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Identification of small peptides arising from hydrolysis of meat proteins in dry fermented sausages

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ABSTRACT

In this study, proteolysis and low molecular weight (LMW) peptides (<3 kDa) from commercial Argentinean fermented sausages were characterized by applying a peptidomic approach. Protein profiles and peptides obtained by Tricine-SDS-PAGE and RP-HPLC-MS, respectively, allowed distinguishing two different types of fermented sausages, although no specific biomarkers relating to commercial brands or quality were recognized. From electrophoresis, α-actin, myoglobin, creatine kinase M-type and L-lactate dehydrogenase were degraded at different intensities. In addition, a partial characterization of fermented sausage peptidome through the identification of 36 peptides, in the range of 1000–2100 Da, arising from sarcoplasmic (28) and myofibrillar (8) proteins was achieved. These peptides had been originated from α-actin, myoglobin, and creatine kinase M-type, but also from the hydrolysis of other proteins not previously reported. Although muscle enzymes exerted a major role on peptidogenesis, microbial contribution cannot be excluded as it was postulated herein. This work represents a first peptidomic approach for fermented sausages, thereby providing a baseline to define key peptides acting as potential biomarkers.

1. Introduction

There has been a renewed interest in traditional fermented meat products, mainly in Europe, where they have a great significance and economic impact (Vignolo, Fontana, & Fadda, 2010). A huge variety of dry fermented products can be found as a consequence of variations applied in raw materials, formulations and manufacturing processes, according to the habits of different countries and cultures.

The typical flavor of dry cured and fermented sausages is the result of a subtle balance between volatile (alcohol, ketones, aldehydes, furans) and non-volatile (amino acids, peptides, sugars, nucleotides) compounds. These molecules come from raw materials (meat, fat, spices, nitrites and other additives) and/or biochemical reactions during fermentation and ripening (Stahnke, 2002). Meat protein degradation is one of the main biochemical events which are catalyzed by enzymes that belong to meat (also called endogenous enzymes) or microorganisms. Meat proteins are known to undergo hydrolysis, first to polypeptides by endogenous muscle enzymes, such as cathepsins and calpains. Then, polypeptides release smaller peptides and amino acids by the action of peptidases and aminopeptidases from both muscle and bacteria. Low molecular weight peptides (<3 kDa) and free amino acids are major components of the non-protein nitrogen fraction in fermented meats. These compounds contribute, directly or indirectly, to the generation of volatile and non-volatile flavor compounds in dry and semi-dry sausages (Fadda, López, & Vignolo, 2010).

Prediction and/or characterization of the quality of fermented meat products is a difficult task since a high number of factors such as the type of raw materials, technologies and starter cultures are involved in the process. Analysis of proteins and peptides has a special interest because they play a major role in the end product quality. Recently, proteomic approaches have been applied to correlate proteolytic profiles with technological parameters in view to detect valuable biomarkers as meat quality predictors (Lametsch et al., 2003). Unlike proteomics, the novel concept of peptidomic aims at the systematic analysis of the small polypeptides content within an organism, tissue or cell (peptidome) in order to identify quantity, structure and function (Soloviev, 2010). Thus, this approach covers the mass range between proteomics and metabolomics. The pacemakers for the development of peptidomic technologies are modern mass spectrometry and bioinformatics. These tools are ideally suited for comprehensive peptide analysis, especially combined with the massive information available in today's genomic and transcriptomic databases. In contrast to proteomics, peptidomic has the potential to uncover cleavage sites of precursor proteins. But also, the peptide analysis should be performed on their native forms. Consequently, searching for peptides in databases of non-tryptic peptides is much less effective due to the lack of charge localization at the peptides N and C termini (Zürbig et al., 2006). Also, the poor fragmentation and the lack of specificity for intrinsic proteolytic enzymes are a problem for naturally occurring peptides analysis

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(Mischak, Julian, & Novak, 2007). However, global profiling approaches have been applied to the study and exploration of complex peptidomes in food science. For example, the identification and quantification of nutritionally relevant peptides (bioactive peptides) as well as the study of peptide fractions evolution, as in Parmigiano-Reggiano cheese samples (Lahrichi, Affolter, Zolezzi, & Panchaud, 2013; Panchaud, Affolter, & Kussmann, 2012; Picariello, Mamone, Addeo, & Ferranti, 2012).

Peptidomic analyses have a great potential for the determination of meat composition in processed foods due to the high stability of peptide material. Indeed, peptide fractions originated from meat have already been identified as biomarkers for meat tenderness, authenticity and sensory attributes (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Ouali et al., 2013; Paredi et al., 2013; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010; Sentandreu et al., 2007). To our knowledge, no substantial information about the characterization of peptides generated during fermentation of dry-cured sausages is available so far. The identification of the protein fragments naturally generated during sausage fermentation and ripening would be beneficial in order to better understand proteolysis and flavor development mechanisms that occur during the processing of fermented products. In this study, proteolysis of commercial Argentinean fermented sausages was characterized by a peptidomic approach in an attempt to evaluate their potential as biomarkers of technological and processing conditions. The role of bacterial enzymes responsible for the hydrolysis of meat proteins was also analyzed.

2. Materials and methods

2.1. Sausage sampling procedures

Ten fermented sausages (FS) of different commercial brands purchased from several local stores and supermarkets of Tucumán, Argentina were analyzed. The samples included three high comminuted salami (FS1, FS7 and FS8), five low comminuted salami (FS3, FS4, FS5, FS9 and FS10) and two fuet-type (FS2 and FS6) from industrial plants localized in northwestern and central regions of Argentina. Low and high comminuted salami refer to the degree of mincing of lean meat and fat used in the manufacture; fuet-type sausages are products with smaller diameter, less fermentation time and high pH. From the label, sausage formulation differed in meat composition; fuet-type were manufactured using pork meat while other samples included pork and beef meat (López, Bru, Vignolo, & Fadda, 2012) (Table 1). For sampling, at least three different batches from each commercial brand were analyzed. From each batch, 3 dry sausages without casings (approximately 500 g depending on the size of sausages) were pooled and thoroughly homogenized in order to obtain a representative sample. Subsequently, 20 g for SDS-PAGE analyses and 2.5 g for RP-HPLC analyses were taken from each batch of sausages, as described in items 2.2.1 and 2.3.1, respectively.

2.2. Protein hydrolysis

2.2.1. SDS-PAGE of sarcoplasmic and myofibrillar proteins

A portion of 20 g of three different batches of each sausage brand was selected for sarcoplasmic and myofibrillar protein extraction (Fadda et al., 1999). Bovine meat (muscle *Semimembranosus*) was used as a non-fermented control since SDS-PAGE profiles of meat proteins are not different among pork, beef and their mix at the post mortem time evaluated (Fadda et al., 1999; Fadda, Vignolo, Aristoy, Oliver & Toldrá, 2001, Sentandreu et al., 2010). Meat from Brangus animals with 9–12 months of age and half-carcass about 100–120 kg was obtained after 48 h post-mortem. Protein concentration of both sarcoplasmic and myofibrillar fractions was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. It was further adjusted with deionized water to 0.5 μg/μl final concentration. Samples were diluted 1:1 with buffer (8 M urea; 2 M thiourea; 0.05 M Tris; 75 mM DTT; 3% SDS; 0.05% bromophenol blue; pH 6.80) and heated at 100 °C for 5 min prior to electrophoresis. Tricine-SDS-PAGE (17% acrylamide-bisacrylamide for the resolving gel and 3% acrylamide-bisacrylamide for the stacking gel) was performed using miniprotean II electrophoresis equipment (Bio-Rad, Hercules, CA) by loading 20 μl of each sample as well as Ultra Low and Wide Range molecular weight markers (Sigma-Aldrich, Buenos Aires, Argentina) (Schägger & Von Jagow, 1987). Electrophoresis was carried out at 50 and 70 V for stacking and resolving gel, respectively. Gels were fixed in 30% methanol, 10% acetic acid and stained with Sypro Ruby Gel Stain (Sigma-Aldrich, Buenos Aires, Argentina). Two technical repetitions for each batch were performed for Tricine-SDS-PAGE analyses. Digitalized images of gels were analyzed by Quanti Scan software (version 2.1) (Biosoft, Cambridge, UK) to visualize band intensities and to estimate molecular weight of bands by using low range (6.500 to 66.000 Da) and wide range (6.500 to 200.000 Da) markers (Sigma, MO, USA).

2.3. Peptide analysis

2.3.1. Peptide extraction

From the fermented sausages (10), five previously described as products with high (3) and low (2) consumer's acceptance (López et al., 2012) were selected for peptide analyses and bovine meat was used as non-fermented control. Portions of 2.5 g from each fermented sausage pool from each batch were homogenized in a stomacher (8 min, in ice) with 12.5 ml 0.01 N HCl and 0.1 N HCl for sausages and meat, respectively (Sentandreu et al., 2003). The meat slurries were then centrifuged (20,000 ×g at 4 °C for 20 min) and supernatants

Table 1
Information of fermented sausages as declared on the label.

	FS[a]1	FS2	FS3	FS4	FS5	FS6	FS7	FS8	FS9	FS10
Manufacture origin	Santa Fe	Buenos Aires	Córdoba	Tucumán	Santa Fe	Buenos Aires	Santa Fe	Santa Fe	Santa Fe	Santa Fe
Product type	Salami[b]	Fuet[c]	Salami	Salami	Salami	Fuet	Salami	Salami	Salami	Salami
Degree of mincing (lean meat and fat)	High	Low	Low	Low	Low	Low	High	High	Low	Low
Sodium (%)	1.69	1.48	ND	0.45	1.42	1.65	1.45	1.57	1.45	1.69
Total fat (%)	36.00	37.50	ND	27.50	27.50	40.00	32.50	32.50	27.5	36.00
Saturated fat (%)	13.00	13.75	ND	12.25	10.00	18.25	15.00	14.25	10.5	13.00
Proteins (%)	19.00	24.75	ND	15.50	20.25	25.00	18.00	17.00	19.25	19.00
Lean meat (%)	83.91	83.52	ND	88.54	87.58	82.35	85.55	85.43	87.55	83.91
Meat type	Beef/pork	Pork	Beef/pork	Beef/pork	Beef/pork	Pork	Beef/pork	Beef/pork	Beef/pork	Beef/pork
Colorant addition[d]	ND	D	ND	ND	ND	D	ND	ND	ND	ND

[a] Fermented sausage samples; ND: not declared; D: declared.
[b] Salami: fermented sausages: 4–5 cm diameter; ripening time, generally, more than 2 weeks.
[c] Fuet type fermented sausages having 2–3 cm diameter whose processing implies minor fermentation and ripening time (max 2 weeks), resulting in products less acidic than the traditional salami-type products.
[d] Cochineal carmine (INS120).