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4 **Perspectives in the use of peptidomics in ham**

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25 **Abstract**

The use of proteomics in food science has permitted both a better characterisation of the food products and the control of their final quality despite the fact that food systems are under continuous changes during processing and consumption. Ham is a very popular meat product frequently consumed in Mediterranean countries after a dry-curing processing that results in the generation of large amounts of peptides that contribute to its texture, flavour, and final quality. Proteolysis is the main biochemical mechanism occurring throughout the dry-cured ham processing and a better knowledge of this phenomenon is essential to give an extra-value to the product and produce regular batches. In this regard, peptidomics has become an important tool for the characterisation of dry-cured hams in order to identify and quantify bioactive peptides and potential biomarkers, study their stability under processing conditions or gastrointestinal digestion, and search for post-translational modifications that could affect the quality of dry-cured hams. This manuscript is reviewing from the first uses of comparative proteomics in dry-cured ham approaches to the most modern peptidomic studies and their contribution to the advance in meat science.

1. Introduction

The application of proteomic approaches in food permits better characterisation of the product as well as improvement and control of its final quality. In fact, due to the fact that food can be considered a biological system that is under continuous changes, studies about protein degradation or post-translational modifications have been in the focus since the last decade. Advances in both mass spectrometry instruments and experimental approaches have permitted the evolution from ‘bottom-up’ approaches, mainly used in proteomics, to more sophisticated ‘top-down’ peptidomic experiments that include the difficult data analysis resulting from unspecific hydrolysis.

Peptidomics has been used to follow proteolysis in natural and complex food processes such as curing or fermentation, characterise the effect of processing, follow the simulated gastrointestinal digestion, search for potential peptides to be used as biomarkers, or detect characteristic peptides to differentiate between genotypes and products [1-4].

Ham is a very popular product that is frequently consumed in Mediterranean countries after dry-curing, a processing step that results in the generation of different peptides that contribute to its texture, flavour, and final quality. One of the main biochemical mechanisms occurring in the processing of dry-cured ham is proteolysis, which mainly occurs through the action of endogenous muscle peptidases. The control of proteolysis is of primary importance in order to produce a consistent, regular, and high quality product, so a better knowledge of this phenomenon is essential to give an extra-value and produce regular batches [5].

The use of peptidomic approaches in dry-cured ham has contributed to an advancement in the meat science field through the knowledge of the generated peptide sequences, which have resulted in an added-value of the product due to the presence of natural bioactive peptides and markers generated during curing or final quality of the product.

In this paper, a review from the first uses of comparative proteomics in dry-cured ham approaches to the most modern peptidomic studies and their contribution to the advancement in meat science has been done.

2. Prospects and limitations in the use of proteomics in studying naturally generated peptides

Proteolysis has been identified as the key phenomenon responsible for the generation of peptides during food processing. Techniques like SDS-PAGE, 2D gel electrophoresis, free solution conjugate electrophoresis (FSCE), SEC and HPLC with reversed phase, hydrophilic interaction liquid chromatography (HILIC) or ion exchange chromatography (IEC) have been frequently used in studies based on the comparison of proteomes [6,7]. These techniques combined with single MS revealed information about the breakdown of proteins that occurs post-mortem and during processing of meat. These approaches also elucidated the importance of the generation of peptides derived from the intense action of muscular enzymes.

Main ‘bottom-up’ approach used in dry-cured ham studies is the Peptide Mass Fingerprint (PMF), which is useful for the follow-up of protein degradation along different times and/or between different samples. In this regard, the combination of 2D SDS-PAGE and MALDI-TOF MS analysis allowed the study of changes in myofibrillar and sarcoplasmic fractions of raw and dry-cured hams at different ripening times [6]. Thus, the progressive degradation of actin, tropomyosin and myosin light chains as well as certain sarcoplasmic proteins was observed during the curing of ham in the 2D gels. In this study, main bands were digested using trypsin and subsequently analysed by MALDI-TOF MS. A similar proteomic approach was used to compare the proteomic maps of two muscles, *biceps femoris* and *semimembranosus* in dry-cured hams, showing that the methodology is very

useful for muscular differentiation [8]. A PMF approach was also applied to evaluate the changes occurring in sarcoplasmic proteins after proteolysis and during the ripening of meat products [9]. In that study, the separation of proteins by 2D AUT-PAGE/SDS electrophoresis, which combines an acetic acid-urea-triton polyacrylamide gel with the classic SDS gel, was used together with a MALDI-TOF mass spectrometer for the identification of 13 sarcoplasmic proteins. Although this technique showed a higher resolution in comparison to standard 2D IPG/SDS-PAGE as well as an easier interpretation of the 2D maps, current tendencies are focused on non-destructive and fast techniques. In this sense, spectroscopic high-resolution magic angle spinning (HRMAS) NMR [10] is a complementary technique for proteomic tissue characterisation in which different spectral peaks are assigned to different protein components. Also the non-destructive quantitative magnetisation transfer magnetic resonance imaging (qMT-MRI) methodology can be employed to characterise dry-cured ham tissues differing in anatomical positions and processing protocols using the binary-spin-bath (BSB) model [11]. Recently, a combination of the immunochemical and MS methodologies SDS-PAGE, Western blotting and blot overlay assays together with an linear trap quadrupole (LTQ) Orbitrap mass spectrometer were used to evaluate the changes occurring in actin and myosin heavy chain proteins in the *biceps femoris* muscle during the processing of three Italian dry-cured hams [12]. Finally, some studies combined qualitative and quantitative proteomics using MS in tandem for proteome characterisation. Quantification using a label-free approach has been applied to determine the changes in water-soluble proteins at different processing times [13] and to evaluate the effect of a pressure treatment applied during the salting step on the processing of dry-cured hams [14]. In this regard, MS quantitative methods have been proven to be precise and effective

alternatives to traditional techniques based on gel electrophoresis that present low specificity, limited dynamic range and inaccurate identification of overlapping proteins. However, all these studies were focused on the proteolysis phenomena from the point of view of protein degradation under different conditions and different samples but no specific sequences of the generated peptides were described. In this sense, a better knowledge of the generation of peptides during the different food processes and their specific sequences is essential to be able to confer an added-value to the final product due to their bioactive properties in the human body or as instruments for quality control. The identification of naturally generated peptides requires the analytical and experimental adaptation of previously used strategies for the identification of proteins such as tandem MS and modern bioinformatics tools; however, different challenges make the identification of natural peptides very difficult. The characteristics of these peptides could be very different depending on the degree of protein proteolysis. The classic PMF approach, which is very frequent in proteomics for the identification of proteins, is not useful in this case because these peptides cannot be trypsin digested due to their small size. Thus, the analysis of di- and tripeptides are sometimes in the limit of some standard MS techniques, requiring an extra effort for their unequivocal identification. On the other hand, longer peptides face up the impossibility of controlling the hydrolysis, resulting in a complex mixture of peptides derived from different proteins with unspecific cleavage sites [15].

3. Peptidomics in the characterisation of dry-cured ham

Peptidomics has become an important tool for the characterisation of dry-cured ham, as this complex matrix contains a great variety of peptides naturally generated due to the action of endogenous proteolytic enzymes during the long processing of this product. This

mixture of peptides shows a wide range of sizes and non-specific cleavage sites, which requires different pretreatments and chromatographic steps such as SEC or HPLC to avoid interferences and isolate peptides prior to their identification and quantification by MS analysis [16].

3.1 Identification of peptides

The use of tandem MS techniques allows a fast and reliable identification of the peptides generated from the degradation of sarcoplasmic and myofibrillar proteins during the dry-cured ham processing. For that, the obtained MS/MS spectra are matched with theoretical peptide sequences contained in databases in order to elucidate the specific sequences. So, numerous peptides derived from myofibrillar proteins such as actin [17], myosin light chain [2,18,19], troponin T [20,21], titin [18,22], LIM domain-binding protein 3 [23], and myozenin [21], as well as peptides from sarcoplasmic proteins such as creatine kinase [24], glycolytic enzymes [21,25], myoglobin [26], and ubiquitin [27] have been identified in Spanish dry-cured ham using mainly MALDI-TOF MS and ESI-LC-Q/TOF MS. Table 1 reports the number of peptides and their proteins of origin identified in Spanish dry-cured ham. Several small peptides were also identified in Chinese dry-cured Jinhua ham using MS in tandem, but their proteins of origin were not elucidated [28]. These studies contribute to the characterisation of peptides in order to understand the role of muscle enzymes in the intense proteolysis that happens during ham processing, which would be affected by the raw material and processing conditions. Sarcoplasmic and myofibrillar proteins are hydrolysed by endopeptidases, mainly calpains and cathepsins, which result in the formation of longer polypeptides and changes in texture of dry-cured hams. Then, polypeptides are degraded by exopeptidases including tri- and di-peptidyl peptidases, dipeptidasas, aminopeptidases, and carboxypeptidases, generating small peptides and free amino acids that contribute to the characteristic sensory properties of the product [29,30].

In this regard, certain dipeptides and free amino acids have been described to influence flavour development in Spanish Serrano dry-cured hams [31], whereas two umami taste peptides CCNKSV and AHSVRFY were identified from Chinese Jinhua ham and Italian Parma ham, respectively [32].

3.2 Quantification of peptides

Peptidomic approaches usually include the absolute or relative quantification of peptides by using labelling or label-free methodologies. The relative quantification is based on the comparison of peptide amounts among two or more samples. Labelled methods provide the most accurate quantitative values, but they require complex experimental protocols and expensive isotope labels. On the other hand, label-free methodologies allow a simple, reliable, versatile, and cost-effective quantification, either on the basis of peak intensity measurements or spectral counting. In quantification based on peak intensity, the signal intensity measurements of extracted ion chromatograms correlate to the concentration of that particular peptide, allowing a precise and accurate evaluation of changes in abundance between samples [33, 34]. This label-free MS approach has been used to identify and quantify the peptides naturally generated during the ham dry-curing process, and study their evolution at different processing times [2].

Absolute quantification measures the total levels of a given peptide in the sample, either by using label or label-free approaches. Among them, MRM has proven to be the most suitable methodology, with a wide linear range. MRM is a highly sensitive and selective method of targeted MS that generally employs tandem quadrupoles for the detection and quantification of small and low abundance peptides in complex mixtures. So, the first quadrupole (Q) act as filter to specifically select certain m/z values of peptide ion, the second Q serves as collision cell for ion fragmentation, and the third Q filters a specific fragment ion of the peptide for its detection. Such precursor/fragment ion pairs

(transitions) are monitored over time and the integration of their chromatographic peaks allows the quantification of the target peptide [35,36]. However, there are several challenges in the analysis due the complexity of the samples especially when peptides have been naturally generated by unspecific enzymes or during processing conditions, as well as in the optimisation of the MS parameters for an accurate and sensitive quantification without interferences from other peptides or signal suppression [37]. MRM methodologies have been recently used to quantify several peptides and amino acids generated during the ripening of dry-cured hams [38,39] as well as to evaluate the degradation of added microbial transglutaminase during the maturing process of dry-cured ham through the use of isotope labelled peptide standards [40].

4. Specific applications of peptidomics in dry-cured ham

The applications of peptidomics include the characterisation of peptides in order to identify and quantify bioactive peptides and potential biomarkers, study their stability under processing conditions or gastrointestinal digestion, and search for post-translational modifications in peptides that can affect the quality of dry-cured hams (Figure 1).

4.1 Discovery and bioavailability study of bioactive peptides

Dry-cured ham has been described as a good source of bioactive peptides showing angiotensin converting enzyme (ACE) inhibition as well as antioxidant, antimicrobial, and dipeptidyl peptidase IV (DPP IV) inhibitory activities. The discovery, bioavailability and monitoring of bioactive peptides in complex food matrices such as dry-cured hams usually require empirical strategies that involve the use of high-resolution separation techniques, *in vitro* assays to detect the most active fractions, identification of peptides by MS, selection and synthesis of potential bioactive peptides, and confirmation of their bioactivity by *in vitro* or *in vivo* assays. Moreover, the combination of this traditional workflow with bioinformatics tools or *in silico* analysis permits to obtain a large amount

of data regarding the chemical structure, activity, enzyme-peptide interactions, and availability of bioactive peptides [41].

Focusing on ACE inhibitory activity, water soluble peptide extracts from Spanish dry-cured hams have been reported to exhibit up to 97.7 % of *in vitro* ACE inhibition in some fractions obtained after SEC [42,43]. Several peptides responsible for this activity have been identified through MS analysis [44,45], reporting some potent ACE inhibitory peptides such as AAPLAP, KPVAAP and KAAAAP. In Parma dry-cured hams, a hybrid *in silico/in vitro* tandem approach was employed for the identification of strong inhibitory peptides such as LGL and SFVTT in fractions obtained after *in vitro* gastrointestinal digestion [46]. On the other hand, a study focused on the characterisation of the peptide profiles in Spanish Teruel, Italian Parma, and Belgian dry-cured hams evidenced the presence of ACE inhibitory and antioxidant peptides, showing higher ACE inhibition values in the Spanish ham probably due to its longer curing time [47]. The sequences of some ACE inhibitory peptides and their IC₅₀ values (peptide concentration that inhibits 50% of ACE activity in the reaction mixture) are shown in Table 2.

Dry-cured hams have also been studied as a source of antioxidant peptides. In Spanish dry-cured hams, the antioxidant activity of water soluble extracts has been evaluated and the sequences of some of these antioxidant peptides have been identified by MS [42,48]. In this regard, the peptide SNAAC has been the most potent antioxidant identified to date, showing an IC₅₀ value of 75.2 µM in DPPH radical scavenging assay and 205 µM in ferric-reducing power analysis [49]. Concerning Chinese hams, the peptide GKFNV showed the highest activity in Jinhua ham extracts [50,55], whereas DLEE would be one of the main peptides playing a key role in the antioxidant activity of Xuanwei hams [51]. Table 2 shows some of the antioxidant peptides identified in dry-cured hams and their activity values.

Regarding other bioactivities, several naturally generated peptides from Spanish dry-cured ham have been described to have antimicrobial action against the pathogenic *Listeria monocytogenes*, being RHGYM the most effective [52]. Also some dry-cured ham peptides and dipeptides such as AAATP and KA have been reported to exert DPP IV inhibitory activity and thus could be act as a natural therapeutic approach for the management of type 2 diabetes [53]. Some examples of the identified peptides showing antilisterial and DPP IV inhibitory activities are shown in Table 2.

Peptidomics has also constituted an indispensable tool to study the bioavailability of bioactive peptides in order to assure that the active form reaches the target site to exert its effect. The use of MS-based techniques has permitted to evaluate possible modifications of the structure and activity of bioactive peptides that can occur during food processing, gastrointestinal digestion and transport in the organism. For that purpose, MS analyses are usually combined with computational tools and studies in cellular and animal models [41]. Several studies have evaluated the generation of novel bioactive peptides after simulated gastrointestinal digestion [50,56] as well as the stability of ACE inhibitory and antioxidant peptides against *in vitro* digestion and different processing/storage conditions such as temperature, salt, pH, and light exposure [39,44,57,58]. Furthermore, a recent study based on a MRM-based methodology was able to quantify the amount of the antioxidant peptide AEEEYPDL that was 0.148 fg per g of Spanish dry-cured ham [39], which is crucial to evaluate the bioavailability and potential effect of the peptide in the organism.

Cellular models provide useful information about intestinal absorption mechanisms and bioavailability of peptides. So, the transepithelial transport of several ACE inhibitory peptides identified in Spanish dry-cured hams was studied through a Caco-2 cell monolayer, evidencing the absorption and generation of novel ACE inhibitory peptide

fragments during the process by MS techniques [59]. Nevertheless, *in vivo* studies are needed to demonstrate the efficacy and physiological effects of bioactive peptides in the organism. The potential antihypertensive action of dry-cured ham peptides have been investigated by measuring changes in systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR). In this regard, the administration of Spanish ham extracts to SHR showed a maximum decrease in SBP of 38.38 mmHg after 8-hour ingestion [42], being AAATP the most potent antihypertensive peptide (-25.62 mmHg at 8 h) [45]. Iberian dry-cured ham extracts decreased SBP by 12 mmHg at 8 h in SHR, and the MS analysis revealed the presence of large amounts of peptides containing the ACE inhibitory fragments PPK, PAP and APP repeated in their sequences [43]. Finally, two recent human studies have reported that the regular consumption of bioactive peptides contained in dry-cured hams impair beneficial effects on cardiovascular health [60,61].

4.2 Biomarker search

In general, dry-cured hams require extensive ripening-drying times that last up to 24 months or even longer in some cases. Long times of curing are related to a more intense proteolysis and higher generation of flavour and aroma compounds, which principally determine the quality and economic value of the product. Peptidomics can contribute to process control by determining some peptides as markers of the time of processing, in order to prevent mislabeling and fraudulent activities resulting in over-valued products. In this respect, some peptides generated from LIM domain-binding protein 3 and ubiquitin protein were suggested as potential markers to estimate and control the processing time of Spanish dry-cured hams [23,27]. Moreover, four peptides derived from titin protein were identified by ESI- LC-Q/TOF MS as potential biomarkers of a minimum processing time of 9 months, and complementarily, MALDI-TOF MS could be employed as a fast and easier technique to check the presence of these peptide markers [22].

Differences obtained in the relative quantification of generated peptides during the processing of dry-cured hams permitted to determine the main peptides and proteins responsible for differences between dry-cured ham samples. So, peptides derived from myosin light chain 1 protein were the most influential at the end of the dry-cured ham processing, and specifically peptides APAPAPAPPKEEKI and PAPAPAPAPAPAPAPPKE were identified as potential markers of 9 months of curing [2]. The quantification of biomarkers can also be used to follow the time course of ripening, being helpful in product development and process optimisation to ensure the quality and safety of dry-cured ham [38]. In this regard, two peptides were detected as potential candidates to be biomarkers of the manufacturing process of Norwegian dry-cured hams [54]. Table 2 shows the sequences of peptides identified as potential biomarkers of the dry-cured ham processing.

The final quality of dry-cured hams also depends on the raw material used in its preparation as differences in pig genotypes result in different action of endogenous enzymes and thus different peptide profiles. In this regard, peptidomics was used to determine peptides derived from proteins myosin light chain 1, creatine kinase, myoglobin, troponin T, and myosin heavy chain 7 that were mainly responsible for quantitative differences between dry-cured hams coming from pigs of three different genetic selections [4]. Moreover, differences in proteolysis among dry-cured hams of different genetics and processing conditions could be assessed through this approach. A study comparing peptide profiles of Iberian dry-cured ham processed for 24 months and traditional Spanish dry-cured ham of 14 months showed that the most influential peptides were derived from creatine kinase, actin, troponin T, and myosin heavy and light chains [43]. In the case of different types of European dry-cured hams, peptides from myosin light chain, glyceraldehyde-3-phosphate dehydrogenase, myosin heavy chain, and

myosin 4 were reported to be responsible for differences between Spanish Teruel, Italian Parma and Belgian hams [47].

4.3 Analysis of post-translational modifications in peptides

Peptidomic approaches are useful tools for detecting post-translational modifications (PTM) such as oxidation, deamidation, phosphorylation, and carbamylation, which can modify physical and chemical properties of proteins and peptides. Novel advances in MS have permitted the analysis of PTM despite the existing difficulties related to the complexity of the involved mechanisms, wide variety of generated products as well as the low abundance and lability of some of them under the conditions of sample preparation and/or analysis. Tandem MS techniques allow the determination of the site and nature of PTM because modified amino acids are mass-shifted and thus identifiable in the fragmentation pattern. In addition, the combination of MS/MS with LC can be employed for the quantification of such modifications [62].

Oxidative processes play an important role in the quality and sensory characteristics of dry-cured hams. Several peptides showing methionine oxidation derived from different myofibrillar proteins such as titin, myosin, and nebulin, among others, have been identified in Spanish dry-cured hams [63]. Moreover, a recent work has studied the evolution of oxidised peptides during the processing of 9 months dry-cured ham, identifying and quantifying peptides with methionine, proline, and tryptophan oxidations, and showing quantitative differences resulting from specific processing conditions [64].

An example of relative quantification of peptides with oxidised methionine is shown in Table 3. Also peptide deamidation, mainly of asparagine and glutamine residues, in 12 months dry-cured hams has been reported to be influenced by processing conditions like temperature, pH, and salting of hams [65]. These studies provide valuable information about PTM mechanisms and their effects at peptide level during the dry-cured ham

processing, which could affect the nutritional and quality characteristics of the final product.

5. Current tendencies and future perspectives

Further improvements in mass spectrometers and novel MS techniques are permitting a significant advance in the field of peptidomics through the use of empirical approaches for the identification and quantification of peptides. However, bioinformatics approaches based on the combination of computational tools and peptide databases are gaining importance in recent years as a time- and cost-effective strategy to study the characteristics of peptides, predict proteolysis and biological activities, and evaluate proteins as potential precursors [41,66]. In this regard, *in silico* analysis including quantitative structure-activity relationship models (QSAR), molecular docking simulations and chemometrics are being widely used for bioactive peptide discovery, allowing a greater knowledge of their characteristics and mechanisms of action as well as the optimisation of proteolysis variables when searching for a specific activity [67,68]. Additionally, current tendencies in the study of bioactive peptides are related to their bioavailability as more information regarding peptide changes in human body during digestion, transport, absorption, and metabolism, as well as final available concentrations are necessary to better understand their effects in the organism. In fact, the amount of active peptide that is ingested and is able to reach its target site will determine its effect and impact in the human system, thus quantification of bioactive peptides is needed in future bioavailability studies. Nevertheless, there are still several challenges for determining the content of bioactive peptides in blood stream and target organs as the low concentration levels make instruments to be in the limits of their range of action [37]. Moreover, the difficulty in the analysis is also due to the complexity of biological matrices,

whose main composition is protein, as well as peptide-matrix interactions that can lead to structural changes in bioactive peptides affecting their release, bioavailability, and bioactivity [69].

The high efficiency of peptidomic strategies combined with the predictive capability of bioinformatics approaches represents a powerful tool for the discovery and analysis of bioactive peptides as well as for a better understanding of proteolysis phenomena, which could be applied in future studies towards the improvement of the final quality and the development of added-value processed meat products.

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Conflicts of interest

Authors declare that none of them have any conflict of interest.

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FIGURE CAPTIONS

Figure 1. Scheme showing the applications of peptidomics in dry-cured ham.

Figure 1.