (0.650 mmol) of diisopropylethylamine (DIPEA) were dissolved in 1.625 mL of DMF, activated for two min and then immediately added to the resin. The reaction vessel was stirred for 30 min, then washed twice with DMF. The completion of the reaction was monitored by standard Kaiser test (Kaiser et al., 1970). The resin was washed again twice with CH_2Cl_2 and dried under vacuum. The peptide was cleaved from the resin, with simultaneous removal of the Boc and benzyl protecting groups, by adding 5 mL of a 6:2:1:1 (by vol) solution of trifluoroacetic (TFA):trifluoromethanesulfonic (TFMSA):m-cresol:thioanisol to the reaction vessel and slightly mixing for 60 min. The reaction mixture was washed several times with TFA and the combined eluates were dried under N_2 flux. Diethyl ether was added and the product was extracted twice with water, then the aqueous phase was dried under vacuum; crude yield was 60 %. The product was redissolved in $\text{H}_2\text{O}:\text{CH}_3\text{CN}:\text{HCOOH}$ (50:50:0.2, by vol) and analyzed by LC/ESI-MS (same conditions as utilized for samples).

γ -L-glutamyl-L-phenylalanine (γ -L-Glu-L-Phe) was synthesized starting from N^α -Boc-L-Glutamic acid α - ^{t} butyl ester (0.30 g, 1 mmol) dissolved in anhydrous DMF together with HBTU

(0.36 g, 0.95 mmol) and benzotriazole (HOBt) (0.13 g, 0.95 mmol). The mixture was stirred at room temperature for 10 min, then L-Phenylalanine ^tbutyl ester (0.21 g, 0.95 mmol) was added together with DIPEA (0.33 ml, 2 mmol) and the reaction was left under magnetic stirring for 3 h at room temperature. DMF was then evaporated under reduced pressure and the residue was dissolved in methylene chloride. The organic solution was washed with a saturated solution of KHSO₄ (three times), NaHCO₃ (three times) and NaCl (three times), dried over Na₂SO₄ and filtered. The solvent was evaporated under reduced pressure and the residue was dissolved in 10 mL TFA/CH₂Cl₂ (1:1, v/v) solution, also containing 2 % (v/v) of m-cresol and thioanisole as carbocation 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 isolated by centrifugation; crude yield was 100%. The product was diluted in H₂O:CH₃CN:HCOOH (50:50:0.2, by vol) and analyzed by LC/MS (same conditions utilized for samples).

2. Synthesis of D-lactoyl-L-phenylalanine and L-lactoyl-L-phenylalanine

D-lactoyl-L-phenylalanine (D-Lac-L-Phe) was synthesized starting from (R)-(+)-2-(Benzyloxy)propionic acid (0.54 g, 3 mmol) dissolved in 6 mL of CH₂Cl₂, together with 1.08 g (2.85 mmol) of HBTU; the mixture was stirred at room temperature for 30 min, then L-phenylalanine benzyl ester hydrochloride (0.88 g, 3 mmol), dissolved in 4 mL of CH₂Cl₂, together with DIPEA (1.23 mL, 7.5 mmol), was added. The reaction was left with magnetic stirring for 4 h at room temperature and it was monitored by Thin layer chromatography (TLC, eluent: ethyl acetate as eluent, UV absorbance detector, R_f of 0.8). The organic solution was washed with saturated solutions of KHSO₄ (three times) and NaHCO₃ (three times), dried over MgSO₄, filtered, and dried under vacuum; crude yield was 73%. The benzyl protective group was removed from the product by hydrogenation in methanol catalyzed by Pd/C (10%). After 6 hours the catalyzer was

removed by filtration and the methanol was removed under vacuum; crude yield was 81%. The dried product was redissolved in H₂O:CH₃CN:HCOOH (50:50:0.2, by vol) and analyzed by LC/ESI-MS (same conditions utilized for samples).

L-lactoyl-L-phenylalanine (L-Lac-L-Phe) was synthesized starting from free phenylalanine. L-phenylalanine methyl ester hydrochloride was first synthesized: 2.00 g of L-phenylalanine (12.05 mmol) were dissolved in 100 mL of methanol and kept under continuous stirring in an ice bath; SOCl₂ was slowly added up to a final concentration of 1 M (corresponding to 24 mL of SOCl₂). The reaction was monitored by TLC (eluent: n-butanol:acetic acid:H₂O, 4:1:1, by vol; UV and ninidrine detection, R_f of 0.6) and took place overnight. The reaction mixture was dried under vacuum, then methanol was added and evaporated again under reduced pressure (4 times) to eliminate completely the HCl; crude yield was 98%. (S)-2-acetoxypionic acid (0.41 g, 3.12 mmol) was then dissolved in 4 mL of CH₂Cl₂ together with 1.12 g (2.96 mmol) of HBTU and the mixture was kept under continuous stirring at room temperature for 30 min, in order to activate the carboxylic function. The L-Phenylalanine-methyl ester hydrochloride previously synthesized (0.68 g, 3.12 mmol) was dissolved in 4 mL of CH₂Cl₂, together with DIPEA (1.54 mL, 9.36 mmol) and then added to the activated acetoxypionic acid. The reaction was left under magnetic stirring for 4 h at room temperature. The reaction was monitored by TLC (eluent: ethyl acetate; UV absorbance detector, R_f of 0.8). The organic solution was then washed with saturated solutions of KHSO₄ (three times) and NaHCO₃ (three times), dried over MgSO₄ and filtered and the product was dried under vacuum; crude yield was 46%. The methyl and the acetyl protecting groups were then removed by reacting the product in a mixture of 0.34 g (1.296 mmol) of Ba(OH)₂·5H₂O in 20 mL of tetrahydrofurane (THF)/H₂O (1:1, v/v) for 20 min at 0 °C. THF was then eliminated under vacuum, and the aqueous solution was acidified to pH 3.0 with HCl. The resulting solution (containing barium salts) was analyzed by LC/ESI-MS (same conditions utilized for samples).

3. Synthesis of D-pyroglutamyl-L-phenylalanine and L-pyroglutamyl-L-phenylalanine

D-pyroglutamyl-L-phenylalanine (D-Pyr-L-Phe) was synthesized by starting from 0.39 g (3 mmol) of D-pyroglutamic acid dissolved in 10 mL of CH₂Cl₂ together with 1.08 g (2.85 mmol) of HBTU. The mixture was kept with continuous stirring at room temperature for 30 min. L-Phenylalanine-methyl ester hydrochloride (0.65 g, 3 mmol, synthesized as reported above) was dissolved in 4 mL of CH₂Cl₂, together with DIPEA (1.48 mL, 9 mmol) and then added to the activated pyroglutamic acid. The reaction was left under magnetic stirring for 5 h at room temperature and monitored by TLC (eluent: ethyl acetate; UV absorbance detector, R_f of 0.65). The organic solution was washed with saturated solutions of KHSO₄ (three times) and NaHCO₃ (three times), dried with MgSO₄ and filtered. The product was dried under vacuum; crude yield was 60 %. The methyl group was then removed by dissolving the ester in 50 mL of THF/H₂O (1:1, v/v) and adding 2 equivalents of Ba(OH)₂, then reacting for 20 min at 0 °C. The THF was eliminated under vacuum, and the aqueous solution was acidified to pH 3.0 with HCl. The product was precipitated by centrifugation and dried under vacuum; yield was 54 %. The product was then dissolved in H₂O:CH₃CN:HCOOH (50:50:0.2, by vol) and analyzed by LC/ESI-MS (same conditions utilized for samples).

L-pyroglutamyl-L-phenylalanine (L-Pyr-L-Phe) was synthesized exactly as described for D-Pyr-L-Phe, but the product did not precipitate after methyl ester deprotection; thus, the entire reaction mixture was analyzed by LC/ESI-MS (same conditions utilized for samples).

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