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The use of label-free mass spectrometry for relative quantification of sarcoplasmic proteins during the processing of dry-cured ham

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ABSTRACT

The aim of this work was to quantify changes in the abundance of the major sarcoplasmic proteins throughout the ham dry-curing process by using a label-free mass spectrometry methodology based on the measurement of mass spectral peak intensities obtained from the extracted ion chromatogram. For this purpose, extraction of sarcoplasmic proteins was followed by trypsin digestion and analysis by nanoliquid chromatography coupled to tandem mass spectrometry (Q/TOF) for the identification and relative quantification of sarcoplasmic proteins through individual quantification of trypsinised peptides. In total, 20 proteins, including 12 glycolytic enzymes, were identified and quantified. The accuracy of the protocol was based on MS/MS replicates, and beta-lactoglobulin protein was used to normalise data and correct possible variations during sample preparation or LC–MS/MS analysis. Mass spectrometry-based proteomics provides precise identification and quantification of proteins in comparison with traditional methodologies based on gel electrophoresis, especially in the case of overlapping proteins. Moreover, the label-free approach used in this study proved to be a simple, fast, reliable method for evaluating proteolytic degradation of sarcoplasmic proteins during the processing of dry-cured ham.

1. Introduction

Dry-cured ham is a high-quality product whose organoleptic characteristics are the result of numerous biochemical reactions that occur during its long processing. Among them, proteolysis is the most important reaction, resulting in extensive degradation of sarcoplasmic and myofibrillar proteins by the action of endogenous muscle peptidases, which generates large amounts of peptides and free amino acids (Sforza et al., 2006; Toldrá & Flores, 1998).

Sarcoplasmic proteins are the water soluble fraction that accounts for 30–35% of total muscle proteins, mainly represented by glycolytic enzymes and myoglobin. This group of proteins does not have structural functions, but their degradation has an important effect on meat quality, affecting parameters such as colour and water holding capacity (Joo, Kauffman, Kim, & Park, 1999; Sayd et al., 2006) and contributing to the organoleptic characteristics of dry-cured ham (Toldrá & Flores, 1998). Several studies based on one-dimensional gel electrophoresis have reported that soluble proteins are intensely hydrolysed throughout the processing of dry-cured ham (Bermúdez, Franco, Carballo, Sentandreu, & Lorenzo, 2014; Córdoba et al., 1994; Larrea, Hernando, Quiles, Lluch, & Pérez-Munuera, 2006; Monin et al., 1997; Toldrá, Rico, & Flores, 1993). In fact, some of these studies have quantified the relative abundance of sarcoplasmic proteins during the processing of dry-cured ham, using the measurement of electrophoretic band density (Bermúdez et al., 2014; Larrea et al., 2006).

Traditional gel electrophoresis methodologies provide good sensitivity and linearity in protein quantification by densitometric analysis of bands, but they have some constraints, such as low specificity and limited dynamic range. Moreover, these techniques cannot identify underlying proteins, and are inaccurate in the case of overlapping proteins (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007; Bendixen, 2005; Szabo, Szomor, Foeldi, & Janaky, 2012). These problems could be overcome by using modern mass-spectrometry techniques, which have allowed faster and more accurate identification and quantification of proteins extracted from complex samples (Aebersold & Mann, 2003; Cravatt, Simon, & Yates, 2007). Thus, a combination of 2-D electrophoresis and mass spectrometry, especially MALDI-TOF MS, provides high resolution separations, protein identifications by peptide mass fingerprinting, and quantification by measuring gel band densities (Di Luccia et al., 2005; Paredi et al., 2013; Théron et al., 2011), although only a few studies follow up the degradation profile of proteins during dry-cured ham processing.

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Table 1
Sarcoplasmic proteins identified and quantified by nESI-LC–MS/MS in dry-cured ham.

ID	Protein name	Theoretical MW (Da)	Peptides [a]	Function	Location SDS–PAGE
PYGM	Glycogen phosphorylase	97232	8	Glycogen metabolism (glycolysis-related)	a
ACON	Aconitate hydratase	85306	4	Tricarboxylic acid cycle, carbohydrate metabolism	b
PFK	Phosphofructokinase	85273	5	Glycolysis	c
PK	Pyruvate kinase	63087	5	Glycolysis	d
PGM	Phosphoglucomutase	61551	5	Glucose and carbohydrate metabolism (glycolysis-related)	e
GPI	Glucose 6-phosphate isomerase	57781	8	Glycolysis	f
ENO	Enolase	47100	9	Glycolysis	g
AATC	Aspartate aminotransferase	46446	3	Carbohydrate and amino acid metabolism	h
PGK	Phosphoglycerate kinase	44530	5	Glycolysis	i
CK	Creatine kinase	43032	12	Creatinine metabolism	j
ALDO	Fructose-bisphosphate aldolase	39323	6	Glycolysis	k
GPD	Glycerol 3-phosphate dehydrogenase	37624	3	Lipid metabolism	l
LDH	Lactate dehydrogenase	36596	9	Pyruvate metabolism (glycolysis-related)	m
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	35814	3	Glycolysis	n
MDH	Malate dehydrogenase	35573	4	Carbohydrate and amino acid metabolism, tricarboxylic acid cycle	o
CAH3	Carbonic anhydrase 3	29393	6	Reversible hydration of carbon dioxide	p
PGAM	Phosphoglycerate mutase	28667	4	Glycolysis	q
TPI	Triosephosphate isomerase	26673	6	Glycolysis	r
GST	Glutathione S-transferase	23482	1	Detoxification of reactive oxygen species	s
MYG	Myoglobin	17074	5	Oxygen transport (colour development)	t

[a] Average of peptides used to calculate the ratio of each protein at different processing times.

More recently, the use of modern proteomic techniques, using either labelled or label-free methods, has improved protein quantification relative to traditional 1-D or 2-D gel electrophoresis. Although protein labelling provides the most accurate quantification, it requires the use of expensive isotopes and complex experimental protocols. In contrast, label-free techniques are simple, reliable, versatile and cost-effective (Bantscheff et al., 2007; Neilson et al., 2011). One of the possible strategies in the label-free MS approach is quantification based on the signal intensity measurements of extracted ion chromatograms, whereby the peak areas of peptides correlate to the concentration of the particular protein from which the peptides were derived. This technique allows more precise and accurate evaluation of changes in protein abundance between samples in comparison with other label-free strategies such as spectral counting, which is useful for the relative quantification of proteins in a single sample (Wang, Wu, Zeng, Chou, & Shen, 2006; Zhu, Smith, & Huang, 2010). Thus the aim of the present study is the application of a label-free methodology based on the measurement of ion peak intensities as a simple and reliable method for the relative quantification of sarcoplasmic proteins during the processing of dry-cured ham.

2. Materials and methods

2.1. Materials

Spanish dry-cured hams originating from 6-month-old pigs (Landrace × Large White) were prepared according to the traditional procedure until they reached a total processing duration of 9 months. The process consisted of a pre-salting stage (using a mixture of salt, nitrate and nitrite for 30 min), a salting period (the hams were buried in salt and piled up at 2–4 °C and 90–95% relative humidity for 12 days), a post-salting stage (at 4–5 °C and 75–85% relative humidity for 60 days), and finally a ripening–drying period (at temperatures increasing from 5 °C to 14–20 °C and relative humidity decreasing to 70%).

For the study, samples from three hams were taken at different processing times: 0 months (raw ham), 2 months (end of the post-salting stage), 3.5, 5 and 6.5 months (during the ripening–drying period), and 9 months (at the end of the dry-cured process). The study was done in triplicate.

2.2. Extraction of sarcoplasmic proteins

For the purpose of sarcoplasmic protein extraction, two grams of *Biceps femoris* of each sample taken from the same location within the muscle were minced and 20 mL of 50 mM Tris–HCl pH 8 was added, mixing with a vortex for 2 min. The homogenate was centrifuged at 4 °C and 12,000*g* for 20 min, and supernatants containing sarcoplasmic proteins were collected. Protein concentration was determined by the Bradford protein assay (Bradford, 1976), using bovine serum albumin (BSA) as protein standard.

2.3. Separation of sarcoplasmic proteins by SDS–PAGE

Sarcoplasmic extracts were diluted five times with bidistilled water, and then 100 μL of each extract was mixed with 100 μL of sample buffer (0.5 M Tris–HCl pH 6.8, 10% w/v SDS, 50% v/v glycerol, 0.2 M DTT and 0.05% v/v bromophenol blue). After the homogenate had been heat denatured at 95 °C for 4 min, 10 μL of each sample was loaded onto the gel, and electrophoresis was carried out at 120 V and 50 W, using a 12% acrylamide separation gel and a 4% acrylamide stacking gel (Laemmli, 1970). The ProteoSilver Plus silver stain kit (Sigma, St. Louis, MO, USA) was employed to develop the gel, and SDS–PAGE molecular weight standards, broad range (161-0317; Bio-Rad Laboratories, Inc., CA, USA), consisting of myosin (200 kDa) β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa), were used to assess the molecular weights of the proteins. The bands were identified according to their molecular weight, and the amount of each protein band was calculated by image processing using ImageJ 1.49v software (MD, USA; http://imagej.nih.gov/ij/).

2.4. In-solution digestion of sarcoplasmic proteins

Sarcoplasmic extract solutions were also subjected to digestion using trypsin enzyme (sequencing grade modified trypsin; Pro-