LABEL-FREE RELATIVE QUANTITATION OF ENOLASE AND GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE ENZYMES DURING DRY-CURED HAM PROCESSING

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Abstract –The aim of this study was to quantify changes in the abundance of two glycolytic enzymes during the dry-cured ham processing by using a fast, reliable, and accurate methodology such as the label-free quantitation by mass spectrometry. For this purpose, extraction of sarcoplasmic proteins and trypsin digestion have been done, followed by identification and relative quantitation by nanoliquid chromatography coupled to tandem mass spectrometry. So, enolase and glyceraldehyde 3-phosphate dehydrogenase enzymes have been quantified with a label-free methodology based on the measurements of mass spectral peak intensities, showing how their abundances decrease by proteolysis along the dry-cured ham processing.

Key Words – peptides, quantitative proteomics, mass spectrometry.

I. INTRODUCTION

Glycolytic enzymes are a group of ten sarcoplasmic proteins involved in the extraction of energy from carbohydrates in the form of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). These enzymes are strongly hydrolysed by the proteolytic reactions that occur along the dry-cured ham processing [1, 2], generating large amounts of peptides and free amino acids that contribute to the development of the organoleptic properties of dry-cured ham [3].

Recent advances in mass spectrometry techniques have become an important tool for a fast and precise identification and quantitation of proteins compared to classical methodologies based on gel electrophoresis [4]. In this sense, quantitation of proteins in complex biological samples can be performed by labelling methodologies, which involve the use of stable isotopes, or label-free methods, which are a simple, versatile, reliable and cost-effective alternative to label techniques [4, 5]. One of the strategies in the label-free approach is the quantitation based on the intensities measured from the extracted ion chromatogram obtained in the spectra, in which it has been established a linear correlation between ion amount and signal, and that allows to accurately evaluate changes in protein abundance between samples [6].

In this study, a label-free methodology based on the measurements of peak intensities has been used for the relative quantitation of two glycolytic enzymes such as enolase (ENO) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in order to evaluate their proteolytic degradation during the processing of dry-cured ham.

II. MATERIALS AND METHODS

The study was carried out with *Biceps femoris muscle* from Spanish dry-cured hams at different times of processing (0, 2, 3.5, 5, 6.5, and 9 months). Sarcoplasmic proteins were extracted (1:10, w/v) by adding 50 mM Tris-HCl (pH 8), homogenising and centrifuging at 4ºC and 12,000 *g* for 20 min. Then, supernatants containing sarcoplasmic proteins were submitted to in-solution digestion with trypsin enzyme and nanoliquid chromatography-tandem mass spectrometry (nLC-MS/MS) according to the methodology described by Gallego *et al.* (2015) [7], but with some modifications. Briefly, 0.5 nmol of beta-lactoglobulin protein was added to 1.5 nmol of sarcoplasmic solutions as normaliser of data, followed by reduction, alkylation and digestion with trypsin enzyme. A total of 50 µL of each digested sarcoplasmic extract were lyophilised and then reconstituted with 100 µL 0.1% TFA. Finally, 5 µL of each sample was injected on the nLC-MS/MS system (TripleTOF® 5600+ system, AB Sciex Instruments, MA, USA), and the data analysis was done using Mascot Distiller v2.5.1. software (Matrix Science, Inc.,

Boston, MA, USA) and SwissProt database. Quantitation parameters were selected using the label-free option, based on replicates of the relative intensities of extracted ion chromatograms, and following the previously optimised method described by Gallego *et al.* (2015) [7].

III. RESULTS AND DISCUSSION

As a result of the intense proteolysis that takes place along the dry-cured ham processing, proteins are degraded as time curing advances [3]. So, in order to study the evolution of proteins during this process, sarcoplasmic extracts derived from dry-cured hams at 0, 2, 3.5, 5, 6.5, and 9 months of processing were analysed by nLC-MS/MS for protein identification and quantitation. This study was focused only on ENO (47.1 KDa), and GAPDH (35.8 KDa) glycolytic enzymes, whose relative quantitation was done by using a label-free methodology. For that, raw ham (0 m) has been used as a reference, and three replicates with an average of 9, and 3 peptides of ENO, and GAPDH, respectively, have been used to calculate the ratio of each protein at different times of processing (see Figure 1).

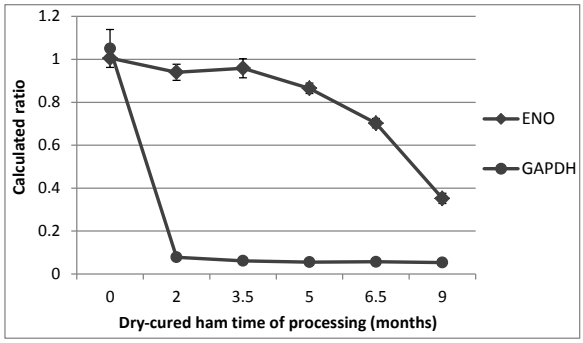

Figure 1. Relative quantitation of ENO and GAPDH glycolytic enzymes along the dry-cured ham processing. Bars represent standard deviations from the replicates (n=3).

Calculated values for ENO enzyme show that the amount of this protein is maintained fairly constant until the 3.5 months of processing and decreases gradually during the dry-curing stage (5, 6.5, and 9 months), reaching a final value of about 0.35. On the other hand, GAPDH is almost completely degraded after the post-salting stage. So, the quantity of this protein diminishes sharply in the first 2 months, keeping a value of about 0.1 until the last time assayed.

The evolution of these two glycolytic enzymes is in agreement with the study done by Larrea *et al.* (2006) [1], in which a semi-quantitation was done based on the measurement of electrophoresis bands density. However, this quantitation method shows some limitations as poor specificity, limited dynamic range and low accuracy in the measurement of protein abundance changes, especially when overlapping of proteins occurs [4]. As an example, the extracted ion chromatogram (XIC) of one of the peptides (YNQLMR) used to quantify the ENO protein is shown in Figure 2. Thus, the label-free methodology employed in this study for relative quantitation allows an accurate study of the protein changes occurring by proteolysis reactions along the dry-cured processing.

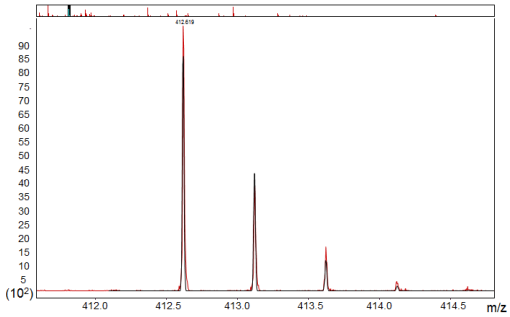

Figure 2. Extracted ion chromatogram (XIC) of the peptide YNQLMR used for the relative quantitation of enolase protein.

Sarcoplasmic proteins are important substrates for proteolysis occurring along the processing of dry-cured ham. Muscle endopeptidases are stable during the whole curing process, except cathepsin D whose activity disappears after 6 months of process, and calpains that are inactivated after the salting stage [3]. Among exopeptidases enzymes, aminopeptidases and dipeptidyl peptidases show good stability throughout the processing [3]. Therefore, these enzymes are responsible for the intense degradation of the studied glycolytic enzymes, which occurs mainly in the post-salting stage and the end of the ripening of dry-cured hams.

IV. CONCLUSION

The relative quantitation based on a label-free approach used in this study has resulted to be a more precise, fast and simple alternative to traditional quantitative methodologies for the evaluation of changes in abundance of enolase and glyceraldehyde 3-phosphate dehydrogenase glycolytic enzymes as well as results very useful to follow-up the proteolysis phenomena that takes place during the processing of dry-cured ham.

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