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Occurrence of non-proteolytic amino acyl derivatives in dry-cured ham

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ABSTRACT

Proteolysis is the most important event occurring during maturation of dry-cured hams: it strongly influences the flavour and the texture of the aged ham by the accumulation of peptides and free amino acids released by protein hydrolysis. Apart from compounds of proteolytic origin, it has been demonstrated that also non-proteolytic amino acyl derivatives (γ-glutamyl amino acids, pyroglutamyl-amino acids and lactoyl-amino acids) may accumulate during ripening of cheese, and they can be also found in fermented soy sauce, where they contribute to the umami taste of the products. Using a semi-quantitative analysis, in this paper we report the occurrence of significant amounts of γ-glutamyl amino acids and, for the first time, pyroglutamyl-amino acids and lactoyl-amino acids, in aged ham. The amino acid counterparts were mainly found to be hydrophobic amino acids. The amount of these compounds was found to increase with time, because they are not degraded by proteolytic activity. They were also found to be stable to simulated gastrointestinal digestion. Angiotensin Converting Enzyme inhibitory activity was also tested, but they were not found to be characterized by significant ACE-inhibitory activity.

1. Introduction

Proteolysis is the most important phenomenon occurring during ageing of dry-cured ham, giving rise to an accumulation of free amino acids and small peptides. These low molecular weight compounds are characteristically formed in ham by the proteolytic activities arising from endogenous meat muscle enzymes such as calpains and cathepsins (Khan, Jung, Nam, & Jo, 2016), acting both on myofibrillar proteins (myosin and actin) as well as on sarcoplasmic proteins (enzymes involved in the glycolytic and respiratory cycles and myoglobin) (Picariello et al., 2006). They positively contribute to the nutritional value and play a fundamental role in flavour and texture development. Moreover, great interest has also been aroused in the last years about the potential functional bioactivities of small peptides (Udenigwe & Aluko, 2011).

Among the low-molecular weight nitrogen fraction of several proteinaceous foods, mainly cheeses but also non-dairy products, a particular group of unusual amino acyl derivatives of non-proteolytic origin, namely γ-glutamyl-amino acids, lactoyl-amino acids and pyroglutamyl-amino acids, collectively known as Non-Proteolytic Aminoacyl Derivatives (NPADs), were found in consistent amount. In these compounds, the carboxylic function of glutamic acid (*via* the carboxylic group of the side chain, γ), pyro-glutamic or lactic acid, forms a peptide bond with the amino group of non-polar (aliphatic or aromatic) amino acids, such as phenylalanine, tyrosine, methionine, leucine, isoleucine or valine.

In particular, γ-glutamyl-phenylalanine (γ-Glu-Phe; broth, salty, and slightly sour), γ-glutamyl-tyrosine (γ-Glu-Tyr; sour and salty) and γ-glutamyl-leucine (γ-Glu-Leu) have been originally reported in Comtè cheese (Roundot-Algaron, Kerhoas, Le Bars, Einhorn, & Gripon, 1994). As demonstrated by subsequent studies, γ-glutamyl-amino acids are widespread in dairy products (Sforza, Cavatorta, Galaverna, Dossena, & Marchelli, 2009; Toelstede & Hofmann, 2009).Their total amounts vary between few mg and > 50 mg per 100 g of cheese.

Properties of these compounds are still largely unknown, apart from their peculiar characteristic taste, which, for most of them, has been described as kokumi taste (Toelstede & Hofmann, 2009).

Indeed, γ-glutamyl-leucine, γ-glutamyl-valine and γ-glutamyl-cysteinyl-alanine having these characteristics have been isolated by edible beans (Dunkel, Köster, & Hofmann, 2007) as well as γ-glutamyl–valyl–glycine discovered in Vietnamese fish sauce (Kuroda, Kato, Kamazaki, & Kageyama 2012; Kuroda et al., 2012). Eight γ-glutamyl peptides were identified as the second dominant class of umami/kokumi taste compounds in fermented corn sauce (Charve, Manganiello,

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& Glabasnia, 2018). Moreover, γ-glutamyl dipeptides with known kokumi taste threshold were also identified in sourdough, in particular with the presence of certain strains of L. *reuterii* during fermentation, suggesting a strain-specific contribution of γ-glutamyl dipeptides to the taste of bread (Zhao & Gänzle, 2016). A patent to prepare these interesting flavour compounds have been registered (Hofmann & Dunkel, 2007).

The conditions that favor the bioproduction and biodegradation of these compounds were investigated using pure enzymes and biological media both on NPADs and their amino acidic precursors (Bottesini, Tedeschi, Dossena, & Sforza, 2014). The obtained data suggested that their production in cheese, and also their partial degradation, might be due to the action of peptidases and γ-glutamyl transpeptidase (γ-GTT) of microbial origin (Sgarbi et al., 2013). Lately, a large number of γ-glutamyl dipeptides have been identified and quantified in Parmesan cheese, correlating their origin mainly to γ-GTT activity from milk (Hillmann, Behr, Ehrmann, Vogel, & Hofmann, 2016).

As well as the other NPADs, pyroglutamyl peptides have been reported in wheat (Higaki-Sato et al., 2003), mushrooms (Altamura, Andreotti, Bazinet, & Long, 1970), and for the first time also in Parmigiano-Reggiano cheese (Sforza et al., 2009), where its formation is promoted by the presence of pyroglutamic acid (Mucchetti et al., 2000). Derivatives of pyroglutamyl peptides have been patented by Nestlè as umami taste enhancers to foodstuffs (Schlichtherle-Cerny & Amadò, 2002).

Finally, lactoyl-amino acids were recently reported for the first time in Parmigiano-Reggiano cheese, formed by microbial enzymatic activities starting from lactic acid and amino acids (Sgarbi et al., 2013).

Lastly, in a recent paper, all these compounds have been found also in fermented soy sauce: in particular, γ-glutamyl and pyroglutamyl dipeptides, as well as lactoyl amino acids, were identified in the acidic fraction of soy sauce (Frerot & Chen, 2013). As far as their properties, the most studied characteristic of all these compounds is their taste enhancing properties both for kokumi and umami taste, as proved by several recent activities and patents (Frerot, Escher, & Firmenich, 1997; Winkel et al., 2008). No data have been reported yet on the occurrence of these compounds in meat products: the only available data are related to the presence of small dipeptides with the composition Glu-Phe, Glu-Ile, Glu-Leu and Glu-Tyr which have already been found in Parma ham, although at that time not recognized as γ-glutamyl derivatives: their content was found to increase during the ripening time and to be possibly linked to the pleasant aged flavour of the product (Sforza et al., 2006). Very recently, N-lactoyl-amino acids have been found to be ubiquitous metabolites that originate from CNDP2-mediated reverse proteolysis of lactate and amino acids in mammalian tissue (Jansen et al., 2015). Non proteolytic amino acyl derivatives contain an unconventional amide bond which is not usually found in peptides and proteins, thus being possibly resistant to the action of common proteases. Indeed, γ-Glu-Phe and Lac-Phe were already demonstrated to be resistant both to *in vitro* digestion and to blood serum proteases (Bottesini et al., 2014). This strong resistance towards enzymatic activities of the gastrointestinal digestion process hints to their possible role as bioactive molecules.

The aim of this work is to evaluate the presence of aminoacyl derivatives in dry-cured hams at different ageing time. γ-glutamyl-amino acids, pyroglutamyl-amino acids and lactoyl-amino acids were chemically synthesized and they were used for their confirmation and quantification by LC-MS/MS analysis.

2. Material and methods

2.1. Samples

Dry-cured ham samples were purchased from the Experimental Station for the Food Preserving Industry (Parma). Samples of *biceps femoris* muscle were analyzed at different processing time: salting, post-salting, 18 and 24 ripening months. The muscles were triturated by a laboratory mill and stored at −22 °C until analysis.

2.2. Extraction of the peptide fraction (MW < 10 kDa)

Extraction of the peptide fraction was carried out as described by Paolella et al. (2015). Five grams of minced ham were extracted with 45 mL of HCl 0.1 M, after the addition of 250 μL of the internal standard (phenylalanine-phenylalanine, 1 mM). After homogenization (1 min) with a fixed rod homogenizer (Ultra Turrax T50 Basic, IKAWerke, Staufen, Germany), samples were centrifuged at 3220*g* for 1 h at 4 °C. The supernatant was subsequently filtered on filters at decreasing pore size (paper, 5 μm type SMWP, 0.45 μm type HPLV). Four millilitres of the obtained extract was evaporated to dryness under vacuum by rotary evaporator and the residue was dissolved with 2 mL of a 0.1% formic acid aqueous solution. This solution was then filtered on 10 kDa cut off filters (Vivaspin 2, Sartorius, Gottingen, Germany). The filtered sample was evaporated to dryness and dissolved in 150 μL of water acidified with 0.1% formic acid.

2.3. Simulated gastro-intestinal digestion

Simulated gastro-intestinal digestion was performed according to the protocol proposed by Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005). Briefly, ground meat from *biceps femoris* muscle (2 g) was incubated with 3 mL of saliva-mimicking juice at 37 °C (pH = 6.8 ± 2) for 5 min, under stirring on a reciprocating shaker. After this first step, 6 mL of the gastric juice (pH = 1.3 ± 0.2) were added and the mixture was incubated for 2 h in the same conditions. Finally, 1 mL of sodium bicarbonate (1 M), 6 mL of duodenal juice (pH = 8.1 ± 0.2) and 3 mL of bile (pH = 8.1 ± 0.2) were added simultaneously and the mixture was left to digest for 2 h. The enzymatic reaction was stopped by heating at 95 °C for 15 min. The mixture was then centrifuged at 3220*g* for 20 min to separate the supernatant (chime) and the pellet (residual matrix). The supernatant was subjected to a final clean-up step by a Sep-Pak C18 cartridge (Waters, Milford, MA, USA): after conditioning the cartridge with methanol (2 mL) and deionized water (3 mL), chime was applied (2 mL) and peptides were eluted with 2 mL of H_2O:CH_3OH (50:50, *v*/v) solution. The eluate (1 mL) was evaporated to dryness under nitrogen flow and the residue was dissolved with 1 mL of aqueous formic acid (0.1% v/v). After centrifugation, samples were analyzed in a UPLC/ESI-MS system. For the semi quantification analysis, 4 μL of internal standard Phe-Phe 1 mM were added to 196 μL of digested sample, just before UPLC/ESI-MS analysis.

2.4. UPLC/ESI-MS analysis and HPLC/ESI-MS/MS analysis

UPLC/ESI-MS analyses and HPLC/ESI-MS/MS analysis were carried out as described by Paolella et al. (2015).

UPLC–ESI-MS analyses were performed with an Acquity Ultraperformance UPLC equipped with a single quadrupole mass spectrometer (Waters, Milford, MA, USA). The chromatographic separation was run in reverse phase using a C18 column (Acquity UPLC BEH 300 Å, 1.7 μm, 2.1 × 150 mm, Waters); eluent A was water with 0.1% formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid. The chromatographic gradient was: 0–7 min 100% A, 7–50 min linear from 100% A to 50% A, 50–52.6 min isocratic 50% A, 52.6–53 min linear from 50% A to 0% A, 53–58.2 min isocratic 0% A, 58.2–59 min linear from 0% A to 100% A, and 59–72 min isocratic 100% A. LC parameters: flow rate, 0.2 mL/min; analysis time, 72 min; column temperature, 35 °C; sample temperature, 18 °C; injection volume, 2 μL for digested samples (with 7 min of solvent delay) and 4 μL for extracted samples (without solvent delay). MS parameters: Full Scan mode, acquisition time 7–58.2 min; ionization type, ESI + (positive ions); scan range, 100–2000 *m/z*; capillary voltage, 3.2 kV; cone