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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
INSTITUTO DE AGROQUÍMICA Y TECNOLOGÍA
DE ALIMENTOS (IATA)

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DE VALÈNCIA

PÉPTIDOS GENERADOS EN JAMÓN CURADO COMO MARCADORES DE CALIDAD

TESIS DOCTORAL

Presentada por:

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HACEN CONSTAR: que el trabajo de investigación titulado *"Péptidos generados en jamón curado como marcadores de calidad*" que presenta Dña Marta Gallego Ibáñez por la Universidad Politécnica de Valencia, ha sido realizado en el Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) bajo nuestra dirección y que reúne las condiciones para optar al grado de doctor.

Y para que conste a los efectos oportunos, firmamos la presente en Paterna a 3 de Julio de 2015.

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Resumen

Durante el proceso de elaboración del jamón curado tienen lugar una serie de reacciones bioquímicas responsables del aroma, color, textura y sabor del producto final. Entre estas reacciones destaca la intensa proteolisis de las proteínas musculares, cuyo resultado es la generación de una gran cantidad de péptidos algunos de los cuales podrían ser utilizados como marcadores de calidad del jamón. En este sentido, las técnicas proteómicas basadas en espectrometría de masas son una herramienta fundamental para la identificación de los péptidos generados de manera natural a lo largo de proceso de elaboración del jamón curado. Así, la presente Tesis Doctoral se ha centrado en estudiar la intensa degradación de tres proteínas presentes en el jamón: la proteína 3 de unión al dominio LIM, la proteína ribosomal ubiquitina-60S y la proteína titina, identificando las secuencias de los péptidos generados a distintos tiempos durante el proceso y evaluando su potencial como marcadores del tiempo de curado del jamón. Además, se han estudiado los cambios oxidativos a nivel peptídico que tienen lugar durante el proceso de curado del jamón, cuya importancia radica en su efecto sobre la calidad y las características tanto nutricionales como sensoriales del producto final, evidenciando la oxidación del aminoácido metionina en numerosos péptidos generados a partir de las principales proteínas miofibrilares.

Además de la identificación de las secuencias peptídicas, los recientes avances en técnicas de espectrometría de masas han permitido la cuantificación precisa de proteínas en muestras complejas. Así, se ha optimizado un método sin marcaje ("label-free") para la cuantificación relativa de las principales proteínas sarcoplásmicas a partir de la cuantificación de los péptidos generados y de esta forma estudiar la degradación proteolítica durante el proceso de elaboración del jamón curado. Esta metodología supone un avance respecto a los métodos cuantitativos utilizados hasta el momento ya que permite evaluar de manera sencilla, precisa y fiable los cambios en la abundancia de proteínas a lo largo del proceso de elaboración del jamón curado.

Varios estudios recientes se han centrado en estudiar el potencial del jamón curado como fuente de péptidos bioactivos, principalmente aquellos que son inhibidores de la enzima convertidora de angiotensina I (ECA). Sin embargo, no se conoce si estos péptidos son capaces de atravesar la barrera intestinal y alcanzar la corriente sanguínea en forma activa. Por ello, en la presente Tesis Doctoral se ha simulado el transporte intestinal mediante la utilización de células Caco-2 de péptidos del jamón inhibidores de la ECA, evidenciado la absorción de péptidos enteros, o fragmentos de los mismos generados por acción de las peptidasas intestinales, que podrían ejercer un efecto antihipertensivo *in vivo.*

Resum

Al llarg del procés d'elaboració del pernil curat tenen lloc una sèrie de reaccions bioquímiques responsables de l'aroma, color, textura i sabor del producte final. Entre aquestes reaccions destaca la intensa proteolisis de les proteïnes musculars, el resultat de la qual és la generació d'una gran quantitat de pèptids alguns dels quals podrien ser utilitzats com a marcadors de qualitat del pernil. En aquest sentit, les tècniques proteòmiques basades en espectrometria de masses són una ferramenta fonamental per a la identificació dels pèptids generats de manera natural al llarg de procés d'elaboració del pernil curat. Així, la present Tesi Doctoral s'ha centrat a estudiar la intensa degradació de tres proteïnes presents en el pernil: la proteïna 3 d'unió al domini LIM, la proteïna ribosomal ubiquitina-60S i la proteïna titina, identificant les seqüències dels pèptids generats a distints temps al llarg del procés i avaluant el seu potencial com a marcadors del temps de curat del pernil. A més, s'han estudiat els canvis oxidatius a nivell peptídic que tenen lloc durant el procés de curat del pernil, la importància dels quals radica en el seu efecte sobre la qualitat i les característiques tant nutricionals com sensorials del producte final, evidenciant l'oxidació de l'aminoàcid metionina en nombrosos pèptids generats a partir de les principals proteïnes miofibrilars.

A més de la identificació de les seqüències peptídiques, els recents avanços en tècniques d'espectrometria de masses han permés la quantificació precisa de proteïnes en mostres complexes. Així, s'ha optimitzat un mètode sense marcatge ("label-free") per a la quantificació relativa de les principals proteïnes sarcoplàsmiques a partir de la quantificació dels pèptids generats i d'esta manera estudiar la degradació proteolítica durant el procés d'elaboració del pernil curat. Esta metodologia suposa un avanç respecte als mètodes quantitatius utilitzats fins al moment ja que permet avaluar de manera senzilla, precisa i fiable els canvis en l'abundància de proteïnes al llarg del procés d'elaboració del pernil curat.

Diversos estudis recents s'han centrat en estudiar el potencial del pernil curat com a font de pèptids bioactius, principalment aquells que són inhibidors de l'enzim convertidor d'angiotensina I (ECA). No obstant això, no es coneix si aquests pèptids són capaços de travessar la barrera intestinal i arrivar al corrent sanguini en forma activa. Per això, en la present Tesi Doctoral s'ha simulat el transport intestinal mitjançant l'utilització de cèl·lules Caco-2 amb pèptids del pernil inhibidors de l'ECA, evidenciant l'absorció de pèptids sencers, o fragments generats per acció de les peptidases intestinals, que podrien exercir un efecte antihipertensiu *in vivo*.

Abstract

A series of biochemical reactions responsible for aroma, color, texture and flavour of the final product occur during the dry-cured ham processing. Among these reactions, proteolysis of muscle proteins is one of the most important, resulting in the generation of a large amount of peptides which could be used as quality markers of dry-cured ham. In this sense, proteomic techniques based on mass spectrometry have become a fundamental tool for the identification of peptides naturally generated throughout the dry-cured ham processing. So, the present Thesis has been focused on the study of the intense degradation of three proteins present in dry-cured ham: LIM domain-binding protein 3, ubiquitin-60S ribosomal protein and titin protein, identifying the peptide sequences generated at different times along the process and evaluating their potential as markers of dry-cured ham processing time. Moreover, peptide oxidation that occurs during dry-curing process has been studied due to its importance on final quality of dry-cured ham as well as its influence on the nutrititional and sensory characteristics, showing oxidation of methionine residues in numerous peptides generated from the degradation of the major myofibrillar proteins.

Besides the identification of peptidic sequences, recent advances in mass spectrometry techniques have allowed the precise quantitation of proteins in complex mixtures. So, a label-free methodology has been optimized for the relative quantitation of major sarcoplasmic proteins from the quantitation of the generated peptides and the study of the proteolytic degradation along the dry-cured ham processing. This methodology represents an advance regarding quantitative methods used to date as it allows a simple, accurate and reliable evaluation of changes in the abundance of proteins throughout the dry-cured ham processing.

Several recent studies have been focused on the study of the potential of dry-cured ham as a source of bioactive peptides, mainly those showing angiotensin I-converting enzyme (ACE) inhibitory activity. However, it is not clear whether these peptides are able to cross the intestinal barrier and reach the blood stream

in an active form. Thus, in the present Thesis the transepithelial transport of dry-cured ham peptides having ACE inhibitory activity has been simulated through a Caco-2 cell monolayer, showing the absorption of intact peptides or fragments generated by the action of intestinal peptidases, which could exert an *in vivo* antihypertensive effect.

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I. Introducción

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1. La carne y los productos cárnicos

1.1 Carne de cerdo

La carne de cerdo está considerada como carne blanca según la Organización Mundial de la Salud (OMS): "Las llamadas carnes rojas (vacuno y ovino/caprino) y carnes blancas (porcino y aves de corral) ofrecen diversas propiedades positivas y una gama de sabores y texturas". Así, la carne de cerdo es un excelente producto a incluir dentro de una alimentación variada y equilibrada, participando de manera relevante en las dietas de muchos países europeos. Además, su contenido en proteínas de alta calidad, minerales, vitaminas y micronutrientes que aportan efectos beneficiosos para la salud hace que sea una carne muy valorada por sus propiedades nutritivas, así como por sus diversas propiedades tecnológicas y sensoriales (Comisión Europea, 2014; FAO, 2015).

1.1.1 Relevancia actual: producción y consumo

La carne de cerdo es la más producida a nivel mundial respecto a la carne de otras especies, con un total de 109 millones de toneladas producidas en 2012. China es el máximo productor mundial de carne de porcino (49,5%), seguida de EEUU (10%) y Alemania (5,3%). España se sitúa en cuarto lugar, con una producción que representa el 3,4% del volumen mundial (Comisión Europea, 2014; MAGRAMA, 2014).

En España, el porcino es el principal sector ganadero, alcanzando el nivel más alto de su historia en 2013 con un valor de 6.273 millones de euros, lo que representa el 39% de la Producción Final Ganadera y el 14,2% de la Producción Final Agraria. La producción de carne de cerdo en 2013 alcanzó cifras de 3,4 millones de toneladas, siendo las principales comunidades que la producen Cataluña (43%) y Castilla y León (13%). Debido a los altos niveles de producción actuales, el sector cárnico español apuesta por el comercio exterior, alcanzando valores de más de un millón de toneladas exportadas de carne de cerdo (un 2,5% más que el año 2012). Las ventas a países de la Unión Europea supusieron dos

terceras partes del total, siendo Francia, Alemania, Portugal e Italia los principales países compradores (MAGRAMA, 2014).

Concretamente en España, las carnes supusieron un 7,9% respecto al consumo de alimentos y bebidas en el hogar en el año 2013, alcanzando casi las 2,4 millones de toneladas consumidas. El consumo de carne fresca se mantuvo prácticamente estable y la carne transformada aumentó un 0,9%, mientras que la carne congelada sufrió un descenso del 1,7% respecto al año anterior. El pollo es la carne más consumida en los hogares españoles, mientras que la carne de cerdo ocupa la segunda posición, alcanzando un volumen de consumo en 2013 de más de 484 millones de kilos. La evolución en el consumo de los principales tipos de carne fresca en el año 2013 respecto al año anterior se muestra en la **Figura 1** (MAGRAMA, 2014).

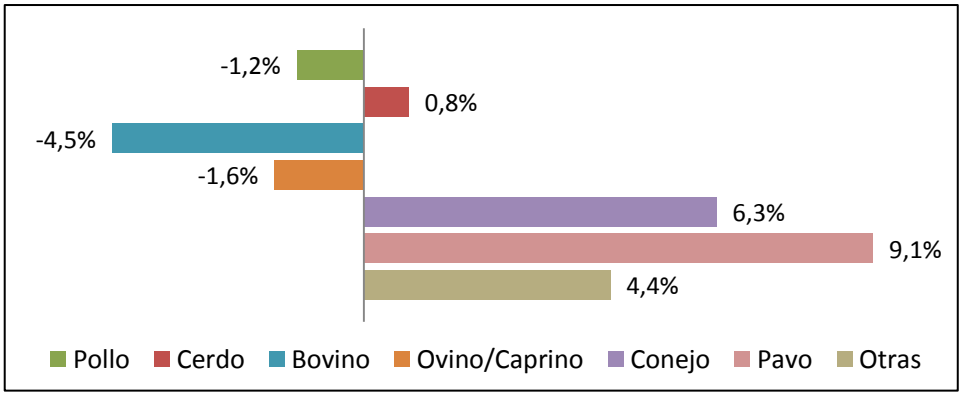

Figura 1. Evolución en el consumo de los principales tipos de carne fresca en 2013 respecto al año 2012.

La actual crisis económica y financiera está condicionando algunos hábitos de consumo y en consecuencia, el mercado de la carne. La menor renta económica disponible en los hogares españoles hace que los consumidores gasten más en productos básicos y sustituyan en muchos casos las carnes de porcino y bovino por la carne de pollo, la cual es más económica. Además, se está observando un aumento en la tendencia de consumo de carne blanca como conejo o pavo, debido a su mayor valor nutricional y propiedades saludables así como a la

creciente preocupación de los consumidores por su alimentación. Durante los últimos años el consumo de carne de porcino se ha mantenido más o menos estable, de modo que se están llevando a cabo campañas gubernamentales para un mayor conocimiento de esta carne blanca, de los productos cárnicos tradicionales y derivados cárnicos, así como de la importancia de la ganadería porcina en España (MAGRAMA, 2015). Asimismo, los requerimientos y aspiraciones actuales de la población a una nutrición óptima, saludable y de calidad han llevado a la industria cárnica a investigar, desarrollar y poner en el mercado una amplia gama de productos que satisfagan esa necesidad de productos cada vez más saludables, seguros y sensorialmente apetecibles.

1.2 Jamón curado

El jamón curado es uno de los productos más populares y valorados de España, debido tanto a sus características organolépticas como nutricionales. En nuestra dieta, este producto representa una fuente importante de proteínas de alto valor biológico y micronutrientes tales como vitaminas del grupo B y minerales biodisponibles (Jiménez-Colmenero et al., 2010).

Existen diferentes tipos de jamones en España, con variadas procedencias y curaciones propias de la región de origen, y en los que especialmente prima la calidad. De este modo, existen diversas denominaciones de origen y de calidad diferenciada (ver **Tabla 1**) creadas para exigir y controlar que los jamones cumplan con unas características propias y diferenciales (MAGRAMA, 2015). Pese a esto, al aumento de fraudes comerciales en el sector del jamón en los últimos años, principalmente en aquellos procedentes de cerdo ibérico, ha llevado a la aprobación de una nueva *Norma de calidad para la carne, el jamón, la paleta y la caña de lomo ibérico (Real Decreto 4/2014, del 10 de enero del 2014)*, con cambios y nuevas normativas respecto a la derogada disposición anterior (*Real Decreto 1469/2007*). El objetivo de esta norma es mejorar la información que aporta el producto en su etiqueta, evitar el engaño del consumidor y la competencia desleal entre empresas, e impulsar la conservación de la raza ibérica (BOE, 2014).

Tabla 1. Denominaciones de origen y de calidad diferenciada del jamón en España (MAGRAMA, 2015).

	NOMBRE	DISTINCIÓN	COMUNIDAD
Jamón de cerdo ibérico	Jamón de Huelva*	Denominación de Origen Protegida	Andalucía y Extremadura
	Los Pedroches*	Denominación de Origen Protegida	Andalucía
	Dehesa de Extremadura*	Denominación de Origen Protegida	Extremadura
	Guijuelo*	Denominación de Origen Protegida	Extremadura, Castilla-La Mancha, Castilla y León
Jamón de cerdo blanco	Jamón de Teruel*	Denominación de Origen Protegida	Aragón
	Jamón de Trevélez*	Indicación Geográfica Protegida	Andalucía
	Jamón de Serón*	Indicación Geográfica Protegida	Andalucía
	Jamón serrano*	Especialidad Tradicional Garantizada	Cataluña, Castilla y León, La Rioja, Castilla-La Mancha, Murcia
	Jamón curado	Marca de calidad "CV" para productos agrarios y agroalimentarios	Comunidad Valenciana

* Inscrita en registro de la Unión Europea.

1.2.1 Relevancia actual: producción y consumo

España es uno de los países con más tradición en cuanto a elaboración y consumo de los más variados embutidos y jamones. De este modo, la producción española de elaborados cárnicos se aproxima a los 1,3 millones de toneladas anuales, siendo el cuarto productor europeo por detrás de Alemania, Italia y Francia. El jamón curado representa el 20% de este volumen de producción

(ver **Figura 2**), con resultados muy positivos tanto en demanda interna como en comercio exterior, y siendo España el primer productor mundial de este tipo de producto (ANICE, 2014).

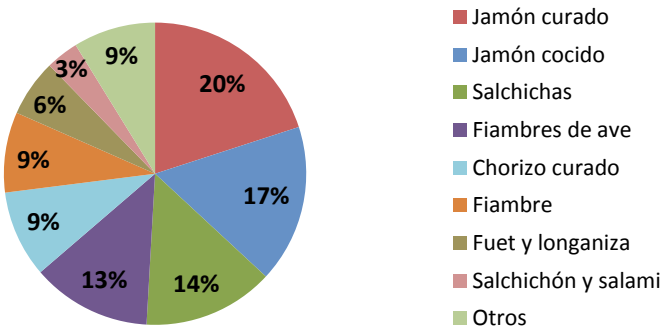

Figura 2. El mercado de elaborados cárnicos en España, expresado en % sobre volumen total de producción.

Dentro de los elaborados cárnicos, el jamón es el preferido por los consumidores españoles (84,5%), consumiéndose durante 2013 cerca de 71 mil toneladas de jamones curados procedentes de cerdo blanco y 19 mil toneladas de jamones de cerdo ibérico, lo que supuso un incremento del 5% respecto al año anterior. Aun así, las exportaciones de jamón curado, que alcanzaron las 31 mil toneladas en 2013, son fundamentales para mantener los elevados niveles de producción en España. Las ventas se realizan principalmente a nivel europeo (86%), siendo Alemania y Francia los principales compradores (ANICE, 2014).

1.2.2 Proceso de elaboración

La elaboración del jamón curado sigue una tradición ancestral basada en la conservación del producto y que consta de dos fases bien diferenciadas. Durante la primera fase se lleva a cabo una etapa de salado, necesaria para la correcta conservación del jamón, mientras que la segunda fase incluye un largo periodo de secado y maduración, durante el cual tiene lugar la estabilización y el desarrollo de fenómenos físicoquímicos y bioquímicos que determinarán las

características organolépticas particulares del producto final. Las principales etapas en la elaboración del jamón curado se resumen en la **Figura 3**.

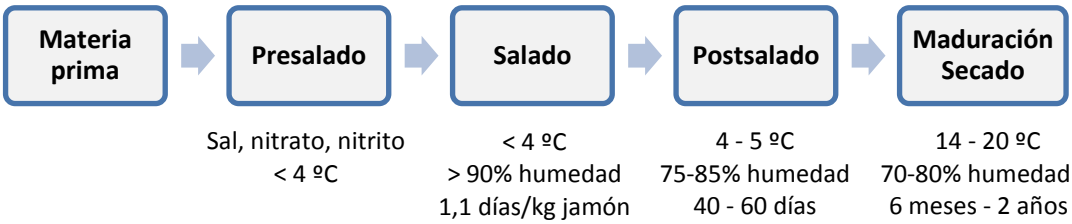

Figura 3. Principales etapas en la elaboración del jamón curado con las condiciones óptimas de cada etapa.

La materia prima utilizada es fundamental para obtener un producto final de calidad, del mismo modo que en la preparación y acondicionamiento de los perniles es esencial la correcta eliminación de la sangre residual de las piezas para reducir posibles problemas microbiológicos. Durante la fase de presalado, se lleva a cabo un frotado superficial de las piezas con sales de curado para favorecer su penetración, principalmente cuando el salado se va a realizar mediante el apilado de los perniles. En el salado tiene lugar la absorción y difusión de las sales de curado dentro de la masa muscular, de forma que la sal contribuye a disminuir la actividad de agua y al desarrollo de características de textura, mientras que los nitratos y nitritos tienen actividad bacteriostática y contribuyen al desarrollo del aroma y color del jamón curado. En la etapa posterior de postsalado se produce el reparto homogéneo de la sal por toda la pieza muscular y tiene lugar una ligera y paulatina deshidratación superficial debido a la difusión de sal hacia el interior y difusión de agua hacia la superficie. El proceso de elaboración acaba con la fase de maduración-secado, cuyo objetivo es la total estabilización del jamón. En esta etapa continúa la lenta deshidratación del producto debido a un aumento gradual de la temperatura y una disminución de la humedad relativa, a la vez que abundan las reacciones enzimáticas responsables de las características organolépticas del jamón curado, las cuales se describen a continuación.

1.3 Principales procesos bioquímicos

Durante el proceso de elaboración del jamón curado tienen lugar una serie de reacciones bioquímicas que condicionan el aroma, color, textura y sabor del producto final. La mayoría de reacciones son de tipo enzimático, destacando la proteolisis, la lipólisis, y en menor medida, la glucólisis y la transformación de nucleótidos. Además, tienen lugar reacciones químicas como las reacciones de Maillard, degradaciones de Strecker y oxidaciones. Estos cambios bioquímicos tienen lugar de forma simultánea y en mayor o menor medida en función de las características y etapa del proceso.

1.3.1 Reacciones enzimáticas

1.3.1.1 Proteolisis

El proceso de proteolisis consiste en la hidrólisis de las proteínas musculares, tanto sarcoplásmicas como miofibrilares, la cual tiene lugar fundamentalmente por las propias enzimas musculares. Es una de las reacciones bioquímicas más importantes en la generación de compuestos responsables del sabor y aroma durante la elaboración del jamón curado (Toldrá y Flores, 1998), y consta de varias etapas:

- En primer lugar se produce la hidrólisis de las proteínas sarcoplásmicas y miofibrilares por acción de las enzimas endopeptidasas, principalmente calpaínas y catepsinas, dando lugar a polipéptidos.
- Estos polipéptidos de tamaño intermedio son posteriormente degradados a péptidos más pequeños por acción de las exopeptidasas, mayoritariamente dipeptidilpeptidasas y tripeptidilpeptidasas.
- En la última etapa de la degradación proteolítica, se generan péptidos muy pequeños y aminoácidos libres debido a la acción de otros grupos de exopeptidasas como las dipeptidasas, aminopeptidasas y carboxipeptidasas.

Las principales endopeptidasas musculares son la μ-calpaína y m-calpaína, las catepsinas B, H, L y D, y el proteasoma. Las enzimas calpaínas tienen un pH de actuación próximo a la neutralidad y son inactivadas tras la etapa de salado (Rosell y Toldrá, 1996). Mientras, las catepsinas actúan a pH ligeramente ácido y

son muy estables durante todo el proceso de curado del jamón, excepto la catepsina D que es inhibida por la sal durante la etapa de maduración (Toldrá et al., 1993). El proteasoma actúa a pH ligeramente alcalino y se desconoce su estabilidad durante el proceso de curado del jamón.

La acción conjunta de calpaínas y catepsinas sobre las principales proteínas del músculo genera la rotura de éstas, provocando un aumento de la terneza de la carne y la generación de nuevos péptidos (Sentandreu et al., 2002; Lametsch et al., 2003). Estos péptidos generados por la acción de las endopeptidasas son posteriormente degradados por las enzimas exopeptidasas, las cuales hidrolizan la cadena peptídica a partir de sus extremos. Los principales grupos de exopeptidasas son los siguientes:

- Las peptidilpeptidasas, que generan dipéptidos y tripéptidos según se trate de dipeptidilpeptidasas o tripeptidilpeptidasas, respectivamente. Ambos tipos actúan a partir del extremo amino terminal y son muy comunes durante el proceso de elaboración del jamón curado.
- Las aminopeptidasas, que liberan aminoácidos libres a partir del extremo amino terminal de la cadena peptídica.
- Las carboxipeptidasas, que también generan aminoácidos libres pero en este caso a partir del extremo carboxilo terminal.
- Otras enzimas menos estudiadas aunque comunes en carne y productos cárnicos son las peptidildipeptidasas, que generan dipéptidos a partir del extremo carboxilo terminal de polipéptidos, y las tripeptidasas y dipeptidasas, que hidrolizan tripéptidos y dipéptidos, respectivamente, liberando como consecuencia aminoácidos libres.

Las aminopeptidasas son las principales responsables de la generación de aminoácidos libres durante el proceso de elaboración del jamón curado (Toldrá et al., 1997). Así, arginil, alanil, piroglutamil, leucil y metionil aminopeptidasas son las más importantes en el músculo esquelético, y presentan buena estabilidad hasta aproximadamente los 8 meses de curado. A partir de este momento, su actividad se ve reducida debido al efecto inhibidor de la sal, la desecación, la propia actividad proteolítica y la presencia de aminoácidos libres

(Toldrá et al., 1992; Flores et al., 1998). De especial importancia es la alanil aminopeptidasa, cuya actividad supone más del 80% del total de actividad aminopeptidasa, ya que actúa de forma específica sobre la alanina pero también sobre sustratos con enlaces aromáticos, alifáticos y aminoacil básicos (Toldrá y Flores, 1998). Así pues, esta aminopeptidasa puede considerarse la principal enzima generadora de aminoácidos libres, los cuales son los máximos responsables, junto con diversos compuestos volátiles, del particular aroma y sabor del jamón curado.

1.3.1.2 Lipólisis

La hidrólisis enzimática de los lípidos musculares y del tejido adiposo tiene una gran contribución al aroma y sabor característico del jamón curado. Afecta principalmente a triglicéridos, que son hidrolizados a diglicéridos y monoglicéridos y posteriormente a ácidos grasos debido a la acción de las lipasas (ácida lisosomal y neutra), así como a fosfolípidos que generan también ácidos grasos como producto final por la actividad de las fosfolipasas. Posteriormente, estos ácidos grasos liberados actúan como sustrato en reacciones oxidativas dando lugar a compuestos volátiles que serán responsables del aroma del producto (Motilva et al., 1993; Toldrá y Flores, 1998).

1.3.1.3 Glucólisis

La hidrólisis de glúcidos, conocida como glucólisis, es la vía metabólica encargada de obtener energía mediante la conversión de la glucosa en piruvato por acción enzimática. En condiciones aeróbicas, el piruvato entra a formar parte del ciclo del ácido cítrico y la cadena de transporte electrónico generando gran cantidad de energía. Mientras, la glucólisis anaeróbica es la única fuente de energía de las células en ausencia de oxígeno, donde el piruvato se convierte en ácido láctico produciendo como consecuencia una bajada del pH. En la carne postmortem, este descenso del pH es responsable de la desnaturalización de las proteínas miofibrilares, la reducción en la capacidad de retención de agua, y la aparición de carnes PSE o DFD en función, respectivamente, de si se ha producido una rápida

acidificación o el pH se ha mantenido próximo a la neutralidad por ausencia de azúcares y glucógeno (Greaser, 1986; Lehninger, 2000; Sheffler y Gerrard, 2007).

1.3.1.4 Transformación de nucleótidos y nucleósidos

El adenosín trifosfato (ATP) es el nucleótido mayoritario en el músculo vivo y la principal fuente de energía. Tras el sacrificio del animal, la generación de ATP va disminuyendo a medida que se agotan las reservas de fosfocreatina y glucógeno e inactivan las enzimas responsables de su formación. Además, comienza una rápida hidrólisis del ATP por medio de una serie de reacciones de defosforilación y desaminación generando diferentes compuestos conocidos como productos de la degradación del ATP, lo que conlleva a la instauración del *rigor mortis* en la carne (Greaser, 1986).

1.3.2 Reacciones químicas

1.3.2.1 Reacciones de Maillard

La reacción de Maillard o de pardeamiento no enzimático es una de las rutas principales en la generación de compuestos aromáticos en la carne y derivados cárnicos. Se produce como resultado de la reacción entre los grupos amino libres de compuestos proteicos y los grupos carbonilos de azúcares, y requiere un aporte de calor o almacenamiento prolongado a temperatura ambiente. En el caso concreto del jamón curado, la temperatura y el tiempo de almacenaje durante la etapa de maduración favorecen este tipo de reacciones (García et al., 1991; Ventanas et al., 1992). La combinación de reacciones de Maillard y procesos oxidativos es muy importante en el desarrollo del típico aroma y sabor del jamón curado debido a la generación de compuestos volátiles como cetonas, aldehídos, ésteres, hidrocarburos, ácidos, alcoholes y pirazinas (Flores et al., 1997).

1.3.2.2 Degradaciones de Strecker

Una de las vías de las reacciones de Maillard es la degradación de Strecker, la cual consiste en la desaminación oxidativa y posterior descarboxilación de aminoácidos en presencia de compuestos dicarbonílicos. Como resultado se

generan numerosos compuestos volátiles que contribuyen al aroma del jamón curado (Mottram, 1998).

1.3.2.3 Degradación y oxidación de lípidos

La degradación de lípidos tiene su origen en la hidrólisis enzimática y generación de ácidos grasos libres, tal como se ha descrito en un apartado anterior. Posteriormente, estos ácidos grasos son susceptibles de oxidación a través de una serie de reacciones en cadena. En primer lugar, se forman radicales libres por acción de diferentes catalizadores como la luz, oxígeno, calor o enzimas oxidativas, dando lugar a la formación de hidroperóxidos. Estos compuestos son muy inestables y reactivos, por lo que van a originar productos secundarios de oxidación, los cuales tendrán un gran impacto sobre el aroma del producto (Shahidi et al., 1986; Toldrá y Flores, 1998). Además, los hidroperóxidos pueden polimerizarse al reaccionar con proteínas, péptidos o aminoácidos (Buscailhon et al., 1993). En el jamón curado es necesario un cierto nivel de oxidación lipídica para el desarrollo del aroma y sabor, mientras que un exceso dará lugar a características organolépticas negativas en el producto.

1.3.2.4 Oxidación de proteínas y péptidos

La oxidación de proteínas y péptidos es importante por el deterioro de calidad que puede provocar en la carne y productos cárnicos durante el procesado y almacenamiento. Así, este proceso puede iniciarse por la reacción de aminoácidos con iones metálicos, especies reactivas de oxígeno o radicales libres derivados de lípidos (Stadtman y Levine, 2003), y puede conllevar tanto a cambios en el color, aroma, sabor y textura, como a la pérdida de aminoácidos esenciales y cambios en la conformación y funcionalidad de las proteínas (Xiong, 2000; Estévez, 2011). Los aminoácidos básicos, los aromáticos y principalmente la cisteína y metionina son los residuos más susceptibles de ser oxidados debido a su estructura (Gatellier et al., 2009; Zhang, et al., 2013). En el jamón curado, la oxidación de proteínas se ve acelerada durante las primeras etapas del proceso por el efecto de la sal, mientras que se estabiliza al final de la etapa de maduración (Koutina et al., 2012).

2. Péptidos generados durante el proceso de elaboración del jamón curado

Como ya se ha comentado anteriormente, durante el proceso de elaboración del jamón se produce una intensa proteolisis de las proteínas sarcoplásmicas y miofibrilares generando gran cantidad de pequeños péptidos (menores de 4,5 kDa) y aminoácidos libres. Diversos estudios han documentado la presencia de fragmentos proteicos generados durante el proceso de curado del jamón (Rodríguez-Nuñez et al., 1995; Hansen-Møller et al., 1997; Sentandreu et al., 2003; Larrea et al., 2006), en los cuales los fragmentos proteicos han sido separados mediante técnicas cromatográficas o de electroforesis en gel. Sin embargo, ha sido el reciente avance en técnicas proteómicas como la espectrometría de masas en tándem, lo que ha permitido la identificación y secuenciación de los péptidos generados durante la proteolisis. De hecho, secuencias de oligopéptidos derivados de proteínas miofibrilares como la actina, miosina, titina o troponina T (Di Luccia et al., 2005; Sentandreu et al., 2007; Mora et al., 2009a; Mora et al., 2010a) y de proteínas sarcoplásmicas como la creatina quinasa, mioglobina o enzimas glicolíticas (Mora et al., 2009b; Mora et al., 2011; Mora y Toldrá, 2012) han sido identificadas recientemente en jamón curado.

Hasta ahora, el interés de estudiar los péptidos generados de manera natural durante el proceso de elaboración del jamón curado ha radicado principalmente en obtener información sobre el proceso de curado y reacciones bioquímicas que tienen lugar durante el mismo. Sin embargo, los principales estudios actuales sobre esta temática se centran en el potencial de los péptidos generados de ser utilizados como marcadores de calidad o del tiempo de curado así como en el estudio de sus posibles propiedades bioactivas con el fin de dar un valor añadido al jamón. Así, la identificación de péptidos marcadores permitiría un mayor control y dirección de los procesos de elaboración del jamón curado, la optimización de las reacciones enzimáticas hacia un producto final de calidad óptima, la disminución de fraudes comerciales, y por tanto, la aportación de beneficios productivos y económicos al sector jamonero.

2.1 Péptidos marcadores de calidad

2.1.1 Influencia en el sabor y aroma del jamón

La intensidad y calidad del aroma y sabor característico del jamón curado están fuertemente relacionadas con la materia prima inicial, actividad enzimática y condiciones durante el proceso de elaboración tales como la cantidad de sal añadida, temperatura, humedad y duración del curado (Toldrá et al., 1997; Toldrá, 2002). La composición en aminoácidos de los péptidos, principalmente de dipéptidos y tripéptidos, determina su contribución al sabor y aroma típico del jamón (Sentandreu et al., 2003, Sforza et al., 2006). Además, los aminoácidos libres generados tras la intensa acción de las aminopeptidasas, que pueden alcanzar varios cientos de miligramos por 100 g de jamón (Toldrá et al., 1997; Toldrá et al., 2000) también son principales responsables. De este modo, los ácidos glutámico y aspártico, que se generan en grandes cantidades, pueden impartir un sabor ácido, aunque sus sales sódicas contribuyen a una mejora del sabor. Además, aminoácidos como alanina, serina, prolina, glicina e hidroxiprolina modulan el sabor aportando dulzor (Aristoy y Toldrá, 1995), mientras que lisina y tirosina aportan el sabor a viejo característico del jamón de Parma (Careri et al., 1993). Por otro lado, una proteolisis excesiva puede dar lugar a péptidos o aminoácidos como triptófano y fenilalanina, que aportan sabores amargos indeseables o retrogusto metálico (Careri et al., 1993; Parolari et al., 1994). Finalmente, algunos aminoácidos libres pueden convertirse en fuente de compuestos volátiles durante el curado o calentamiento, por ejemplo metionina y cisteína pueden generar compuestos volátiles azufrados, mientras que leucina, valina e isoleucina pueden generar aldehídos por reacciones de degradación de Strecker (Toldrá et al., 2000). Así pues, el sabor y aroma final del jamón curado va a depender de la cantidad y composición en aminoácidos de los péptidos generados por proteolisis durante el proceso de elaboración, así como de la cantidad y proporción de aminoácidos libres.

2.1.2 Influencia en la textura del jamón

La sal añadida en la etapa de salado durante la elaboración del jamón y los cambios bioquímicos que tienen lugar durante este proceso son los principales condicionantes de la textura del jamón curado. En este sentido, la dureza ha sido relacionada con la pérdida de humedad y aumento de la concentración de sal a lo largo del curado (Virgili et al., 1995; Ruiz-Ramírez et al., 2005), mientras una excesiva proteolisis debido a la intensa acción de las endopeptidasas musculares se relaciona con terneza y pastosidad (Palorari et al., 1994; Toldrá y Flores, 2000). Así pues, la relación entre la textura del jamón y la hidrólisis de proteínas sarcoplásmicas y miofibrilares durante su proceso de elaboración (en especial las proteínas estructurales titina y troponina T por su importancia en la contracción muscular), con la consecuente generación de péptidos, ha sido ampliamente estudiada para el establecimiento de marcadores de calidad del jamón (Tabilo et al., 1999; Mora et al., 2009a; Mora et al., 2010a; Del Olmo et al., 2013; Marcos et al., 2013).

2.2 Péptidos marcadores del tiempo de curado

El estudio de compuestos que puedan ser utilizados como marcadores del tiempo de curado del jamón no ha sido muy extenso hasta el momento. Los ensayos realizados se basan en perfiles proteolíticos y cambios significantes de proteínas o fragmentos derivados, a largo del curado del jamón, como marcadores del proceso. Los métodos electroforéticos han sido los más utilizados en estos análisis, de modo que la aparición o desaparición de bandas o cambios cuantitativos en el transcurso del proceso de curado pueden permitir la discriminación entre diferentes tiempos del proceso (Di Luccia et al., 2005; Larrea et al., 2006; Picariello et al., 2006; Théron et al., 2011).

Por otro lado, otros estudios se han centrado en la evolución de compuestos volátiles generados (Pinna et al., 2012) o la concentración de compuestos musculares como creatina, creatinina, nucleótidos y sus compuestos de degradación (Mora et al., 2010b; Hernández-Cázares et al., 2011) para poder determinar estimadores del tiempo de proceso. Así por ejemplo, la ratio

hipoxantina/inosina puede ser útil para indicar un tiempo mínimo de curado de 5 meses (Escudero et al., 2011).

Sin embargo, el uso de péptidos para estimar y controlar el tiempo de curado del jamón sólo se ha estudiado en esta tesis, y se comentará en detalle en los capítulos correspondientes.

2.3 Péptidos bioactivos

Los péptidos bioactivos son compuestos derivados de los alimentos, genuinos o generados, que, además de su valor nutricional, ejercen un efecto fisiológico en el organismo (Vermeirssen et al., 2004). Por ello, la importancia de su estudio radica en conocer mejor sus propiedades y actividades *in vivo* para su uso potencial como ingredientes en alimentos funcionales o nutracéuticos.

La carne, además de constituir una fuente importante de proteínas con un gran valor nutricional, asociado principalmente a su contenido en aminoácidos esenciales, presenta un potencial significativo como fuente de péptidos bioactivos. Estos péptidos están inactivos dentro de la secuencia de la proteína original y necesitan ser liberados para ejercer su actividad. En el caso de proteínas cárnicas, los péptidos bioactivos pueden generarse por acción de enzimas como la pepsina, tripsina o quimiotripsina durante la digestión gastrointestinal (Arihara et al., 2001; Escudero et al., 2010), por la proteolisis que tiene lugar durante la maduración, fermentación y curado de la carne (Sentandreu y Toldrá, 2007; Arihara y Ohata, 2008; Broncano et al., 2012) o de manera artificial mediante el tratamiento con proteasas utilizadas durante el proceso, por ejemplo enzimas vegetales como la papaína o bromelina utilizadas para tenderizar la carne (Korhonen y Pihlanto, 2003; Saiga et al., 2003).

Los péptidos bioactivos tienen normalmente entre 2 y 20 residuos de aminoácidos, y en función de su secuencia y composición pueden ejercer diferentes funciones fisiológicas en el organismo tales como actividad antihipertensiva, antimicrobiana, antioxidante, antidiabética, antitrombótica, etc. (Korhonen y Pihlanto, 2003; Arihara, 2006). Pero para ejercer su actividad, los péptidos deben resistir la completa degradación por las enzimas

gastrointestinales, ser absorbidos intactos a través de la pared intestinal, y alcanzar el sistema circulatorio en forma activa (Vermeirssen et al., 2005). En otros casos, los péptidos interaccionan con receptores expresados en el tracto gastrointestinal, sin necesidad de alcanzar el torrente circulatorio para ejercer su función (Yamada et al., 2002).

Los péptidos antihipertensivos son el grupo de péptidos bioactivos más ampliamente estudiado (Vercruysse et al., 2005) y en los que también nos centraremos en la presente tesis doctoral, por ello que sean explicados con más detalle en el siguiente apartado.

2.3.1 Péptidos antihipertensivos

Los péptidos antihipertensivos son aquellos capaces de inhibir a la enzima convertidora de angiotensina I (ECA). Esta enzima es una dipeptidil carboxipeptidasa que juega un papel esencial en la regulación de la presión sanguínea a través de su acción sobre el sistema renina-angiotensina. La ECA convierte la angiotensina I en un potente vasoconstrictor, la angiotensina II, y además induce la liberación de aldosterona e inactiva al vasodilatador bradiquinina (Skeggs et al., 1956; Ondetti et al., 1977). La acción de la enzima ECA sobre estos dos sistemas provoca un aumento de la tensión arterial, dando lugar a la enfermedad cardiovascular más común en la actualidad, la hipertensión (Unger, 2002).

La unión de los péptidos antihipertensivos a la ECA está fuertemente influenciada por el extremo C-terminal de sus secuencias, de modo que aquellos péptidos con aminoácidos hidrofóbicos en las últimas tres posiciones del C-terminal tienen más predisposición a unirse al sitio activo de la enzima y por tanto, presentan un mayor poder de inhibición (Yokohama et al., 1992; Brown y Vaughan, 1998; Matsui y Matsumoto, 2006).

Como ya se ha comentado anteriormente, los péptidos bioactivos deben permanecer activos durante la digestión y absorción gastrointestinal y alcanzar la corriente sanguínea para ejercer su acción. En la **Figura 4** se muestran las barreras potenciales que pueden activar o inactivar los péptidos durante las

etapas de digestión y absorción intestinal. En este sentido, son muy importantes además de los estudios *in vitro* de la inhibición de la ECA mediante el control del sustrato utilizado, los ensayos con células Caco-2 como modelo del epitelio intestinal, lo que permite predecir *in vitro* el transporte de los péptidos a través del intestino. La línea celular Caco-2 son células derivadas de adenocarcinoma de colon humano que una vez diferenciadas como monocapas celulares mantienen la morfología y la función de los enterocitos maduros. Además, estas células expresan las peptidasas propias del borde de cepillo, las cuales pueden hidrolizar los péptidos y modificar su actividad (Hidalgo et al., 1989; Yee, 1997; Vermeirssen et al., 2004). Es por ello que la estabilidad de los péptidos a la hidrólisis enzimática y absorción intestinal determina su biodisponibilidad y actividad, siendo la principal causa de las diferencias que a veces se obtienen entre ensayos *in vitro* e *in vivo* (Pihlanto-Leppälä, 2000; Vermeirssen et al., 2004; Bejjani y Wu, 2013).

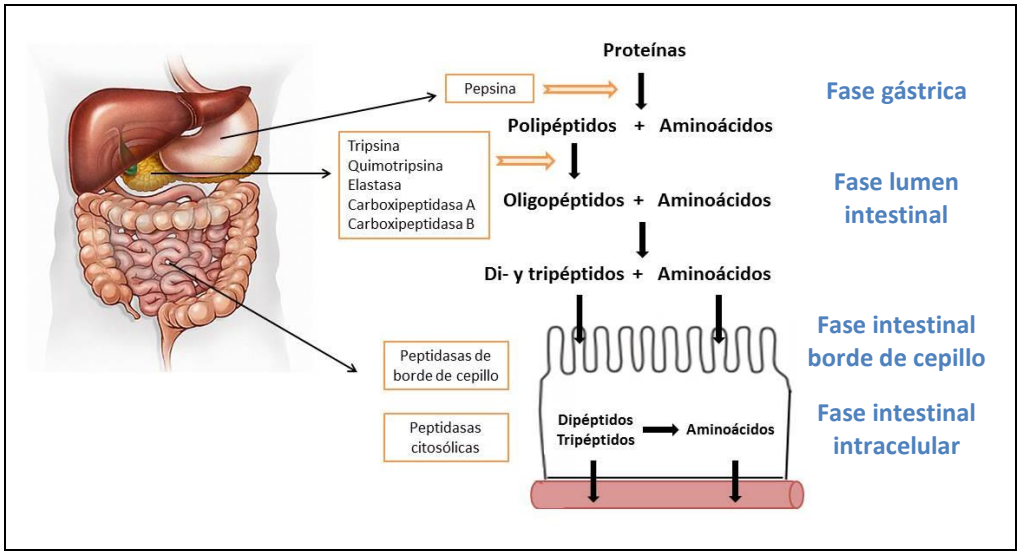

Figura 4. Barreras potenciales de activación o inactivación de los péptidos durante la digestión y absorción gastrointestinal.

Existen diversos estudios sobre péptidos antihipertensivos en carne de cerdo y jamón curado. Por ejemplo, se han identificado péptidos con actividad inhibidora de la ECA generados a partir de carne de cerdo tras simulación de la digestión humana (Escudero et al., 2010), y que además mostraron un efecto *in vivo* cuando se administraron oralmente a ratas espontáneamente hipertensas (Escudero et al., 2012a). En jamón, decenas de péptidos inhibidores de la ECA generados por la acción proteolítica de enzimas musculares durante el proceso de curado han sido identificados hasta el momento (Sentandreu y Toldrá, 2007; Escudero et al., 2013), evaluándose además su actividad antihipertensiva *in vivo* (Escudero et al., 2012b; Escudero et al., 2013) y su estabilidad a la digestión gastrointestinal *in vitro* o a tratamientos térmicos (Escudero et al., 2014).

3. Métodos analíticos para la determinación de péptidos generados en el jamón curado como marcadores de calidad.

3.1 Extracción de péptidos

Para el análisis de péptidos de la carne y productos cárnicos la extracción es una etapa fundamental. Una vez eliminada la grasa y tejido conectivo del tejido muscular, la muestra se tritura por métodos mecánicos y se toma una muestra representativa para el posterior análisis. Esta muestra es homogeneizada con la solución de extracción, que suele ser agua bidestilada, soluciones acuosas ácidas (clorhídrico, acético o perclórico), soluciones salinas diluidas, tampón fosfato neutro e incluso mezclas de agua con disolventes orgánicos. En el caso de muestras de carne y productos cárnicos se suelen emplear soluciones ácidas como el ácido clorhídrico 0.01N (Rodríguez-Nuñez et al., 1995; Mora et al., 2008). Tras la centrifugación del extracto, se recoge el sobrenadante y se desproteiniza añadiendo 3 volúmenes de acetonitrilo, metanol o etanol. En ocasiones, la extracción y desproteinización se realizan en un solo paso extrayendo la muestra con un solvente desproteinizante como el ácido tricloroacético (Hughes et al., 2002) o el ácido perclórico 0.6N (Dunnett y Harris, 1997; Martín et al., 2001). La muestra desproteinizada contiene un extracto de péptidos y aminoácidos, el cual se concentra para la siguiente etapa del análisis.

3.2 Técnicas de separación

En la mayoría de las ocasiones, el análisis de péptidos requiere de un fraccionamiento previo para simplificar la muestra y eliminar posibles sustancias del extracto que puedan causar interferencias en el análisis posterior. Los métodos de separación se basan en diferentes principios como el fraccionamiento por tamaño, carga o polaridad, y para ello se utilizan diferentes técnicas que serán descritas a continuación.

3.2.1 Ultrafiltración

Se trata de una técnica preparativa con la que es posible aislar la fracción peptídica de interés según su tamaño molecular. Además, se puede utilizar para concentrar extractos de péptidos. Existe una gran variedad de membranas para separar péptidos de diferentes tamaños moleculares, entre las cuales las membranas de nanofiltración han supuesto una gran mejora del proceso al separar en función del tamaño y carga de las moléculas (Rautenbach y Gröschl, 1990).

3.2.2 Cromatografía de exclusión molecular

Esta cromatografía, también llamada cromatografía de filtración en gel, permite separar proteínas y péptidos en función de su tamaño molecular. La fase estacionaria es un gel con un tamaño de poro determinado, de forma que las moléculas más pequeñas quedan retenidas en las partículas del gel y las moléculas de tamaño mayor al diámetro del poro son excluidas y eluyen rápidamente. Así pues, los péptidos eluyen de manera ordenada de mayor a menor tamaño molecular (ver **Figura 5**).

El intervalo de tamaños a fraccionar depende del tipo de gel utilizado. Por ejemplo, un gel Sephadex G-25 (GE Healthcare) es adecuado para separar péptidos de tamaños entre 500 y 5.000 Daltons (Spanier et al., 1988; Rodríguez-Nuñez et al., 1995; Hughes et al., 2002), mientras que un gel Sephadex G-10 sería adecuado para separar péptidos menores de 700 Daltons (Cambero et al., 1992; Hughes et al., 2002).

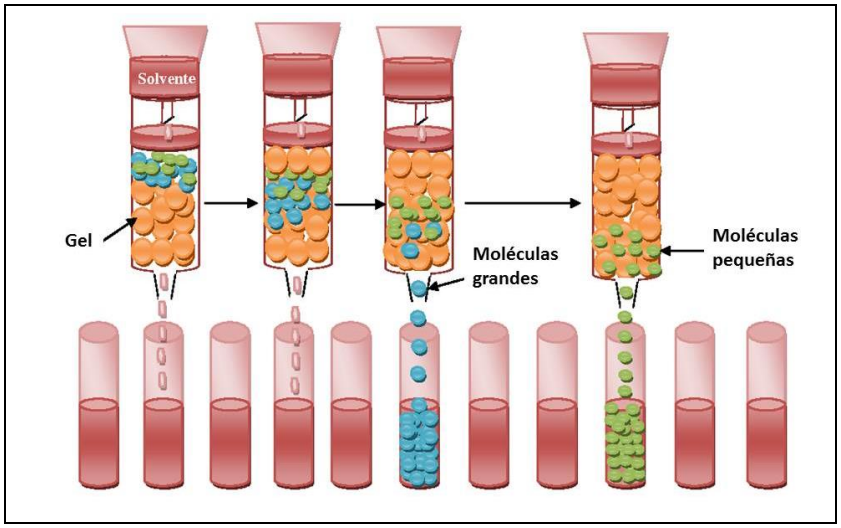

Figura 5. Esquema del funcionamiento de la cromatografía de exclusión molecular.

La elución de los péptidos se suele realizar con ácido clorhídrico 0.01N o con tampones fosfato diluidos, y a velocidades de flujo muy bajas (por ejemplo, 15 mL/hora). Las fracciones se colectan y se monitorizan por absorción ultravioleta a 214, 254 o 280 nm para controlar la elución de los compuestos de interés. En la **Figura 6** se muestra un cromatograma típico de un extracto de jamón curado.

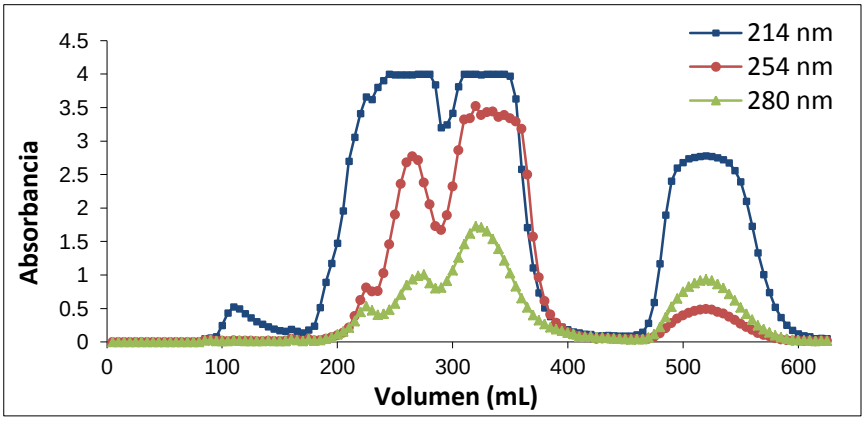

Figura 6. Cromatografía de filtración en gel con una columna Sephadex G-25 de un extracto de jamón curado desproteinizado.

3.2.3 Electroforesis en gel

La técnica más utilizada para la separación de proteínas y péptidos es la electroforesis en gel de poliacrilamida (PAGE), tanto de una dimensión, donde la separación se realiza en función de la masa molecular en presencia de dodecil sulfato sódico (SDS) (ver **Figura 7A**), como bidimensional, la cual está basada en una primera separación en función del punto isoeléctrico utilizando un gradiente de pH, seguida de una separación en función de la masa molecular (ver **Figura 7B**).

El uso más común de la PAGE es la separación de proteínas de tamaño entre 30-500 kDa mediante el uso de glicina como componente fundamental del tampón. Pero además, es posible la separación de pequeñas proteínas y péptidos de entre 1-30 kDa si se ajusta la concentración de acrilamida en el gel y se sustituye la glicina del tampón por tricina. Además, este sistema es también útil para la separación de péptidos muy hidrofóbicos, y se ha utilizado tanto en electroforesis unidimensional como bidimensional (Schägger, 2006).

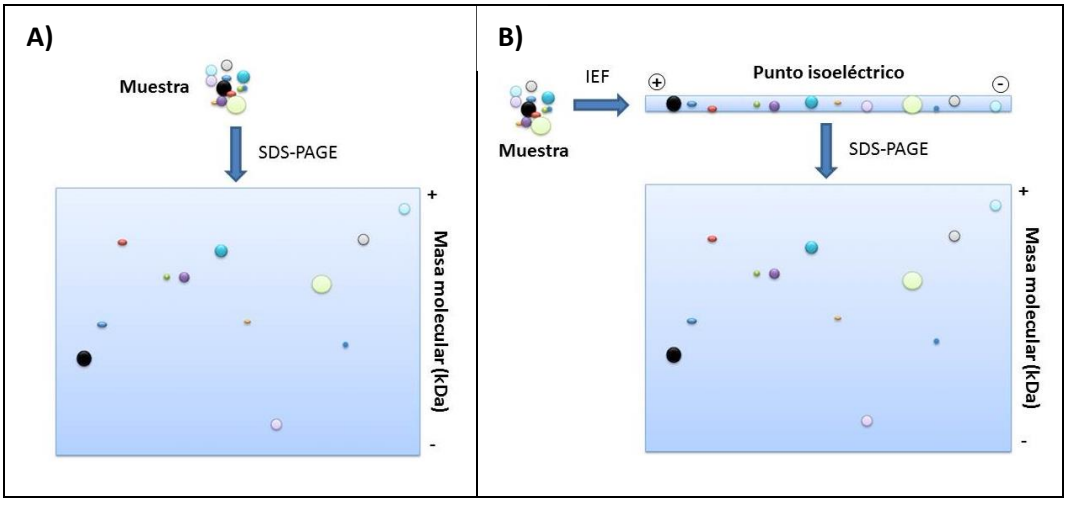

Figura 7. A) Esquema de electroforesis unidimensional. B) Esquema de electroforesis bidimensional.

La electroforesis en gel se ha utilizado mucho en el estudio de cambios post-mortem del músculo esquelético, lo que ha contribuido a un mejor conocimiento de los mecanismos bioquímicos que tienen lugar durante el periodo de almacenamiento de la carne (Lametsch y Bendixen, 2001; Jia et al., 2006a). Además, también se ha utilizado para relacionar los cambios postmortem del proteoma con el desarrollo de la terneza en la carne de cerdo (Lametsch et al., 2003; Hwang et al., 2005) y con cambios en el color de la misma (Sayd et al., 2006). En el caso del jamón curado, diversos estudios han utilizado electroforesis en gel para evaluar la degradación de proteínas durante el proceso de elaboración (Larrea et al., 2006) o estudiar como este proceso puede variar en función de la calidad de la materia prima inicial (Tabilo et al., 1999).

3.2.4 Cromatografía líquida de alta resolución en fase reversa (RP-HPLC)

Este tipo de cromatografía es la técnica más utilizada para analizar extractos peptídicos, ya que permite su separación en base a diferencias de hidrofobicidad debidas a la composición en aminoácidos de los péptidos. Existen diferentes tipos de columnas de fase reversa, siendo las más utilizadas aquellas que consisten en un soporte de sílice con cadenas n-alquil enlazadas con grupos octadecil (C18) y octil (C8). En la RP-HPLC, la fase estacionaria es apolar mientras que la fase móvil es polar. Las típicas fases móviles utilizadas en este tipo de separaciones son acetonitrilo como fase orgánica y una fase acuosa compuesta generalmente de un 0,1% de ácido trifluoroacético o ácido fórmico en agua (Sforza et al., 2006). Además, para una separación óptima de los péptidos se suele utilizar una elución en gradiente, en la que se va aumentando la hidrofobicidad de la fase móvil. De esta manera, los péptidos se reparten entre la fase estacionaria y la fase móvil en función de su polaridad, de modo que los péptidos más polares eluyen en primer lugar de la columna, mientras que los más hidrofóbicos, que han sido retenidos en la columna, eluyen más tarde. La detección de los péptidos puede realizarse a diferentes longitudes de onda (214, 254 o 280 nm), o si el sistema cuenta con un detector de diodos se puede obtener el espectro de absorción completo para cada péptido. El resultado es un cromatograma donde se representa la señal de absorbancia frente al tiempo de

elución, y donde la calidad y tiempo de separación dependen, respectivamente, del tamaño de las partículas de la fase estacionaria y la longitud de la columna.

Esta técnica de RP-HPLC permite la obtención de los denominados mapas peptídicos de manera muy eficaz (Colilla et al., 1991). De esta forma, a partir de digestiones proteicas o extractos de péptidos se consigue el aislamiento de péptidos para su posterior estudio o identificación (Recio et al., 2000; Sentandreu et al., 2003; Mora et al., 2009a), así como el seguimiento de su evolución durante la proteolisis que tiene lugar durante la elaboración del jamón curado (Toldrá y Aristoy, 1993; Rodríguez-Nuñez et al., 1995).

3.2.5 Cromatografía de intercambio iónico (CII)

Este tipo de cromatografía permite la separación de los péptidos en función de la carga de las moléculas, por lo que es una técnica complementaria a la descrita anteriormente. La fase estacionaria muestra en la superficie grupos funcionales iónicos que interactúan con iones de carga opuesta del analito. Así pues, la cromatografía de intercambio catiónico retiene cationes cargados positivamente debido a que la fase estacionaria muestra un grupo funcional cargado negativamente como el ácido fosfórico o sulfónico, mientras que la cromatografía de intercambio aniónico retiene aniones usando grupos funcionales cargados positivamente como un catión de amonio cuaternario o una amina. Como resultado, los péptidos de carácter ácido se separan mejor en columnas de intercambio aniónico (Pizzano et al., 1998) y aquellos de carácter básico lo hacen en columnas de intercambio catiónico (Recio et al., 2000; Sentandreu et al., 2003).

Los mejores resultados se suelen obtener utilizando sales no volátiles como el cloruro sódico, pero esto dificulta posteriores análisis por espectrometría de masas. Para superar este problema, la separación por intercambio iónico suele ir seguida de una cromatografía normal, de fase reversa o de interacción hidrofílica para eliminar la sal y así posteriormente analizar los péptidos de interés por espectrometría de masas.

3.2.6 Cromatografía de interacción hidrofílica (HILIC)

Este tipo de cromatografía constituye una alternativa a la cromatografía de fase reversa y a la de intercambio iónico, las cuales presentan limitaciones en el análisis de compuestos polares debido a su baja retención. La HILIC utiliza fases móviles similares a las utilizadas en RP-HPLC, con la consecuente ventaja sobre la solubilidad del analito, sin embargo, no precisa del uso de agentes derivatizantes o pares iónicos, necesarios en fase reversa cuando se analizan compuestos muy polares. Por otro lado, se trata de una cromatografía líquida totalmente compatible con espectrometría de masas, eliminando el paso de desalado necesario cuando se utiliza la CII.

La cromatografía de interacción hidrofílica ha sido utilizada para la separación de pequeños péptidos presentes en carne (Mora et al., 2007; Broncano et al., 2012) como método complementario a la cromatografía de fase reversa. Compuestos que presentan poca o ninguna retención en RP-HPLC suelen presentar una fuerte retención en columnas HILIC, las cuales requieren fases móviles con un alto contenido en componente orgánico para favorecer las interacciones hidrofílicas entre el analito y la fase estacionaria. Además, el tiempo de retención aumenta con la polaridad del péptido, la polaridad de la fase estacionaria y al disminuir la concentración de disolvente orgánico de la fase móvil, al contrario que en RP-HPLC.

3.3 Identificación y secuenciación

Para la identificación de proteínas existen dos estrategias, la identificación mediante huella peptídica para la que únicamente se necesita análisis por espectrometría de masas (MS), y la identificación mediante espectrometría de masas en tándem (MS/MS).

La huella peptídica de una proteína es el conjunto de péptidos generados tras la digestión de la proteína con una enzima específica que tiene un patrón de corte conocido. La tripsina es la enzima más frecuentemente utilizada para este fin, la cual es una endopeptidasa que escinde específicamente los residuos de lisina y arginina a partir de sus extremos C-terminal, generando péptidos de masas

moleculares adecuadas para el análisis por espectrometría de masas y que serán capaces de ionizarse debido a su capacidad de atrapar y retener un protón (Brown y Wold, 1973; Olsen et al., 2004). Las masas experimentales de los péptidos obtenidos de la hidrólisis proteica pueden determinarse mediante espectrometría de masas acoplada a un detector adecuado, y son comparadas con las masas peptídicas teóricas de proteínas contenidas en bases de datos (James et al., 1993; Pappin et al., 1993). De esta forma, para que la identificación de la proteína sea correcta, es necesario que las masas absolutas de un gran número de péptidos coincidan con las masas teóricas y que cubran gran parte de la secuencia de la proteína presente en la base de datos.

En el caso de la identificación de péptidos presentes en matrices complejas como el jamón curado y que han sido generados a partir de procesos proteolíticos en los que participan mezclas de enzimas el estudio se complica, puesto que los péptidos no presentan sitios de corte específicos de una enzima conocida y el perfil peptídico no es contrastable con las masas teóricas de las bases de datos proteicas. En estos casos, los péptidos deben ser identificados por elucidación de su secuencia de aminoácidos mediante espectrometría de masas en tándem, técnica que se explicará más adelante.

El gran avance durante las últimas décadas de las técnicas de espectrometría de masas ha hecho que éstas sean las más frecuentemente utilizadas para la identificación de péptidos. Sin embargo, la degradación de Edman ha sido hasta entonces el método tradicionalmente utilizado para este fin, por ello que sea también explicado a continuación.

3.3.1 Secuenciación por degradación de Edman

La degradación de Edman consiste en la degradación progresiva del péptido a partir del aminoácido localizado en el extremo N-terminal y la identificación posterior, mediante cromatografía líquida de alta resolución, de cada aminoácido liberado. Así se puede conocer la secuencia de los aminoácidos que componen el péptido. Esta técnica se basa en la reacción del fenilisotiocianato con el grupo amino libre terminal del péptido, de modo que el aminoácido de

este extremo es eliminado como un derivado feniltiohidantoína, el cual puede ser identificado por HPLC. El ciclo se repite para la identificación de cada uno de los aminoácidos del péptido (Bermodso et al., 1970).

Esta técnica de secuenciación ha sido muy útil durante décadas, pero presenta varias limitaciones como la necesidad de tener libre el extremo N-terminal del péptido o que el péptido a secuenciar sea de elevada pureza. Además hay que considerar que la sensibilidad disminuye y las interferencias aumentan en cada paso del ciclo, y que es un proceso muy lento. El uso de secuenciadores automáticos permiten realizar la degradación de Edman y la posterior identificación mediante su acoplamiento a un HPLC, y han sido utilizados para la identificación de péptidos en productos cárnicos curados (Hughes et al., 2002; Sentandreu et al., 2003; Sforza et al., 2003).

3.3.2 Identificación por espectrometría de masas

El reciente avance en espectrometría de masas (MS) ha permitido el análisis de proteínas y péptidos gracias al desarrollo de métodos de ionización suave que permiten convertir biomoléculas grandes, polares y no volátiles en iones en fase gaseosa. Esta ionización puede ser por electrospray (ESI) o por desorción/ionización láser asistida por matriz (MALDI).

Los espectrómetros de masas son instrumentos capaces de separar los iones que componen una muestra según su relación masa/carga (*m/z*). Están formados al menos por una fuente de iones, un analizador de masas y un detector (ver **Figura 8**), y normalmente trabajan a vacío con el fin de no alterar la trayectoria de los iones y evitar colisiones con moléculas neutras hasta su llegada al detector.

La técnica consta de los siguientes pasos:

- Introducción de la muestra, directamente o por acoplamiento a técnicas de separación cromatográficas.
- Vaporización e ionización de la muestra, para convertir los péptidos en iones en fase gaseosa, mediante MALDI o ESI.
- Separación de los iones según su relación *m/z* en un analizador de masas, que puede operar en modo continuo (sector magnético, cuadrupolo),

pulsado (tiempo de vuelo) o por captura de iones (trampa iónica, transformada de Fourier, orbitrap).

- Medida en un detector (copa de Faraday o fotomultiplicador), obteniendo un espectro de masas que muestra la abundancia de los iones frente a su valor *m/z*.

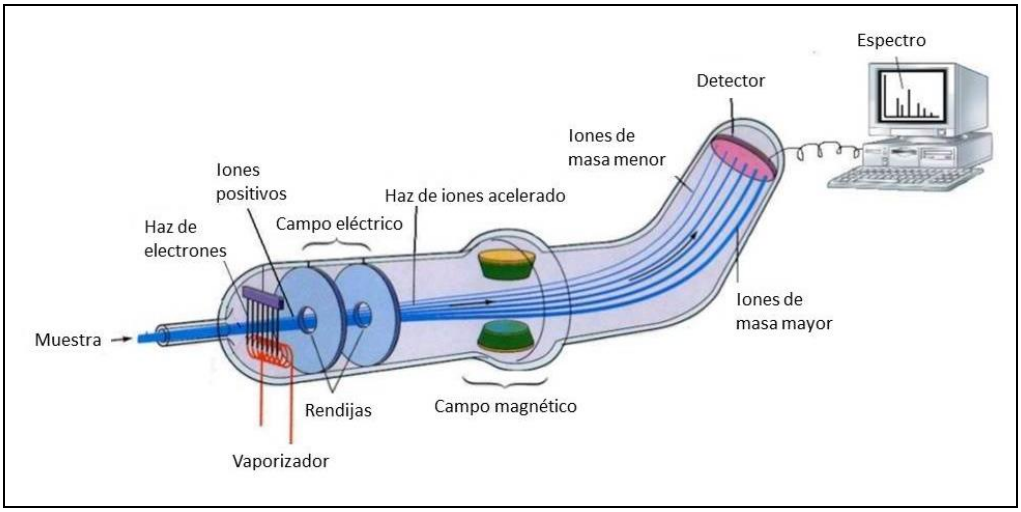

Figura 8. Esquema de un espectrómetro de masas.

En ocasiones, antes de la entrada al detector se realiza una fragmentación opcional de determinados iones seleccionados mediante descomposición metaestable o disociación inducida por colisiones (CID). Para ello se utilizan espectrómetros de masas en tándem, que implican el acoplamiento de dos analizadores iguales o diferentes separados por una cámara de fragmentación, y los cuales serán explicados con más detalle más adelante.

El gran uso actual de la espectrometría de masas se debe a sus características únicas como son: 1) su aplicación a todo tiempo de muestras ya sean volátiles o no, polares o apolares, y en cualquier estado físico, 2) la gran especificidad molecular que aporta, debido a su habilidad para medir de forma muy precisa masas moleculares, 3) su enorme sensibilidad de detección, llegando al orden de attomoles y zeptomoles, 4) su rapidez y versatilidad, ya que permite determinar

las estructuras de la mayoría de compuestos, y 5) su capacidad de analizar muestras complejas en combinación con técnicas de separación de alta resolución. No obstante, la calidad de los experimentos con espectrometría de masas depende de diferentes factores tales como los métodos de preparación de muestras, la composición de la matriz, las características de la muestra analizada (hidrofobicidad, secuencia de aminoácidos, estructura 3D), la cantidad de proteína, pureza y complejidad de la muestra, las características de los instrumentos (precisión, resolución), y la disponibilidad de estándares de calibración internos o externos.

En el caso de muestras complejas de proteínas, la purificación previa mediante electroforesis en gel o cromatografía permite la obtención de mezclas de péptidos menos complejas para un análisis más eficiente y sensible, ya que existe menos riesgo de solapamiento de picos y de supresión de iones, lo cual ocurre cuando muchos iones diferentes compiten por las cargas durante la ionización.

3.3.2.1 Fuentes de ionización

Para que una muestra pueda ser analizada por espectrometría de masas es necesario que ésta puede ser ionizada para generar iones en fase gaseosa, y que éstos sean estables. Además, la cantidad de iones generados debe ser proporcional a la cantidad de componentes de la muestra, y debe haber una mínima o nula fragmentación de los componentes durante la ionización, a menos que queramos analizar estos fragmentos. Para ello existen métodos de ionización suave tales como desorción/ionización por láser asistida por una matriz (MALDI) o electrospray (ESI).

<u>Ionización por MALDI</u>

La mezcla de péptidos se coloca sobre una placa metálica a la que se añade la matriz, que normalmente es ácido α-ciano-hidroxicinámico o ácido 2-5-dihidroxibenzoico. Al evaporarse el solvente se forman cristales de la mezcla analito-matriz, y la placa es entonces introducida en el analizador, donde la muestra es irradiada con un láser de nitrógeno pulsado para su ionización

(Karas et al., 1989; Tanaka, 2003). Los iones generados por esta técnica son mayoritariamente monocargados $(M+H)^+$ (ver **Figura 9**).

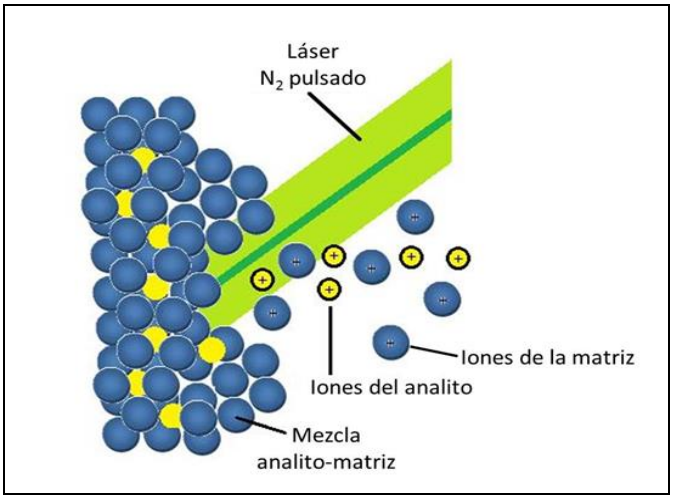

Figura 9. Esquema de ionización por MALDI.

En esta técnica, es imprescindible que la matriz sea capaz de absorber una gran cantidad de energía procedente de la radiación láser y posteriormente se la transmita, de manera controlada, a las moléculas de la muestra para la ionización. Además, la matriz debe ser completamente solvente con el analito y se debe conseguir una mezcla homogénea para permitir la correcta formación de los cristales, ya que el proceso de cristalización es una etapa crítica en este tipo de ionización. De hecho, las intensidades de señal obtenidas tras la ionización por MALDI dependen de la adecuada incorporación de los péptidos en los cristales, de la probabilidad de captura y/o retención de un protón durante el proceso de desorción, y de otros factores como los efectos de supresión en mezclas de péptidos. Por ello, es difícil relacionar la altura de pico del espectro con la cantidad de péptido, a menos que se use un patrón interno (Mann et al., 2001).

La ionización por MALDI es muy efectiva en el análisis de péptidos, pero cuando las moléculas son muy pequeñas (< 500 Da) el ruido de fondo de la matriz puede interferir en el rango de masas de estos compuestos. En estos casos,

existen recientes alternativas como la supresión de la señal de la matriz, el uso de matrices de alta masa molecular o la ionización en superficie, sin matriz (Dass, 2007a).

Ionización por ESI

La muestra en solución se vaporiza como un fino aerosol a través de su paso por una aguja hueca sometida a un campo eléctrico. Como resultado, se forma una nube de gotas cargadas eléctricamente que se mueven a contracorriente a través de una corriente de gas inerte, el cual evapora parte del solvente en un proceso de desolvatación. Como consecuencia, las gotas disminuyen su volumen y los iones presentes en la superficie de las gotas se aproximan entre sí. En un determinado momento, la repulsión de las cargas positivas de los iones vence la tensión superficial que mantiene unidos los iones en las gotas provocando que éstas se rompan (Explosión de Coulomb). El proceso se repite hasta que se evapora todo el solvente y sólo quedan los iones en fase gaseosa, los cuales pasarán al analizador de masas (ver **Figura 10**). Los iones generados pueden presentar varias cargas $(M + nH)^{n+}$, de modo que el espectro de masas que se obtiene puede mostrar varios picos correspondientes a la misma secuencia peptídica pero con diferentes cargas (Fenn et al., 1990). Este tipo de ionización se puede aplicar a una gran variedad de muestras líquidas y acoplar fácilmente a cromatografía líquida, por lo que es el método más usado.

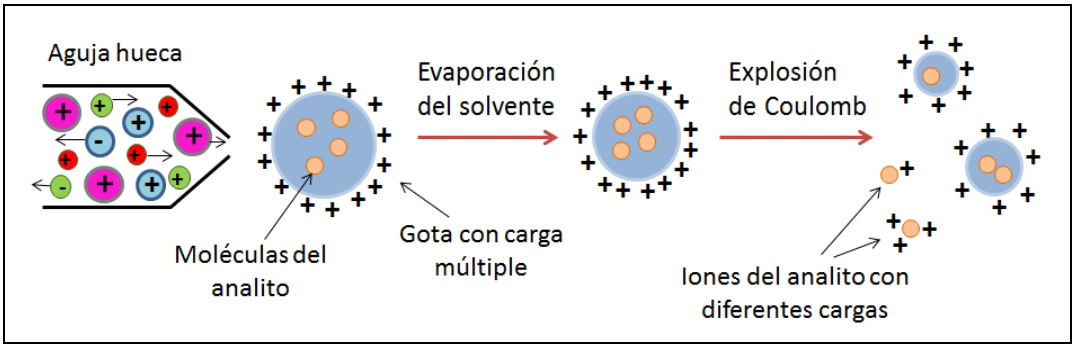

Figura 10. Esquema de la ionización por electrospray (ESI).

El último avance de la ionización ESI ha sido el desarrollo de microcapilares de borosilicato recubiertos de oro para la inyección de la muestra en el espectrómetro de masas. Esta técnica se denomina nanoelectrospray (nanoESI) y permite trabajar con volúmenes de muestra de 1-2 µL de manera muy rápida. Debido a que la intensidad se ve aumentada conforme disminuye el flujo de trabajo, la ionización por nanoESI permite una mayor optimización de la señal y una sensibilidad del orden de femtomoles (Wood et al., 2003).

Aunque se han desarrollado diversas combinaciones de fuentes de ionización y analizadores de masas, MALDI suele acoplarse a un analizador tiempo de vuelo (TOF) o TOF/TOF, mientras que ESI normalmente lo hace con un cuadrupolo, una trampa iónica, o instrumentos híbridos cuadrupolo-trampa iónica, cuadrupolo-TOF o triple cuadrupolo.

3.3.2.2 Analizadores de masas

Espectrómetro de masas de tiempo de vuelo (TOF)

Tras salir de la fuente de ionización, los iones formados son acelerados por un campo eléctrico (10^3 a 10^4 V) hacia el detector, alcanzando distintas velocidades en función de sus relaciones masa/carga. De este modo, los iones más ligeros viajan a mayor velocidad a lo largo de la zona de deriva y llegarán antes al detector, el cual registra los "tiempos de vuelo" de los distintos iones (ver **Figura 11**). El resultado es un espectro de masas en el que quedan representadas las masas moleculares (*m/z*) de los péptidos presentes en la muestra.

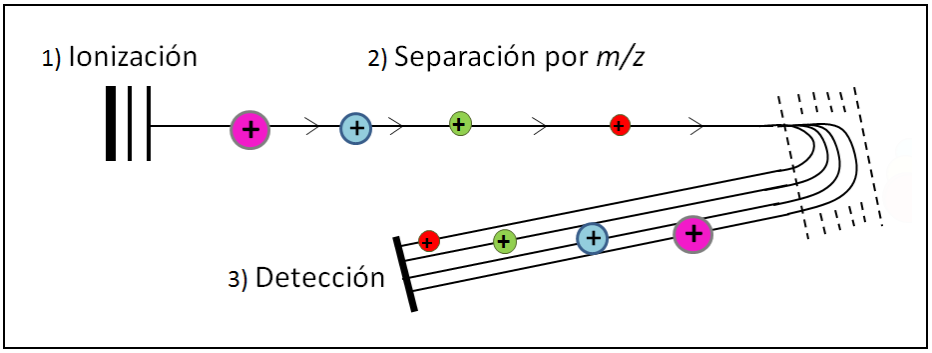

Figura 11. Esquema de funcionamiento del analizador de tiempo de vuelo (TOF).

En muchas ocasiones, este tipo de analizador suele ir acoplado a una fuente de ionización por MALDI. En este sentido, la espectrometría de masas MALDI-TOF es una técnica altamente efectiva para determinar masas moleculares en un amplio rango, presenta alta resolución y precisión, y además es económicamente accesible, rápida y puede ser automatizada. Por ello, este método ha sido utilizado para identificar cambios proteicos mediante huella peptídica en carne y jamón curado (Lametsch et al., 2002; Lametsch et al., 2004; Di Luccia et al., 2005; Théron et al., 2011), así como para determinar las masas moleculares de péptidos generados durante la proteolisis del jamón (Mora et al., 2009a; Mora et al., 2009b).

Espectrómetro de masas cuadrupolar

Este tipo de analizador es un instrumento compacto, robusto y con elevada velocidad de barrido. Consta de cuatro barras cilíndricas paralelas que actúan como electrodos. Las barras opuestas se conectan eléctricamente, un par lo hacen al polo positivo y el otro al negativo, y se aplican potenciales variables de corriente continua y de radiofrecuencia. Los iones se aceleran en el espacio entre las barras mediante un potencial de 5 a 10 V, mientras las tensiones de corriente continua y alterna se incrementan simultáneamente manteniendo constante su relación. Esto provoca un movimiento ondulatorio de los iones, sincronizados en cada momento con el voltaje aplicado, de modo que sólo aquellos de una determinada *m/z* que oscilan de una manera moderada serán capaces de atravesar el canal central del analizador y llegar al detector. De este modo, se puede seleccionar la *m/z* que corresponde al analito a determinar o todos los iones de un determinado rango de *m/z* para analizar los iones de interés (Dass, 2007b).

Espectrómetro de masas de trampa iónica

Una trampa de iones es un dispositivo en el que los cationes o iones en estado gaseoso se forman y quedan retenidos por acción de campos eléctricos y/o magnéticos. Este tipo de analizador consta de un electrodo anular y un par de electrodos colectores. Al electrodo anular se le aplica un potencial de

radiofrecuencia variable mientras que los dos electrodos colectores están conectados a tierra. De este modo, los iones con un adecuado valor de *m/z* circulan en una órbita estable dentro de la cavidad rodeada por el anillo. Cuando el potencial de radiofrecuencia se incrementa, las órbitas de los iones más pesados llegan a estabilizarse, mientras que las de los iones más ligeros se desestabilizan y salen hacia el detector (Dass, 2007b). Normalmente, los iones son expulsados en orden creciente de *m/z* mediante cambios graduales en los potenciales.

3.3.2.3 Espectrometría de masas en tándem

La espectrometría de masas en tándem (MS/MS) se basa en el acoplamiento de dos espectrómetros de masas por medio de una cámara de fragmentación o colisión. Tras la ionización por ESI o MALDI, el primer espectrómetro de masas separa los iones de acuerdo a su relación *m/z.* A continuación, se selecciona un determinado ion precursor, el cual pasa a la cámara de colisión donde se fragmenta debido a la colisión con un gas inerte. El ion precursor y los iones fragmento pasan entonces al segundo espectrómetro de masas para su análisis y llegan finalmente al detector, el cual registrará los resultados como un espectro de masas.

Debido a que los péptidos pueden fragmentarse por múltiples sitios, existe una nomenclatura para indicar el tipo de fragmentos generados (Roepstorff y Fohlman, 1984). Así, la rotura del esqueleto carbonado de un péptido puede originar seis tipos de fragmentaciones, que se clasifican en series del extremo N-terminal (a_n, b_n, c_n) o del C-terminal (x_n, y_n, z_n) en función de cuál de los extremos del péptido original se conserva en sus estructuras. El subíndice (n) corresponde a la cantidad de residuos de aminoácidos que posee el ion fragmento. En general, la fragmentación a nivel del enlace peptídico genera mayoritariamente dos tipos de iones, los de tipo *b* y los de tipo *y*, los cuales son complementarios en la información sobre la secuencia. Los espectros de fragmentación MS/MS aportan información de las diferencias de masas entre dos iones consecutivos del mismo tipo, permitiendo conocer la identidad y posición de los aminoácidos y por tanto la secuencia completa del péptido si la fragmentación ha sido de buena

calidad. Otras veces, tiene lugar una doble fragmentación y no todos los iones fragmento están a niveles detectables los que permite obtener solamente una secuencia parcial del péptido (Mann et al., 2001).

La información obtenida de la secuencia parcial o total, la masa del péptido y la localización exacta de los aminoácidos puede permitir la identificación de la proteína de origen en las bases de datos (Henzel et al., 1993; Mann et al., 1993), lo cual será comentado en el apartado correspondiente.

Los espectrómetros de masas en tándem pueden ser de varios tipos, los cuales se detallan a continuación.

Tiempo de vuelo en tándem (TOF/TOF)

Este tipo de analizador consta de un TOF lineal y un TOF reflector separados por una cámara de colisión como fuente de iones. En el primer TOF los iones precursores son acelerados y dirigidos hacia un dispositivo selector de iones (TIS), que permite seleccionar un determinado ion e impide la entrada en la celda de colisión de iones con *m/z* mayores o menores que éste. Los iones seleccionados son decelerados antes de la entrada en la cámara de colisión, donde son fragmentados por disociación inducida por colisión (CID). Posteriormente, los iones fragmento son reacelerados en el segundo TOF, y se lleva a cabo la separación en función de su relación *m/z* (Yergey et al., 2002). Recientemente, este diseño ha sido modificado para introducir un reflectrón de campo curvilíneo como TOF 2, que puede aceptar iones de un amplio rango de energía y elimina la necesidad de decelerar los iones antes de la entrada a la cámara de colisión y de reacelerar los iones fragmento.

En otro diseño de TOF/TOF en tándem, además de la celda de colisión existe un sistema TIS que actúa como selector de iones, una celda LIFT que eleva la energía potencial para la postaceleración de los iones, y un dispositivo de supresión metaestable para eliminar los precursores no fragmentados (ver **Figura 12**). Este tipo de analizador consta de un solo tubo de tiempo de vuelo con un acelerador de iones intermedio, de forma que no produce fragmentación sino que

aprovecha los iones metaestables generados durante la ionización y aceleración (Suckau et al., 2003).

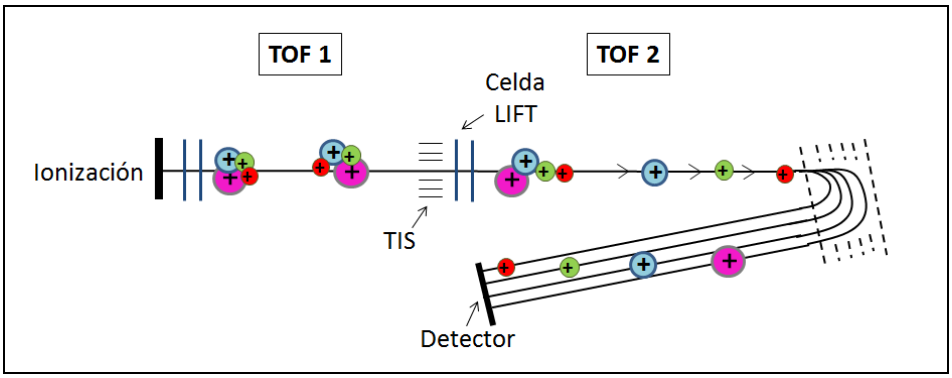

Figura 12. Esquema de un analizador tipo TOF/TOF con celda LIFT.

Este tipo de analizador de masas en tándem suele ir acoplado a una fuente de ionización por MALDI, permitiendo una buena resolución y precisión de masas y un tiempo de análisis muy rápido. Así, la técnica MALDI-TOF/TOF ha sido utilizada en el estudio de cambios proteicos postmortem en carne de bovino (Jia et al., 2006b), la identificación de péptidos antihipertensivos generados tras la digestión *in vitro* de carne de cerdo (Escudero et al., 2010) o la identificación de péptidos resultantes de la degradación de la troponina T durante el curado del jamón (Mora et al., 2010a).

Adicionalmente, este tipo de instrumentos MALDI-TOF/TOF también pueden ir acoplados a un sistema de cromatografía líquida. De modo que tras la separación, las fracciones peptídicas que eluyen de la columna se van depositando de forma automática en la placa MALDI para su ionización.

Cuadrupolo – Trampa iónica

Se caracteriza porque todos los pasos se llevan a cabo en el mismo espacio pero siguiendo una secuencia temporal. En primer lugar se lleva a cabo el aislamiento del ion, a continuación la excitación/disociación de éste, y finalmente el análisis de masas de los iones producidos. El espectro de masas se obtiene mediante

variaciones del campo eléctrico aplicado para que los iones salgan de manera secuencial hacia el detector (Dass, 2007b).

Cuadrupolo – Tiempo de vuelo (Q-TOF)

En el primer cuadrupolo se aísla un determinado ion (ion precursor), el cual se fragmenta en la cámara de colisión, formada por un segundo cuadrupolo o un hexapolo, al aplicar una determinada energía por medio de un gas inerte (normalmente helio). Finalmente, los fragmentos generados se analizan en el analizador de tiempo de vuelo, que presenta una inyección de iones ortogonal. En muchos casos existe un cuadrupolo o hexapolo adicional, tras la fuente de ionización, con el fin de aportar humedad (Dass, 2007c).

Triple cuadrupolo (QQQ)

En la **Figura 13** se muestra un esquema de funcionamiento de este tipo de espectrómetro de masas en tándem. En el primer cuadrupolo (Q1) los iones son acelerados o separados de los iones precursores. Los iones separados más rápidos pasan al segundo cuadrupolo (Q2) que actúa como cámara de colisión, produciéndose la fragmentación por las colisiones entre los iones precursores con los átomos del gas de la cámara, que es normalmente helio. Los iones fragmento resultantes son separados en el tercer cuadrupolo (Q3). Muchas veces el segundo cuadrupolo que actúa como cámara de colisión es un hexapolo u octapolo. La adquisición mediante QQQ presenta una gran versatilidad, por eso es uno de los espectrómetros de masas en tándem más utilizados.

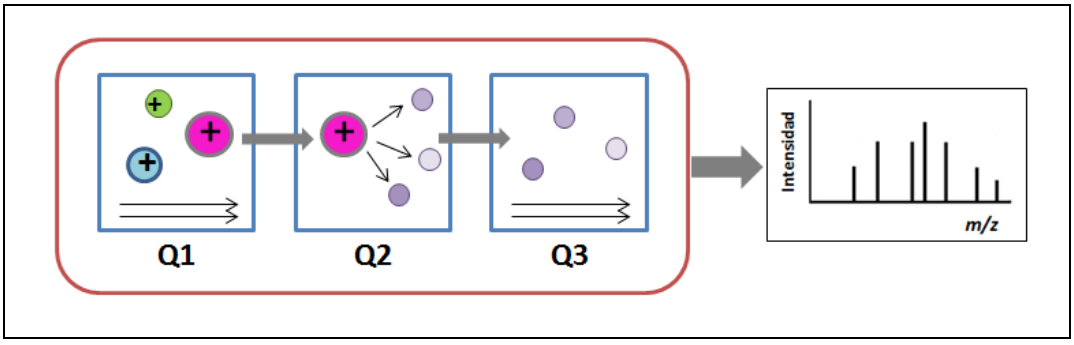

Figura 13. Esquema de un espectrómetro triple cuadrupolo (QQQ).

Cromatografía líquida acoplada a espectrometría de masas en tándem

El uso de cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS) está muy extendido para el análisis de proteínas y péptidos. La técnica de separación más comúnmente empleada en este caso es la cromatografía líquida de alta resolución (HPLC), que permite la purificación y separación de los péptidos por hidrofobicidad. A continuación, y de forma automática, el eluyente es ionizado y transferido al espectrómetro de masas. De este modo, se consigue una eficiente separación por LC y una sensible identificación de los péptidos de la muestra por MS, generando una gran cantidad de datos en forma de espectros MS/MS que aportan información sobre la masa molecular y cantidad de los iones generados. En este sentido, el cromatograma de iones totales (TIC) representa la suma de intensidades de los iones en todo el rango de masas detectadas en el análisis, mientras que el cromatograma de iones extraídos (XIC) muestra sólo un particular valor *m/z*, correspondiente al analito de interés y extraído de todo el conjunto de datos.

La LC-MS/MS permite la identificación de una gran cantidad de péptidos en un solo análisis, incluso de aquellos con la misma masa molecular pero que difieren en hidrofobicidad. Además, permite el análisis directo de muestras complejas con gran cantidad de proteínas incluso cuando la concentración de éstas difiere en varios órdenes de magnitud (Mann et al., 2001).

El método de ionización más frecuentemente empleado cuando se utiliza LC-MS/MS es la ionización por electrospray, y más recientemente por nanoelectrospray. Los últimos avances han permitido el acoplamiento de la fuente de ionización por nanoelectrospray con LC de nano flujo (nLC), que trabaja con flujos de nanolitros por minuto, en orden de obtener la máxima sensibilidad posible.

La **Figura 14** muestra el esquema de funcionamiento de la cromatografía líquida acoplada a espectrometría de masas en tándem con fuente de ionización por electrospray (ESI).

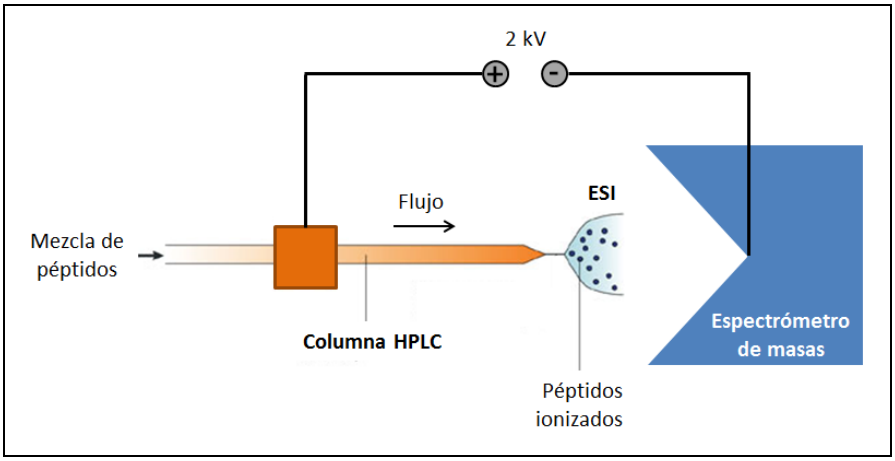

Figura 14. Esquema de cromatografía líquida acoplada a espectrometría de masas.

3.4 Cuantificación

Existen diferentes estrategias en proteómica cuantitativa, las más tradicionales se basan en medidas de intensidad de bandas de proteínas separadas en geles, mientras que las más recientes se basan en el uso de la espectrometría de masas y destacan por su precisión y exactitud.

3.4.1 Cuantificación por electroforesis en gel

Los métodos clásicos de cuantificación se basan el uso de electroforesis en gel, unidimensional o bidimensional, en donde la separación y cuantificación de proteínas se lleva a cabo mediante la medida de las intensidades de bandas obtenidas. La medida de la densidad de las bandas se realiza mediante escáner densitométrico, medidas de espectrometría o fluorescencia, o análisis de imagen por ordenador. De este modo, se generan imágenes digitales de los geles consistentes en píxeles, cada uno de los cuales tiene un valor de brillo o intensidad de señal que a través de programas especiales permiten la cuantificación de las proteínas. Este tipo de cuantificación suele ser relativa a un patrón interno añadido a la muestra (por ejemplo, albúmina de suero bovino) o comparando la densidad de unas bandas respecto a otras para evaluar la

abundancia relativa de proteínas en un gel o entre varios de ellos. Esta técnica aporta buena sensibilidad y linealidad, sin embargo no revela la identidad de las proteínas subyacentes, presenta baja especificidad y limitado rango dinámico, y aporta resultados menos precisos en proteómica cuantitativa que los métodos basados en espectrometría de masas, principalmente cuando existe solapamiento de bandas (Bendixen, 2005; Bantscheff et al., 2007).

La medida de intensidades de bandas mediante densitometría ha sido utilizada para la cuantificación relativa de proteínas miofibrilares y sarcoplásmicas a lo largo del proceso de elaboración del jamón (Larrea et al., 2006). Sin embargo, debido a las limitaciones comentadas, es frecuente la combinación de la electroforesis en gel, como medio de separación, con la espectrometría de masas para la identificación y cuantificación de proteínas, técnica que permite una mayor robustez, sensibilidad y rango dinámico. Así, existen diversos estudios que evalúan los cambios en la expresión de proteínas cárnicas y su abundancia entre muestras mediante electroforesis en gel seguida de espectrometría de masas en tándem, basada principalmente en el uso de métodos de marcaje con isótopos para la cuantificación relativa de las proteínas (Bouley et al., 2004; Doherty et al., 2004; Bjarnadóttir et al., 2012).

3.4.2 Cuantificación por espectrometría de masas

La espectrometría de masas ofrece una alta sensibilidad, precisión y fiabilidad cuando se utiliza con fines cuantitativos, principalmente gracias a la alta resolución y velocidad de escaneo, exactitud, y buena precisión de masa de los instrumentos modernos. Como ya se ha comentado, la intensidad de la señal obtenida en un espectro de masas no siempre puede relacionarse directamente con la cantidad de analito presente en la muestra, debido principalmente a la variabilidad en cuanto a eficiencia de la ionización. Por ello, el desarrollo de técnicas modernas basadas en espectrometría de masas ha sido de gran importancia y utilidad en la cuantificación de mezclas complejas de proteínas. La cuantificación puede ser relativa o absoluta en función del objetivo a alcanzar, aunque la primera suele ser la más frecuente.

3.4.2.1 Cuantificación relativa

Este tipo de cuantificación, también llamada comparativa, se basa en estudiar diferencias en la abundancia de proteínas entre diferentes muestras, dando como resultado una proporción cuantitativa o cambio relativo entre ellas. Para ello, el marcaje con isótopos estables constituye el método más preciso, los cuales pueden ser incorporados a las proteínas o péptidos de forma metabólica, química o enzimática. Todas estas metodologías se basan en que dos formas de una molécula, que difieren sólo en una sustitución con un isótopo estable (no radioactivo), se comportan idénticamente durante un análisis por espectrometría de masas, y solamente existirá una diferencia de masa entre ellas. Así pues, la comparación de la intensidad de señal obtenida para cada tipo de molécula (el control con el isótopo ligero presente de forma natural, y la muestra con el isótopo pesado que ha sido incorporado) permite determinar la proporción entre ellas y por tanto, la cuantificación relativa (Steen y Mann, 2004). Sin embargo, los métodos con marcaje precisan de procedimientos experimentales complejos y presentan varias limitaciones como son el alto coste de los isótopos utilizados, el restringido número de muestras que pueden ser analizadas y de marcadores disponibles que impide el uso de esta técnica en todos los tipos de muestras, y la necesidad de una gran cantidad de muestra inicial especialmente preparada para el análisis (Aebersold y Mann, 2003; Bantscheff et al., 2007; Neilson et al., 2011).

Por otro lado, existen métodos sin marcaje, conocidos también como "label-free", que son algo menos precisos que los anteriores pero permiten de forma simple y con gran fiabilidad la cuantificación relativa de proteínas. Además, son métodos versátiles y económicos ya que no requieren marcadores, y permiten el análisis de un número ilimitado de muestras minimizando el número de pasos de preparación de las mismas (Zhu et al., 2010; Neilson et al., 2011). Por todo ello, su uso se está extendiendo en la actualidad de manera muy significativa frente a los métodos basados en marcaje isotópico, para comparar perfiles proteicos entre muestras.

Un esquema del procedimiento típico utilizado para cuantificación relativa de proteínas mediante métodos con marcaje y sin marcaje se muestra en la **Figura 15**.

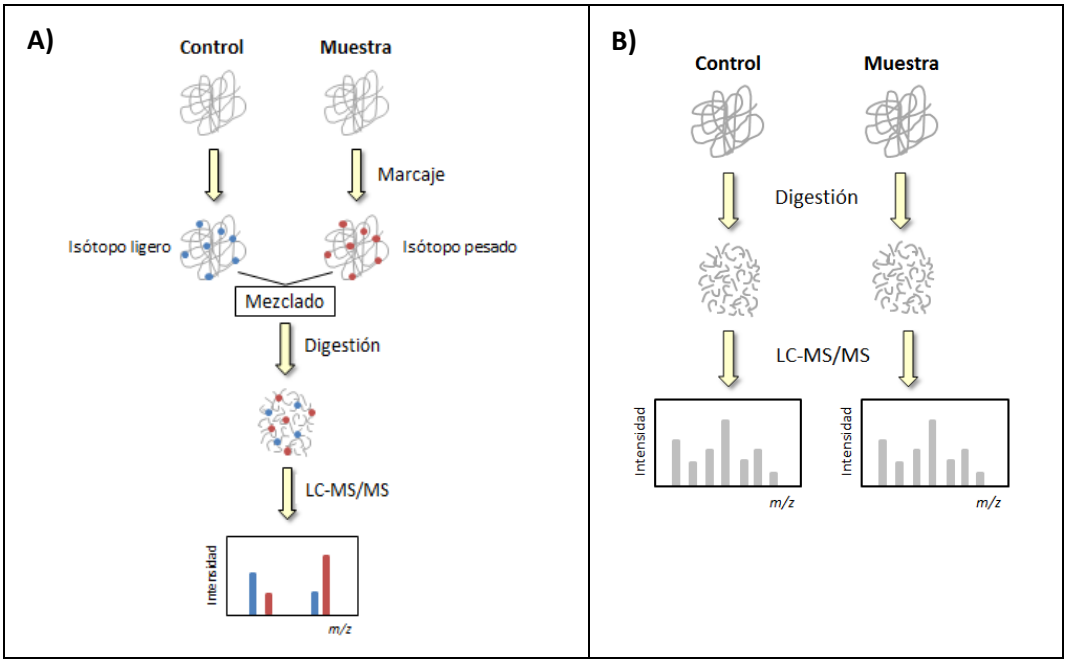

Figura 15. Esquema general de proteómica comparativa mediante A) método con marcaje y B) método sin marcaje. (Adaptación de Zhu et al., 2010).

3.4.2.1.1 Métodos con marcaje

Marcaje metabólico

Este tipo de marcaje se denomina SILAC (marcaje isotópico estable con aminoácidos en cultivo celular) y se basa en la incorporación *in vivo* de isótopos estables en todas las proteínas de un organismo. Es un método simple y preciso en el que uno o varios aminoácidos marcados se introducen en el medio de cultivo de células para que éstas lo incorporen en su proteoma. Posteriormente, se mezcla la misma cantidad de células procedente de los medios a comparar, se extraen y digieren las proteínas, y se analiza por espectrometría de masas (ver **Figura 16**). Cada par de péptidos estará separado en el espectro por la

diferencia de masa introducida, la cual es variable en función del aminoácido marcado, de modo que las intensidades de señal de los péptidos aportarán la información cuantitativa (Ong et al., 2002).

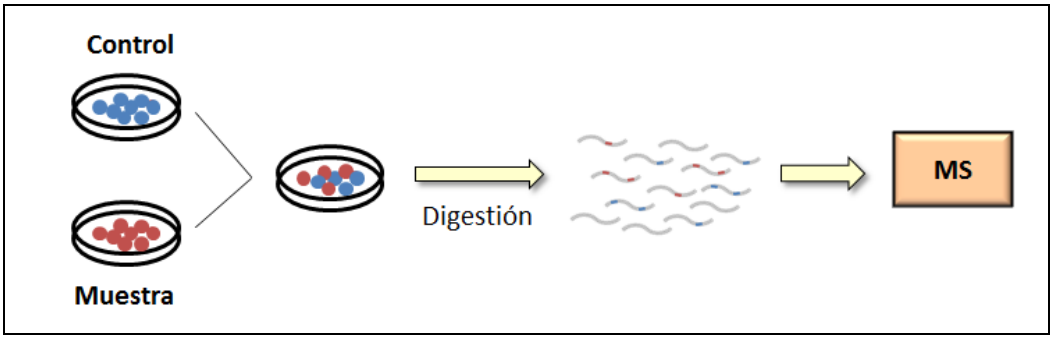

Figura 16. Esquema de cuantificación por marcaje metabólico (SILAC).

Marcaje químico

Este método se basa en el uso de reactivos químicos marcados isotópicamente que se unen covalentemente a las proteínas o péptidos a través de las cadenas laterales de determinados aminoácidos o aminas primarias. Uno de los métodos de marcaje químico utilizado de manera más frecuente es el llamado ICAT (etiqueta por afinidad codificada por isótopo), el cual se compone de un grupo reactivo de unión específica a residuos de cisteína, un marcador de biotina y un enlazador que contiene el isótopo ligero o pesado. El reactivo ICAT se une a los residuos de cisteína de las proteínas, las muestras se mezclan y digieren, y los péptidos resultantes son purificados por cromatografía de afinidad con avidina y analizados por espectrometría de masas (ver **Figura 17**). La cantidad relativa de cada proteína se determina midiendo la intensidad de cada pico de masa, que difiere entre la muestra marcada y la muestra no marcada una o más veces la diferencia de masa codificada en el reactivo. Este método presenta el inconveniente de que sólo puede aplicarse a proteínas que contienen residuos de cisteína (Gygi et al., 1999; Tao y Aebersold, 2003).

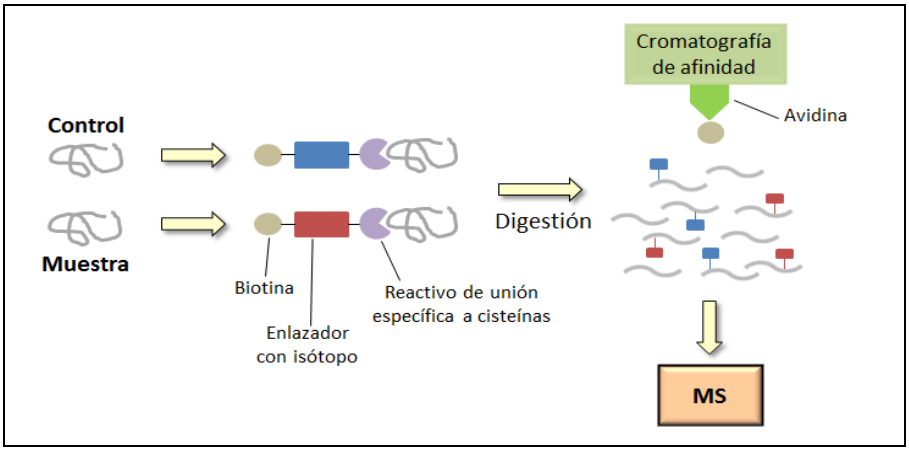

Figura 17. Esquema de cuantificación por marcaje químico ICAT.

Más recientemente, se ha desarrollado una alternativa al marcaje con ICAT basada en el reactivo iTRAQ (etiquetas isobáricas para cuantificación relativa y absoluta). Esta técnica realiza el marcaje mediante isótopos isobáricos, que tienen una estructura química semejante pero difieren en la composición del isótopo estable. Existen kits con cuatro y ocho formas isotópicamente diferentes de reactivos iTRAQ, los cuales reaccionan con el grupo amino de las lisinas y con el extremo N-terminal de los péptidos. A diferencia de los otros métodos, en éste el marcaje se realiza a nivel de péptido, de forma que las proteínas de distintas muestras son digeridas y los péptidos resultantes son entonces marcados con los diferentes reactivos iTRAQ. Tras la reacción química, las distintas muestras se mezclan y se analiza por espectrometría de masas en tándem (ver **Figura 18**). En el espectro obtenido, los péptidos marcados con cualquiera de los reactivos iTRAQ tienen el mismo peso molecular en el espectro de masas y por tanto, se fragmentan a la vez. Durante la fragmentación, la rotura del reactivo iTRAQ libera iones monocargados específicos de cada una de las muestras, permitiendo la cuantificación relativa en función de la intensidad de los mismos, y de forma simultánea para cuatro u ocho muestras en función del kit de reactivos químicos utilizado (Chee et al., 2007; Wiese et al., 2007). Este tipo de marcaje químico también puede ser utilizado para cuantificación absoluta de proteínas.

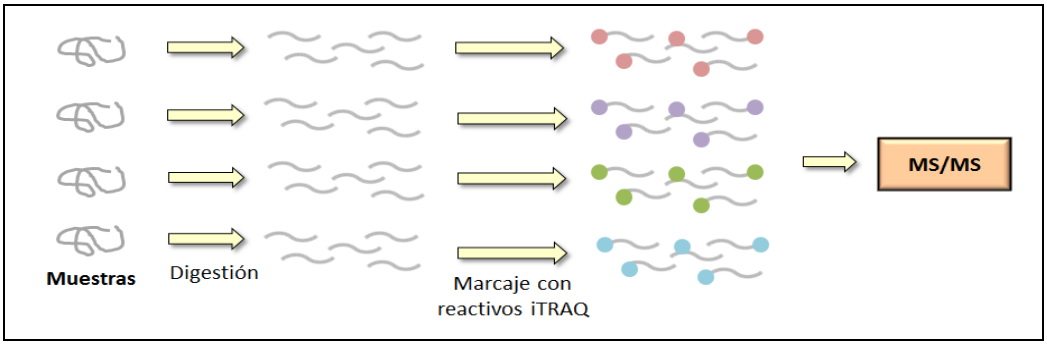

Figura 18. Esquema de cuantificación por marcaje químico iTRAQ para la cuantificación relativa de cuatro muestras.

Marcaje enzimático

Este método se conoce también como marcaje ^{18}O proteolítico, y se basa en el marcaje con isótopos estables durante la digestión enzimática (ver **Figura 19**). Una muestra se digiere en agua normal, normalmente con la enzima tripsina, mientras en otra muestra la digestión se realiza en agua enriquecida con ^{18}O. De este modo, los péptidos trípticos incorporan normalmente dos átomos del isótopo ^{18}O en sus extremos C-terminal. Las dos muestras se mezclan y analizan por espectrometría de masas, de modo que la cuantificación se realiza a partir de la medida de las intensidades de pico de los péptidos marcados y sin marcar. La diferencia de masas entre los pares de péptidos es de solamente 4 Daltons, lo que hace más difícil la cuantificación que con los anteriores métodos de marcaje descritos. Además, la incorporación de ^{18}O puede ser incompleta, lo que dificulta el análisis de datos (Yao et al., 2001).

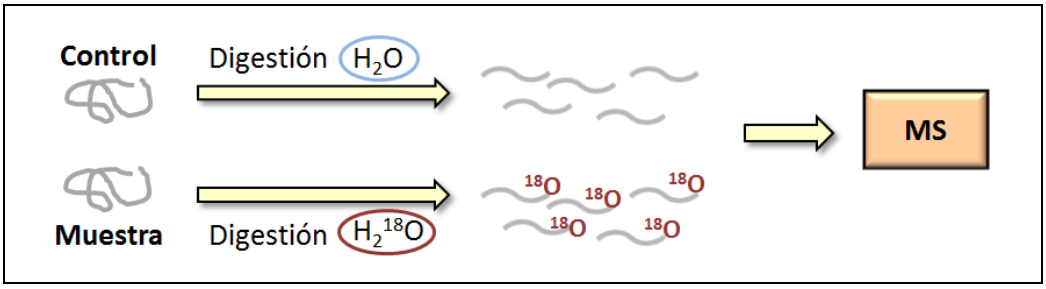

Figura 19. Esquema de cuantificación por marcaje enzimático.

3.4.2.1.2 Métodos sin marcaje (“label-free”)

En esta metodología, las muestras cuyas proteínas han sido previamente digeridas, son analizadas por espectrometría de masas. Este tipo de análisis cuantitativo incluye habitualmente las siguientes etapas: 1) preparación de la muestra, en la que se realiza la extracción de las proteínas, reducción, alquilación y digestión enzimática; 2) análisis por cromatografía líquida acoplada a espectrometría de masas en tándem; y 3) análisis de los datos incluyendo la identificación de péptidos y proteínas, la cuantificación y el análisis estadístico.

Normalmente, las técnicas de cuantificación “label-free” implican el uso de espectrometría de masas en tándem con una fuente de ionización por electrospray. De esta forma, se obtiene una alta resolución, precisión, reproducibilidad y linealidad, así como una gran exactitud y fiabilidad de los datos obtenidos a partir de proteomas complejos. Sin embargo, hay que considerar que para lograr una cuantificación que se ajuste lo máximo posible a la realidad, la preparación de la muestra, su inyección el sistema LC-MS/MS y la separación cromatográfica deben ser altamente reproducibles. De hecho, se necesitan estrategias de normalización de los datos para controlar posibles errores introducidos a lo largo del proceso experimental, utilizando estos normalizadores para la mejora del alineamiento de los picos obtenidos en el espectro, corrigiendo la posible variabilidad y falta de exactitud del análisis. Además, debido a la gran complejidad que presenta el proteoma de la muestra y la enorme cantidad de datos recogidos tras el análisis por LC-MS/MS, se requiere que los datos sean analizados de manera automática mediante programas de software especializado (Zhu et al., 2010).

La cuantificación puede llevarse a cabo mediante dos estrategias diferenciadas, que son la medida de la intensidad de señal basada en el espectro del ion precursor o el conteo espectral, las cuales se detallan a continuación y cuyos esquemas de funcionamiento pueden observarse en la **Figura 20**.

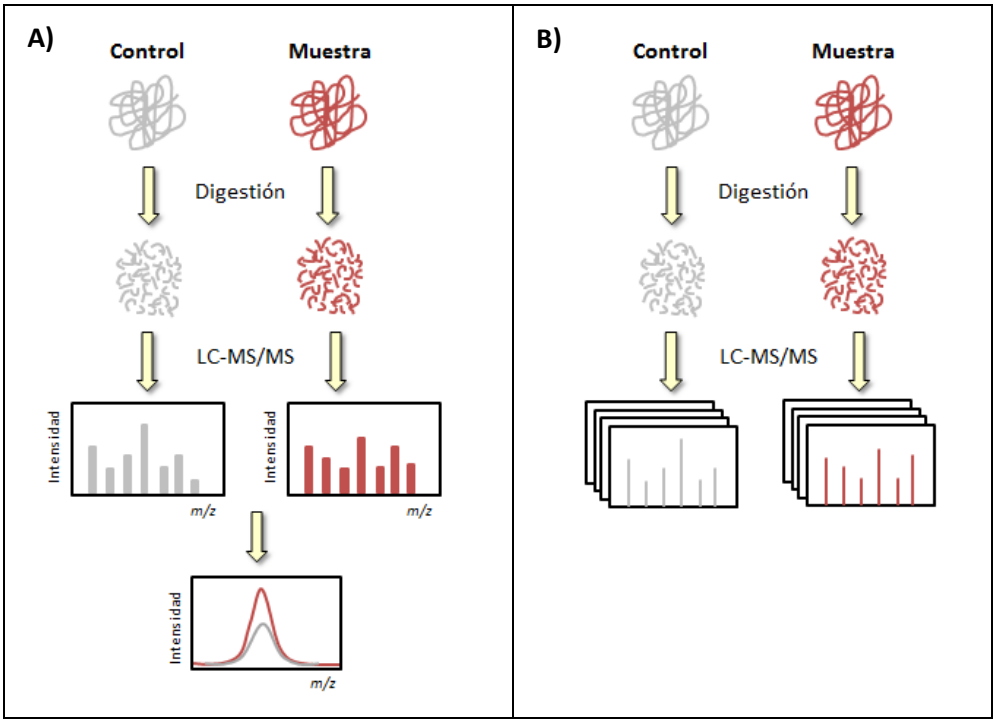

Figura 20. Esquema de cuantificación por métodos sin marcaje por A) intensidad de señal y B) conteo espectral.

Intensidad de señal del ion precursor

Se basa en el alineamiento e integración de los picos cromatográficos para cada péptido ionizado, obtenidos en los cromatogramas de iones extraídos (XICs) tras un análisis de LC-MS/MS, de modo que la medida de la abundancia de sus iones permite determinar la concentración del péptido medido. Así pues, las áreas de los picos de los péptidos se correlacionan linealmente con la cantidad de proteína a partir de la cual derivan dichos péptidos (Wiener et al., 2004; Zhu et al, 2010).

Este tipo de metodología permite evaluar la abundancia relativa de proteínas entre muestras complejas de manera muy precisa, con un amplio rango dinámico, e incluso si existen numerosos iones de péptidos solapados en el espectro de masas. Además, requiere pequeños volúmenes de muestra para el

análisis y no tiene límites en cuanto al número de muestras o condiciones que pueden ser comparadas.

Conteo espectral

Este método consiste en el contaje del número de espectros asignados a los péptidos más abundantes de una proteína, tras los análisis por MS/MS, como medida de su abundancia relativa. Esto es posible porque un aumento en la abundancia de una proteína conlleva normalmente un aumento del número de péptidos trípticos generados, y por tanto, una mayor cobertura de la secuencia proteica, un mayor número de péptidos identificados y un aumento del número de espectros MS/MS totales obtenidos para cada proteína (Liu et al., 2004). Este método permite una cuantificación fiable y de forma simple y reproducible, pero la linealidad de respuesta y precisión son algo menores que utilizando la anterior metodología de intensidad de señal, debido a que la dinámica de exclusión de iones utilizada para la fragmentación de los mismos va en detrimento con una cuantificación exacta. A pesar de ello, el conteo espectral es muy útil para estimar la cantidad relativa de proteínas en una muestra (Neilson et al., 2011) y, como se verá más adelante, también puede utilizarse para cuantificar proteínas de manera absoluta.

3.4.2.2 Cuantificación absoluta

Consiste en determinar la concentración o cantidad absoluta de una proteína en una muestra, lo que aporta más información que la cuantificación relativa y permite la comparación entre conjuntos de datos sin necesidad de que posean la misma muestra de referencia entre ellos. Especialmente en este caso, la etapa de digestión enzimática es crítica, ya que la falta de escisiones de la enzima sobre el péptido resultará en una disminución no real de las cantidades obtenidas en la cuantificación del mismo. La cuantificación absoluta, al igual que la relativa, puede realizarse mediante métodos con o sin marcaje.

3.4.2.2.1 Métodos con marcaje

Uno de los métodos más frecuentes consiste en sintetizar péptidos de forma química marcados isotópicamente y añadirlos en una cantidad determinada en la muestra para ser usados como patrones internos. Son conocidos como péptidos para cuantificación absoluta (AQUA), y consisten en péptidos idénticos a aquellos generados por proteolisis pero con una ligera diferencia de masa, que pueden añadirse a la muestra antes de que ésta sea sometida a digestión enzimática o a la mezcla de péptidos, y que son usados principalmente para la cuantificación de pequeñas moléculas (Steen y Mann, 2004). El análisis se realiza comúnmente mediante SRM (monitorización de reacciones selectivas), que consiste en trabajar con espectrometría de masas en tándem seleccionando una única masa (*m/z*) del espectro que corresponde a un fragmento iónico del péptido a cuantificar (Kondrat et al., 1978). Como resultado del análisis, los picos del espectro están separados una determinada *m/z* correspondiente al marcaje y la cuantificación se realiza a partir de la intensidad de señal de los picos obtenidos y de la cantidad conocida de péptido marcado añadido (Gerber et al., 2003; Lange et al., 2008).

El método SRM se denomina MRM (monitorización de reacciones múltiples) cuando se aplica a múltiples iones fragmento de uno o más iones precursores (Kondrat et al., 1978). Habitualmente el análisis se lleva a cabo en un triple cuadrupolo (QQQ) que está acoplado a un sistema HPLC conectado en línea a una fuente de ionización por electrospray y que opera en modo SRM. El proceso consiste en la selección del ion precursor (Q1), fragmentación (Q2), selección del ion fragmento de interés (Q3) y registro de la señal en el detector (ver **Figura 21**). De esta forma, se permite un rápido y continuo monitoreo de los iones de interés con un menor límite de detección que utilizando un análisis completo MS/MS (Ludwing y Aebersold, 2014).

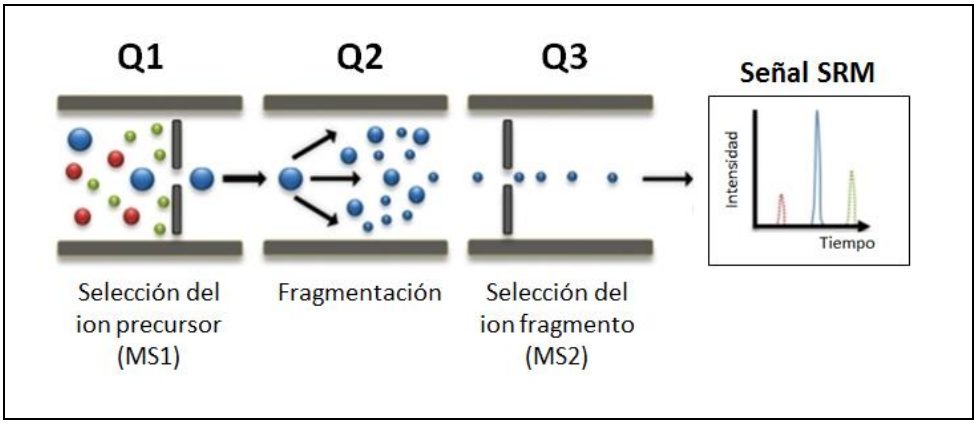

Figura 21. Esquema de la monitorización de reacciones selectivas (SRM).

3.4.2.2.2 Métodos sin marcaje ("label-free")

El índice de abundancia de proteína (PAI) se define como el número de péptidos observados en el análisis dividido por el número de péptidos trípticos observados para cada proteína en un rango de masas dado del espectrómetro de masas (Rappsilber et al., 2002). Este índice fue exponencialmente modificado para dar el índice emPAI, que es la forma exponencial del PAI menos uno, y el cual es directamente proporcional al contenido absoluto de proteína en la muestra. Esta técnica permite obtener una aproximación precisa de la cantidad absoluta de proteínas en un análisis a gran escala, pero hay que tener en cuenta que la correlación entre este índice y la abundancia de proteína disminuye conforme lo hace la resolución del espectrómetro de masas utilizado, y que la presencia de proteínas muy abundantes en la muestra reduce la sensibilidad cuantitativa del análisis (Ishihama et al., 2005).

Por otro lado, la técnica de expresión de proteína absoluta (APEX) consiste en una modificación del contaje espectral de modo que tiene en cuenta el número de péptidos observados en el espectro de masas para una proteína y la probabilidad de los péptidos de ser detectados por el instrumento. De esta forma y mediante la introducción de factores de corrección para mejorar la precisión de la cuantificación, APEX permite estimar la cantidad absoluta de una proteína al multiplicar el factor de abundancia relativa por una estimación, en

función de los péptidos esperados, de la concentración total de la proteína. El inconveniente que presenta esta técnica es que requiere resultados previos de MS sobre el proteoma estudiado para conocer los factores de corrección a aplicar en la cuantificación (Lu et al., 2007).

3.5 Análisis de datos

El análisis de datos es esencial tanto en proteómica cualitativa como cuantitativa, ya que los datos obtenidos tras un experimento de espectrometría de masas en tándem consisten en una gran cantidad de iones y sus espectros de masas asociados, de los cuales debe extraerse la información para el posterior análisis de resultados. Para ello, se lleva a cabo el procesado de los espectros de masas y la transformación de los datos en una lista de picos, la cual es una forma elaborada de los datos que puede contener los valores *m/z* observados, la intensidad, o el estado de carga para cada uno de los iones detectados y posteriormente fragmentados por MS/MS, entre otros.

En el caso de espectros de MS, la identificación de péptidos y proteínas se lleva a cabo por huella peptídica a partir de bases de datos. Como ya se ha explicado anteriormente, la proteína se digiere con una enzima específica y el conjunto de péptidos generados, que es característico para cada proteína, se analiza por espectrometría de masas para obtener sus masas moleculares. Estos valores de masas se comparan con las digestiones teóricas existentes en bases de datos para su identificación. Esta técnica es útil para proteínas aisladas, ya que a medida que aumenta la complejidad de la muestra se hace más complicada la identificación.

Para la interpretación de los espectros de MS/MS existen diversas estrategias:

- Secuenciación *de novo*: en esta aproximación se obtienen las secuencias de los péptidos directamente a partir de la información del espectro de fragmentación, sin recurrir a bases de datos. Se usa para la identificación de proteínas de organismos que no están secuenciados y por tanto no incluidos en bases de datos.

- Búsqueda en bases de datos: los espectros de MS/MS obtenidos experimentalmente se correlacionan con los espectros de MS/MS teóricos contenidos en las bases de datos. Los iones fragmentos de un péptido son característicos de su secuencia, de modo que la búsqueda de un péptido idéntico en las bases de datos permite identificar la proteína correspondiente. Puede seleccionarse la enzima específica utilizada para la digestión y generación de péptidos, o puede utilizarse en el caso de péptidos generados por digestión no controlada para identificar sus secuencias.
- Etiqueta de secuencia: se basa en la extracción de una pequeña secuencia parcial (de entre tres y cinco aminoácidos) del espectro MS/MS de uno o varios péptidos, y el posterior rastreo en bases de datos en busca de la proteína que comparta tales secuencias específicas. Esta estrategia es útil en los espectros de péptidos trípticos y en organismos con proteomas limitados e incluidos en bases de datos, ya que a medida que el proteoma es más complejo disminuye la probabilidad de asignar correctamente un péptido a una determinada proteína.

El procedimiento más frecuente para la identificación de péptidos es la búsqueda en bases de datos, para lo que existen diversas herramientas bioinformáticas. Entre ellas, MASCOT y SEQUEST son los motores de búsqueda comerciales más utilizados, pero existen otros como X!Tandem, OMSSA y ProbID que son de libre distribución. Estos programas utilizan mayoritariamente NCBInr y Uniprot (que consiste en SwissProt y su suplemento TrEMBL) como bases de datos de proteínas, aunque existen múltiples de ellas disponibles (Eng et al., 1994; Perkins et al., 1999). Así, estas herramientas bioinformáticas permiten la interpretación de los espectros de fragmentación de un péptido al compararlos con patrones teóricos de fragmentación de péptidos contenidos en bases de datos proteicas, tras la optimización de los parámetros de búsqueda en base a los utilizados en el ensayo. Como resultado, se obtiene una lista de péptidos clasificados en función del nivel de coincidencia entre los espectros teóricos y los observados experimentalmente, y es este método de calificación el que principalmente diferencia entre cada uno de los motores de búsqueda. Tras la identificación de

las secuencias peptídicas, cada péptido es asignado a una proteína de origen, dando un valor que indica el grado de confianza de esa asignación. De hecho, a mayor número de péptidos sean identificados para una determinada proteína, la confianza de que correspondan a esa proteína será mayor.

En el caso de proteómica cuantitativa, existen diferentes herramientas bioinformáticas en función del método de cuantificación utilizado, con o sin marcaje, y optimizando la metodología en función de las características del ensayo realizado. Algunos de los múltiples programas disponibles para este fin son MASCOT, VEMS, MSQuant, Abacus o ProteinPilot.

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II. Objetivos

II. Objetivos

El objetivo principal de la presente tesis doctoral es el estudio de los péptidos generados de manera natural durante el proceso de elaboración del jamón curado y su caracterización como marcadores de calidad. El planteamiento experimental se ha basado en técnicas proteómicas, lo que permite profundizar en el conocimiento de los procesos bioquímicos que tienen lugar durante el proceso, y más concretamente en la evaluación de péptidos para su uso como marcadores del tiempo de curado y en la identificación de péptidos bioactivos presentes en el jamón.

Para conseguir este objetivo general se han planteado los siguientes objetivos parciales:

- Identificación de los péptidos generados a partir de la degradación de la proteína 3 de unión al dominio LIM, proteína ribosomal ubiquitina-60S y titina a diferentes tiempos del proceso del jamón curado mediante técnicas proteómicas, y evaluación de su potencial como péptidos marcadores del tiempo de curado del jamón.
- Estudio de la oxidación de metionina en péptidos generados a partir de la degradación de las principales proteínas miofibrilares del jamón curado mediante ESI-LC-MS/MS.
- Optimización de una metodología de espectrometría de masas sin marcaje ("label-free") para la cuantificación relativa de proteínas como paso previo a la cuantificación de péptidos y evaluación de cambios en la abundancia de las principales proteínas sarcoplásmicas a lo largo del proceso de elaboración del jamón curado.
- Identificación de péptidos del jamón curado con actividad inhibidora de la enzima convertidora de angiotensina I (ECA), medida de su efectividad mediante simulación del transporte intestinal a través de células Caco-2 y de su posible degradación por las peptidasas intestinales, así como la evaluación de su potencial efecto antihipertensivo.

III. Resultados

Capítulo 1

Degradation of LIM domain-binding protein three during processing of Spanish dry-cured ham

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Degradation of LIM domain-binding protein three during processing of Spanish dry-cured ham

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Abstract

Extensive proteolysis takes place during the processing of dry-cured ham due to the action of muscle peptidases. The aim of this work was to study the degradation of LIM domain binding protein 3 (LDB3), which is located at the Z-lines of the sarcomere, at different times during the Spanish dry-cured ham processing (2, 3.5, 5, 6.5, and 9 months). A total of 107 peptides have been identified by mass spectrometry, most of them generated from the first region of the protein sequence (position 1–90) providing evidence for the complexity and variability of proteolytic reactions throughout the whole process of dry-curing. Methionine oxidation has been observed in several peptides by the end of the process. The potential of some of the identified peptides to be used as biomarkers of dry-cured ham processing has also been considered.

Keywords: Dry-cured ham; LIM domain-binding protein 3; Peptides; Proteolysis; Mass spectrometry; Biomarker; Peptide oxidation.

INTRODUCTION

Spanish dry-cured ham is a high-quality product for its organoleptic and nutritional characteristics. During the dry-curing process of ham occurs a series of biochemical reactions among which proteolysis of muscle proteins is one of the most important. Peptides and free amino acids are generated in large amounts from the progressive enzymatic degradation of sarcoplasmic and myofibrillar proteins, contributing to flavour, texture, and final quality of dry-cured ham (Aristoy & Toldrá, 1995; Toldrá & Flores, 1998; Lametsch et al., 2003).

LIM domain binding protein 3 (LDB3), also called Z-band Alternatively Spliced PDZ-motif protein (ZASP) or protein cypher, is located in the sarcomere and it is essential for maintaining Z line structure and muscle integrity (Zhou et al., 2001). Although it is a minor component of the Z line (about 1 per 400 α-actinin), this protein is very important for myofibrillar development and mechanotransduction during muscle contraction (Leung, Hitchen, Ward, Messer, & Marston, 2013). Thus, LDB3 may act as an adapter in signaling of the striated muscle, as it binds protein kinase C via its three C-terminal LIM domains to the cytoskeleton, and it may also interact via its N-terminal PDZ domain with α-actinin-2, which helps anchor the myofibrillar actin filaments providing mechanical strength to the Z-lines (Klaavuniemi, Kelloniemi, & Ylänne, 2004; Luther, 2009).

Enzymes involved in protein changes are muscle endopeptidases such as cathepsins and calpains, which hydrolyse sarcoplasmic and myofibrillar proteins giving rise to large polypeptides, and exopeptidases like aminopeptidases, carboxypeptidases, peptidyl peptidases and peptidases, which degrade polypeptides to smaller peptides and free amino acids (Toldrá, Aristoy & Flores, 2000; Toldrá & Flores, 1998).

Protein degradation as well as the generation of several peptides and free amino acids during dry-cured ham processing have been described in some studies, in which electrophoretic techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC) have been used to separate the protein fragments for their subsequent identification (Rodríguez-Nuñez, Aristoy, & Toldrá, 1995;

Hansen-Møller, Hinrichsen, & Jacobsen, 1997; Sentandreu et al., 2003). However, the identification of naturally generated peptides has become possible only through the use of the latest generation proteomic techniques such as tandem mass spectrometry. In fact, recent proteomic studies have identified peptides derived from myofibrillar proteins such as actin, myosin light chain, titin, or troponin T (Luccia et al., 2005; Sentandreu et al., 2007; Mora et al., 2009b; Mora, Sentandreu, & Toldrá, 2010), and sarcoplasmic proteins like creatine kinase, myoglobin or glycolytic enzymes (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009a; Mora & Toldrá, 2012; Mora, Valero, Sánchez del Pino, Sentandreu, & Toldrá, 2011). Some studies have investigated how some muscle compounds such as the ratio hypoxanthine/inosine could be possible markers of the ripening time (Escudero, Mora, Aristoy, & Toldrá, 2011). However, to the best of our knowledge, there are no studies based on the evolution of naturally generated peptides from proteins degradation during the dry-curing process to date.

The main goal of this study was the proteomic characterization of LDB3 protein to identify the peptides naturally generated at different times (2, 3.5, 5, 6.5, and 9 months) during the dry-cured ham processing in order to study its evolution and describe some of the proteolytic changes taking place during the process. Additionally, the potential of some of the identified peptides as biomarkers for processing time has been also studied.

MATERIALS AND METHODS

Dry-cured ham preparation

This study was carried out in triplicate using raw hams from 6 months old pigs (Landrace x Large White) for the production of Spanish dry-cured ham. Hams were directly provided under refrigeration from the slaughterhouse (Vaquero Meat Industry, Madrid, Spain) at 24 hour post-mortem. Once received, hams were bled and prepared in our pilot plant according to traditional procedures consisting on the pre-salting stage using a mixture of salt, nitrate and nitrite for 30 min; the salting period where hams were entirely buried in salt, piled up, and placed in a cold room for 10-12 days at 2-4 ºC and 90-95% relative humidity; the

post-salting stage where hams were kept for 60 days at 4-5 ºC and 75-85% relative humidity; and finally the ripening-drying period at temperatures increasing from 5 ºC to 14-20 ºC and relative humidity decreasing to 70%. The total length of the dry-curing process was 9 months.

Test samples were selected at different times of processing: 2 months (at the 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).

Dry-cured ham extraction and deproteinisation

A total of 50 g of *Biceps femoris* muscle coming from each sampling time of the processed Spanish dry-cured hams were minced and homogenised with 200 mL of 0.01 N HCl for 8 minutes in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged at 4 ºC and 12000*g* for 20 min, filtered through glass wool and then the solution was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 ºC for 20 hours. Afterwards, the sample was centrifuged again at 4 ºC and 12000*g* for 10 min and the supernatant was dried in a rotatory evaporator. Finally, the dried deproteinised extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA) and stored at -20 ºC until use.

Size-exclusion chromatography

To fractionate deproteinised cured ham extracts according to molecular mass, a 5 mL aliquot of each extract was subjected to size-exclusion chromatography. A Sephadex G25 column (2.5 x 65 cm, Amersham Biosciencies, Uppsala, Sweden), previously equilibrated with 0.01 N HCl and filtered through a 0.45 µm nylon membrane filter (Millipore), was employed for this purpose. The separation was performed using 0.01 N HCl as mobile phase, at a flow rate of 15 mL/h and 4 ºC. Fractions of 5 mL were collected using an automatic fraction collector and further monitored by ultraviolet absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England).

Fractions corresponding to elution volumes from 125 to 160 mL were pooled together and aliquots of 100 µL were lyophilised.

Peptide identification by nanoliquid chromatography and mass spectrometry in tandem (nLC-MS/MS)

The nanoLC-MS/MS analysis was performed with an Ultimate 3000 RSLC nano system from Dionex (Thermo Fisher Scientific Ltd, Leicestershire, UK), coupled to an AmaZon ETD ion-trap mass spectrometer equipped with a nanoelectrospray ionization source (Bruker Daltonik GmbH, Bremen, Germany).

Lyophilised samples were resuspended in 100 µL of H_2O with 0.1% of formic acid (FA). Ten microliters of the solution were taken and 10 µL of H_2O: ACN (98:2, v/v) with 0.1% (v/v) of FA were added. Sample was kept in cold for 30 min and centrifuged at 10000*g* for 15 min. 2 µL of the supernatant were injected into the LC-MS system through the autosampler. Samples were then preconcentrated on a Dionex Acclaim® PepMap 100 column (100 µm x 2 cm, C18, 5 µm, 100Å) (Dionex Corporation, LC Packings), at a flow rate of 4 µL/min and using 0.1% (v/v) FA as mobile phase. After 3 min of preconcentration, the trap column was automatically switched in-line onto a Dionex Acclaim® PepMap RSLC nano-column (75µm x 15cm, C18, 2µm, 100Å) (Dionex Corporation, LC Packings). The mobile phases consisted of solvent A, containing 0.1% (v/v) FA in water, and solvent B, containing 0.1% (v/v) FA in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 40% of solvent B over 70 min at a flow rate of 0.250 µL/min and running temperature of 30 ºC.

The column outlet was directly coupled to a nanoelectrospray ion source. The ion trap mass spectrometer was used in positive polarity mode. Typical ion spray voltage was in the range of 1.8-2.0 kV, and nitrogen was used as collision gas with a nebulizer pressure of 15 psi and a capillary temperature of 200 °C. The ion trap was used in MS2 in order to enhance scan mode resolution in the range from *m/z* 50 to 3000, at a speed of *m/z* 8100 per second. The first scan of MS scan was from *m/z* value 300 - 1500, with a maximum accumulation time of 50

ms and an average of 5. Other source parameters were optimised with a tryptic digest of BSA (bovine serum albumin) protein.

Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com). Oxidation of methionine was used as variable modifications. The identification of protein origin of peptides was done using UniProt and NCBInr protein databases, with a significance threshold $p < 0.05$, and a tolerance on the mass measurement of 100 ppm in MS mode and 0.3 Da for MS/MS ions.

RESULTS AND DISCUSSION

Each deproteinised sample extract was fractionated by size-exclusion chromatography, and fractions corresponding to the first peak eluted (a total of 7 fractions of 5 mL) were pooled together and lyophilised. The analysed fractions contained the highest size peptides that remained after protein precipitation with ethanol.

Fractions from each time of processing were analysed by nLC-MS/MS mass spectrometry for the identification of their peptidic content. A total of 107 peptides generated from the LDB3 protein have been identified at five different times of processing, as reported in **Tables 1-3**. These tables list the characterised amino acid sequences, the position of the identified peptides in the parent protein, amino acid residues preceding and following the sequence, the observed and calculated masses together with the charge states, and the process times when peptides have been identified.

All the peptide sequences identified in this work by using the protein database UniProt came according to this database from LIM domain-binding protein 3 of *Homo sapiens* species. All the identified peptides have been searched in BLAST and revealed 100% homology with a *Sus scrofa* uncharacterised protein. This is because LDB3 protein has not been characterised in porcine species, but it has in human and bovine (*Bos taurus*) species. So, **Figure 1** shows the sequence

alignment of LDB3 protein between *S. scrofa*, *H. sapiens* and *B. taurus* species. BLAST sequence similarity searches revealed 87% homology between LDB3 sequences of porcine and human (Uniprot database with accession numbers F1SEN8 and O75112, respectively). However, LDB3 has not been completely sequenced in bovine species (accession number Q3ZBC9 in Uniprot database), so *S. scrofa* and *B. taurus* only share a sequence fragment corresponding to the 42% of the full-length sequence of this protein in which BLAST revealed 93% homology between sequences of both species.

An extensive proteolysis has been reported during the processing of dry-cured ham and this comprises the action of endopeptidases and exopeptidases. Muscle endopeptidases are stable during the full process (Toldrá, Rico, & Flores, 1993), except calpains that are inactivated after the salting stage (Rosell & Toldrá, 1996), and cathepsin D whose activity disappears after 6 months of process (Toldrá et al., 1993). Regarding the activity of exopeptidases, aminopeptidases and dipeptidyl peptidases have shown good stability along the curing process (Sentandreu & Toldrá, 2001; Toldrá, Flores, & Sanz, 1997; Toldrá et al., 2000).

Figure 2 shows the LDB3 protein sequence and the action of muscle peptidases at each processing time: 2, 3.5, 5, 6.5 and 9 months. The sequence region comprised between 1 and 90 amino acid residues is the one showing the highest amount of cleavage sites probably due to a more intense action of the proteolytic enzymes in this section of the sequence. There are evidences about the role of aminopeptidases and carboxypeptidases in the generation of the peptides identified from position 1 to 90 of the LDB3 protein sequence and the release of free amino acids (**Table 1**). However, cathepsins would be responsible for the protein breakdown and the generation of most of the peptides identified in the remaining sequence (position 91-715) at all times of processing tested, because calpains do not remain active after the salting stage (Rosell & Toldrá, 1996).

Table 1. Peptides identified by nanoliquid chromatography and mass spectrometry in tandem from position 1 to 90 of the LIM domain-binding protein 3 in Spanish dry-cured ham (UniprotKB/TrEMBL protein database accession number F1SEN8).

Peptide	Position[a]	P_0[b]	Sequence	P_f[c]	Observed[d] (*m/z*)	Charge (+)	Calculated[e] (*m/z*)	Time of processing (months)[f] 2	3.5	5	6.5	9
1	4-22	Y	SVTLTGPGPWGFRLQGGKD	F	658.23	3	1972.02		x			
2	7-22	T	LTGPGPWGFRLQGGKD	F	562.49	3	1684.87		x			
3	4-19	Y	SVTLTGPGPWGFRLQG	G	836.75	2	1671.87		x			
4	7-19	T	LTGPGPWGFRLQG	G	693.40	2	1384.73		x			
5	4-17	Y	SVTLTGPGPWGFRL	Q	744.22	2	1486.79	x	x			
6	7-17	T	LTGPGPWGFRL	Q	600.73	2	1199.65	x	x	x	x	
7	7-15	T	LTGPGPWGF	R	466.12	2	930.46		x			
8	7-15	T	LTGPGPWGF	R	931.23	1	930.46	x	x			
9	7-12	T	LTGPGP	W	540.79	1	540.29		x			
10	11-23	P	GPWGFRLQGGKDF	N	488.84	3	1463.73	x				
11	23-53	D	FNMPLTISRITPGSKAAQSQLSQGDLVVAID	G	1086.60	3	3256.71	x	x	x		
12	24-53	F	NMPLTISRITPGSKAAQSQLSQGDLVVAID	G	1037.66	3	3109.64		x			
13	25-53	N	MPLTISRITPGSKAAQSQLSQGDLVVAID	G	999.39	3	2995.60		x			
14	27-53	P	LTISRITPGSKAAQSQLSQGDLVVAID	G	923.60	3	2767.51	x	x	x	x	x
15	28-53	L	TISRITPGSKAAQSQLSQGDLVVAID	G	885.56	3	2654.42		x			x
16	29-53	T	ISRITPGSKAAQSQLSQGDLVVAID	G	851.94	3	2553.38					x
17	31-53	S	RITPGSKAAQSQLSQGDLVVAID	G	785.24	3	2353.26		x	x	x	x
18	39-53	A	AQSQLSQGDLVVAID	G	772.21	2	1542.79		x	x	x	x
19	42-53	S	QLSQGDLVVAID	G	629.20	2	1256.66					x
20	44-53	L	SQGDLVVAID	G	1016.27	1	1015.52					x
21	47-53	G	DLVVAID	G	744.23	1	743.41			x		x
22	48-53	D	LVVAID	G	629.26	1	628.38					x
23	23-51	D	FNMPLTISRITPGSKAAQSQLSQGDLVVA	I	1010.75	3	3028.60	x	x			x
24	47-51	G	DLVVA	I	516.01	1	515.30					x
25	23-50	D	FNMPLTISRITPGSKAAQSQLSQGDLVV	A	986.59	3	2957.56		x			
26	23-48	D	FNMPLTISRITPGSKAAQSQLSQGDL	V	920.90	3	2759.43	x	x	x	x	x
27	23-47	D	FNMPLTISRITPGSKAAQSQLSQGD	L	882.87	3	2646.34	x	x	x	x	x
28	27-47	P	LTISRITPGSKAAQSQLSQGD	L	719.97	3	2157.14	x				x
29	31-47	S	RITPGSKAAQSQLSQGD	L	581.96	3	1742.89					x
30	23-46	D	FNMPLTISRITPGSKAAQSQLSQG	D	844.41	3	2531.32		x			
31	23-45	D	FNMPLTISRITPGSKAAQSQLSQ	G	825.63	3	2474.30	x	x	x		x
32	31-45	S	RITPGSKAAQSQLSQ	G	524.49	3	1570.84					x
33	23-43	D	FNMPLTISRITPGSKAAQSQL	S	753.91	3	2259.20	x		x	x	x
34	23-43	D	FNMPLTISRITPGSKAAQSQL	S	1130.81	2	2259.20		x			x
35	24-43	F	NMPLTISRITPGSKAAQSQL	S	705.25	3	2112.14					x

36	27-43	P	LTISRITPGSKAAQSQL	S	590.86	3	1770.00					x
37	31-43	S	RITPGSKAAQSQL	S	678.71	2	1355.75					x
38	32-43	R	ITPGSKAAQSQL	S	401.25	3	1199.65			x		
39	23-42	D	FNMPLTISRITPGSKAAQSQ	L	716.44	3	2146.12		x			x
40	23-40	D	FNMPLTISRITPGSKAAQ	S	644.52	3	1931.03			x		x
41	23-38	D	FNMPLTISRITPGSKA	A	578.21	3	1731.93	x	x	x	x	x
42	23-38	D	FNMPLTISRITPGSKA	A	866.76	2	1731.93	x	x	x		x
43	27-38	P	LTISRITPGSKA	A	415.15	3	1242.73					x
44	29-38	T	ISRITPGSKA	A	515.18	2	1028.60					x
45	31-38	S	RITPGSKA	A	415.17	2	828.48					x
46	23-37	D	FNMPLTISRITPGSK	A	554.64	3	1660.90					x
47	32-37	R	ITPGSK	A	301.34	2	601.34			x		
48	23-30	D	FNMPLTIS	R	461.62	2	921.46					x
49	23-30	D	FNMPLTIS	R	922.24	1	921.46	x	x	x	x	x
50	23-28	D	FNMPLT	I	722.20	1	721.35					x
51	23-27	D	FNMPL	T	621.18	1	620.30					x
52	28-58	L	TISRITPGSKAAQSQLSQGDLVVAIDGVNTD	T	1047.57	3	3140.63				x	
53	38-58	A	AQSQLSQGDLVVAIDGVNTD	T	677.36	3	2029.00		x			
54	42-57	L	SQGDLVVAIDGVNT	D	694.58	2	1386.70					x
55	48-63	D	LVVAIDGVNTDTMTHL	E	849.72	2	1697.87		x			
56	61-67	M	THLEAQN	K	406.61	2	811.38		x			
57	67-77	Q	NKIKSASYNLS	L	612.68	2	1223.65		x			
58	64-75	L	EAQNKIKSASYN	L	676.67	2	1351.67		x			
59	67-75	Q	NKIKSASYN	L	512.64	2	1023.53		x			
60	64-74	L	EAQNKIKSASY	N	619.68	2	1237.63		x			
61	67-74	Q	NKIKSASY	N	455.64	2	909.49	x	x	x	x	x
62	68-74	N	KIKSASY	N	398.64	2	795.45	x	x			x
63	68-74	N	KIKSASY	N	796.25	1	795.45		x			
64	78-90	S	LTLQKSKRPIPIS	T	494.10	3	1479.91				x	
65	78-86	S	LTLQKSKRP	I	535.72	2	1069.66		x			

[a] Position of the identified peptides inside the LDB3 sequence.
[b] Position of the amino acid residue preceding the peptide sequence.
[c] Position of the amino acid residue following the peptide sequence.
[d] Molecular ion mass observed in the nLC-MS/MS system calculated in Daltons.
[e] Calculated relative molecular mass in Daltons of the matched peptide.
[f] Peptides identified at each time of ham processing.

Table 2. Peptides identified by nanoliquid chromatography and mass spectrometry in tandem from position 91 to 715 of the LIM domain-binding protein 3 in Spanish dry-cured ham (UniprotKB/TrEMBL protein database accession number F1SEN8).

Peptide	Position[a]	P_0[b]	Sequence	P_1[c]	Observed[d] (*m/z*)	Charge (+)	Calculated[e] (*m/z*)	Time of processing (months)[f] 2	3.5	5	6.5	9
66	92-97	T	AVPPIQ	S	625.34	1	623.36		x			
67	112-128	L	DANSSLAAPSPHPEARA	S	845.71	2	1689.81				x	
68	112-116	L	DANSS	L	493.68	1	492.18	x				
69	126-140	E	ARASPGTPGTPEHGL	T	723.77	2	1446.72			x		
70	187-191	P	LGSSQ	P	490.82	1	490.24	x				
71	221-226	G	KASGAG	L	490.85	1	489.25				x	
72	212-225	A	QMYQMSLRGKASGA	G	509.76	3	1526.73	x				
73	226-230	A	GLLGG	S	417.12	1	415.24		x			
74	239-243	T	VDSAS	P	478.56	1	477.21		x			
75	262-271	D	EWARRSSNLQ	S	623.43	2	1245.62		x			
76	304-308	E	HAPVC	T	525.05	1	525.24			x		
77	311-319	S	QAATPLLPA	S	440.88	2	880.50			x		
78	320-324	A	SAQPP	A	498.51	1	498.24			x		
79	322-326	A	QPPAA	A	482.34	1	482.25			x		
80	346-351	T	ASAAAP	A	487.77	1	486.24				x	
81	361-366	R	PQASAY	S	637.25	1	635.29		x			
82	384-395	E	APAAPAPKPRVV	T	587.61	2	1172.70					x
83	387-391	A	APAPK	P	482.29	1	482.29	x				
84	385-390	A	PAAPAP	K	522.70	1	522.28		x			x
85	398-402	T	ASIRP	S	542.95	1	542.32	x				
86	406-410	Y	QPVPA	S	511.14	1	510.28	x				
87	408-413	P	VPASTY	S	637.12	1	636.31					x
88	435-444	Y	TPSPAPTYSP	S	340.20	3	1016.48	x		x	x	
89	446-450	S	PAPAY	T	518.14	1	517.25	x	x		x	
90	467-472	G	PAESAS	R	560.19	1	560.24					x
91	499-510	Q	SLPRGAPAYTPP	L	409.20	3	1225.65		x			
92	506-511	P	AYTPPL	Q	662.40	1	660.35				x	
93	508-513	Y	TPPLQG	P	612.78	1	611.33	x				
94	596-604	A	PVCAKCNTK	I	963.77	1	962.47		x			
95	665-669	V	EAGDK	F	519.97	1	518.23		x			

[a] Position of the identified peptides inside the LDB3 sequence.

[b] Position of the amino acid residue preceding the peptide sequence.

[c] Position of the amino acid residue following the peptide sequence.

[d] Molecular ion mass observed in the nLC-MS/MS system calculated in Daltons.

[e] Calculated relative molecular mass in Daltons of the matched peptide.

[f] Peptides identified at each time of ham processing.

Table 3. Peptides identified by nanoliquid chromatography and mass spectrometry in tandem from LIM domain-binding protein 3 in Spanish dry-cured ham with a methionine oxidation (UniprotKB/TrEMBL protein database accession number F1SEN8).

Peptide	Position[a]	P_0[b]	Sequence	P_f[c]	Observed[d] (*m/z*)	Charge (+)	Calculated[e] (*m/z*)	Time of processing (months)[f] 2	3.5	5	6.5	9
96	23-53	D	FNMPLTISRITPGSKAAQSQLSQGDLVVAID	G	1091.71	3	3272.71		x	x		x
97	23-47	D	FNMPLTISRITPGSKAAQSQLSQGD	L	888.30	3	2662.34					x
98	23-45	D	FNMPLTISRITPGSKAAQSQLSQ	G	830.88	3	2490.29					x
99	23-43	D	FNMPLTISRITPGSKAAQSQL	S	759.28	3	2275.20					x
100	23-40	D	FNMPLTISRITPGSKAAQ	S	649.77	3	1947.02					x
101	23-38	D	FNMPLTISRITPGSKA	A	583.53	3	1747.93					x
102	59-67	D	TMTHLEAQN	K	353.97	3	1059.47		x			
103	59-63	D	TMTHL	E	309.63	2	617.28			x	x	
104	197-219	N	NPIGLYSAETLREMAQMYQMSLR	G	906.56	3	2717.30		x			
105	207-226	T	LREMAQMYQMSLRGKA	L	733.99	3	2200.06	x				
106	593-611	Q	FFAPVCAKCNTKIMGEVMH[g]	A	1079.59	2	2156.99		x			
107	602-613	C	NTKIMGEVMHAL	R	680.83	2	1358.67			x		

[a] Position of the identified peptides inside the LDB3 sequence.
[b] Position of the amino acid residue preceding the peptide sequence.
[c] Position of the amino acid residue following the peptide sequence.
[d] Molecular ion mass observed in the nLC-MS/MS system calculated in Daltons.
[e] Calculated relative molecular mass in Daltons of the matched peptide.
[f] Peptides identified at each time of ham processing.
[g] Peptide identified with two methionine oxidations.

Thus, peptides from 11 to 17 show the sequential loss of phenylalanine (F), asparagine (N), methionine-proline (MP), leucine (L), threonine (T) and isoleucine-serine (IS) from their N-terminal end probably by the action of aminopeptidases and dipeptidyl peptidases that remain active during all dry-curing process. Moreover, carboxypeptidases generate free amino acids from C-terminal position of peptides, as occurs with the amino acids leucine (L; in peptide 27), aspartic acid (D; in peptide 30), and glycine (G; in peptide 31). In fact, these peptides share the common sequence FNMPLTISRITPGSKAAQSQLSQ (peptide 31), as it is shown in **Table 1**. On the other hand, tripeptidyl peptidases could be responsible for the N-terminal loss of tripeptides like serine-valine-threonine (SVT) and thus generate the peptides 2, 4, and 6 from the peptides 1, 3, and 5, respectively, as well as the loss of the tripeptide asparagine-methionine-proline (NMP) from peptide 35 to generate peptide 36, or the loss of glutamic acid-alanine-glutamine (EAQ) from the peptides 58 and 60 to generate peptides 59 and 61, respectively.

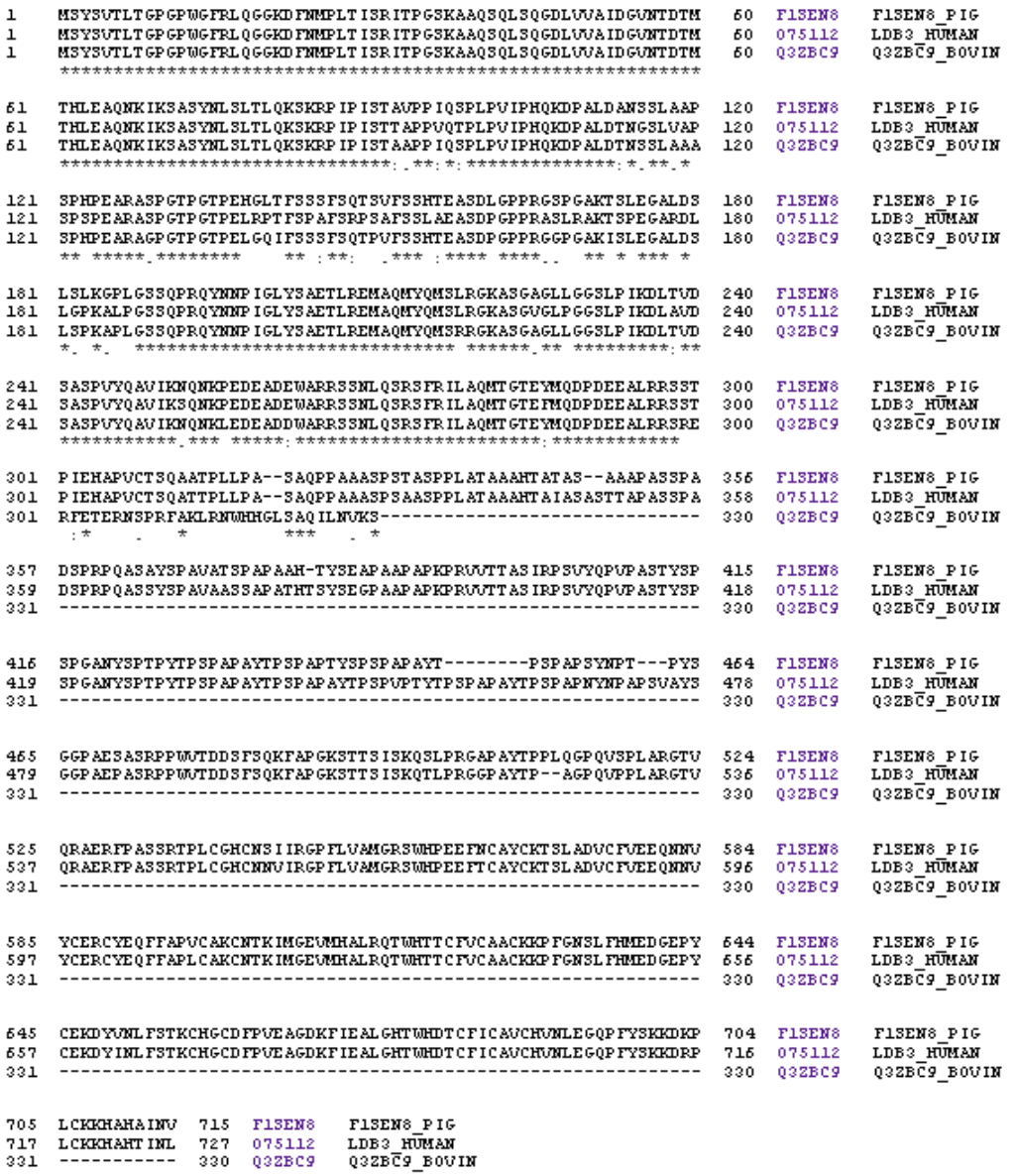


Figure 1. Alignment of LIM domain binding protein 3 sequences of *S. scrofa*, *H. sapiens* and *B. taurus* using UniprotKB/TrEMBL protein database (accession numbers F1SEN8, O75112, and Q3ZBC9, respectively). The same sequences also correspond to accession numbers XP_003359314.1 (*S. scrofa*), NP_009009.1 (*H. sapiens*) and NP_001030493.1 (*B. taurus*) in the NCBInr database.

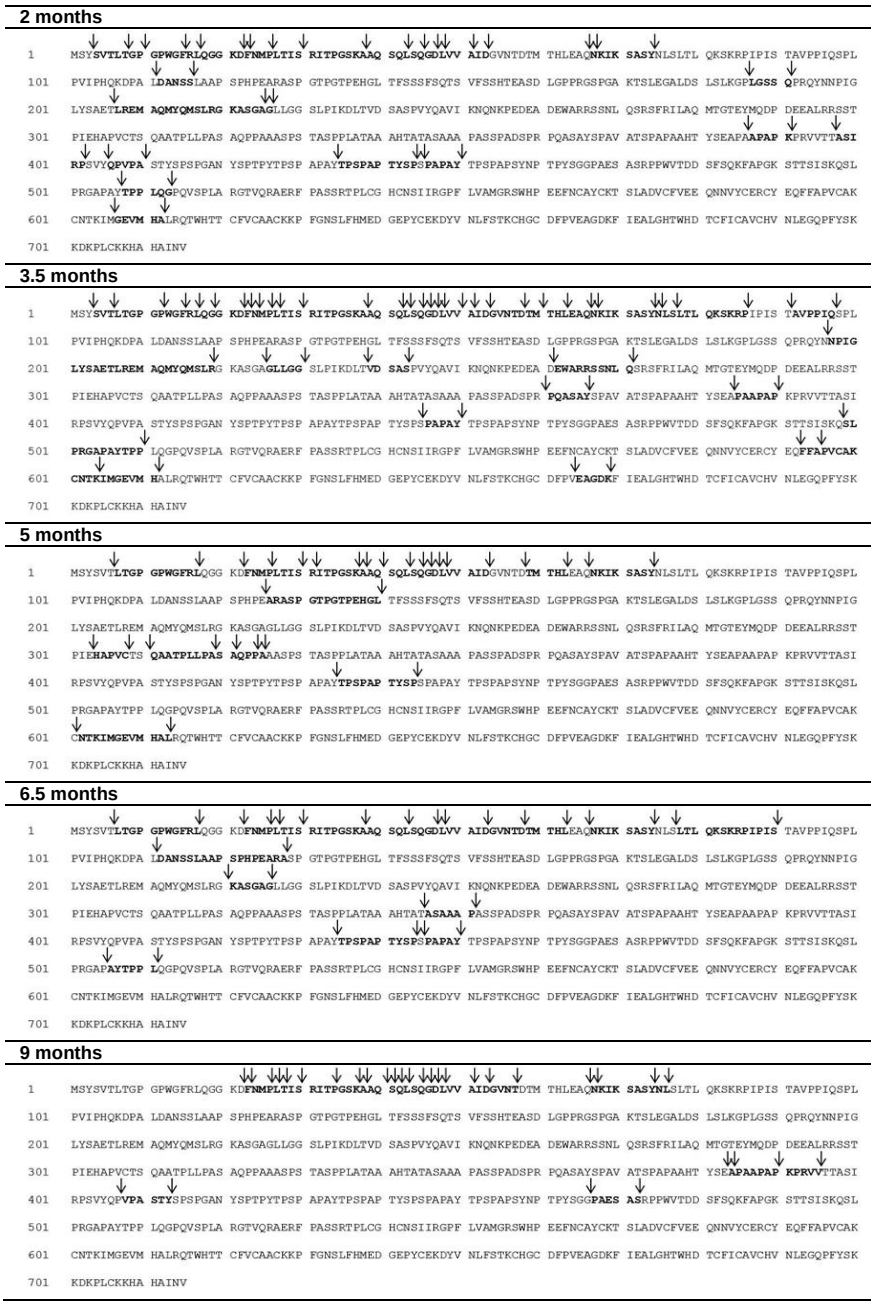


Figure 2. Primary sequence of porcine LDB3 protein (UniprotKB/TrEMBL protein database accession number F1SEN8). Cleavage sites of the identified peptides at each time of processing are indicated with black arrows.

As an example, **Figure 1 of Supplementary material** shows the MS/MS spectra of peptides 6 and 7 demonstrating the loss of arginine-leucine (RL) dipeptide from the C-terminal site, and **Figure 2 of Supplementary material** shows the consecutive loss of the tripeptide serine-glutamine-glycine (SQG) (peptide 21) and the amino acid aspartic acid (D) (peptide 22) from the N-terminal site of peptide 20.

The generation of most peptides shown in **Table 2** could be the result of the action of endopeptidases on LDB3 protein. For example, peptides like 66, 74, 77, 81, 90, and 95, or peptide 64 from **Table 1**, which have been identified during dry-curing stage, would have been generated by the action of cathepsins. After the action of these enzymes, peptides may be further degraded by exopeptidases giving rise to smaller peptides and free amino acids, as happens in peptide 65 which is generated from peptide 64, or peptides 83 and 84 from peptide 82.

Proteolysis is a dynamic and variable process where muscle proteases carry out their action randomly during the process of dry-curing. The complexity of the proteolysis is remarkable as new peptides are continuously being generated and hydrolysed by the action of both endo- and exopeptidases to create new ones (Rodríguez-Nuñez et al., 1995; Toldrá et al., 1997), as can be observed in **Tables 1 and 2**. Thus, the elevated number of enzymes acting during proteolysis would result in the generation of a complex mixture of peptides with unspecified cleavage sites. Some of these peptides are present at certain times of processing at low concentrations due to the constant degradation and are not able to be detected by MS techniques. This fact would explain the unidentification of some peptides at a certain time of processing but its identification again in later times, as occurs for peptides 33, 42, and 88, among others.

The oxidation of essential amino acids such as methionine has been a concern because of its effect on meat quality but also on the nutritive value due to the decreased availability of essential amino acids and poorer digestibility of oxidised proteins (Lund, Heinonen, Baron, & Estévez, 2011; Strange, 1984). Most studies have been focused on protein oxidation mechanisms and the development of

new analysis methodologies to assess the protein oxidation in dry-cured and cooked meat products (Armenteros, Heinonen, Ollilainen, Toldrá, & Estévez, 2009). However, no studies have described the identification of naturally generated peptides showing amino acid modifications along the processing of meat products. In this study, methionine oxidation has been observed in twelve peptides (**Table 3**), and six of these peptides show such modification at the end of dry-cured ham processing (9 months). The oxidation of methionine tends to occur throughout the production of dyr-cured ham, but this process happens more notably at 3.5 months (after the post-salting stage) and then it is maintained toward the final stages of processing. Thus, peptides showing methionine oxidation identified at the end of the dry-cured period could come from the degradation of oxidised peptides at earlier stages. These results are in agreement with a previous study showing that protein oxidation increases throughout the ham time of processing but seems to decelerate toward the final stages of maturation (Koutina, Jongberg, & Skibsted, 2012).

The fact that the identification of some peptides remains stable during the first stages of the dry-curing process such as the case of peptide 6 (MS/MS spectrum is shown in **Figure 1 of Supplementary material**), which has been identified from 2 to 6.5 months of processing, or peptide 11 that has been identified from 2 to 5 months (MS/MS spectrum is shown in **Figure 3 of Supplementary material**), as well as the fact that some peptides have only been detected after 9 months of processing (e.g. case of peptides 35, 36, and 37 or peptides from 43 to 46), would suggest the potential use of these peptides as a way to estimate and control the time of processing. Nevertheless, further studies would be needed in order to consider and confirm these peptides as biomarkers of the dry-curing ham process.

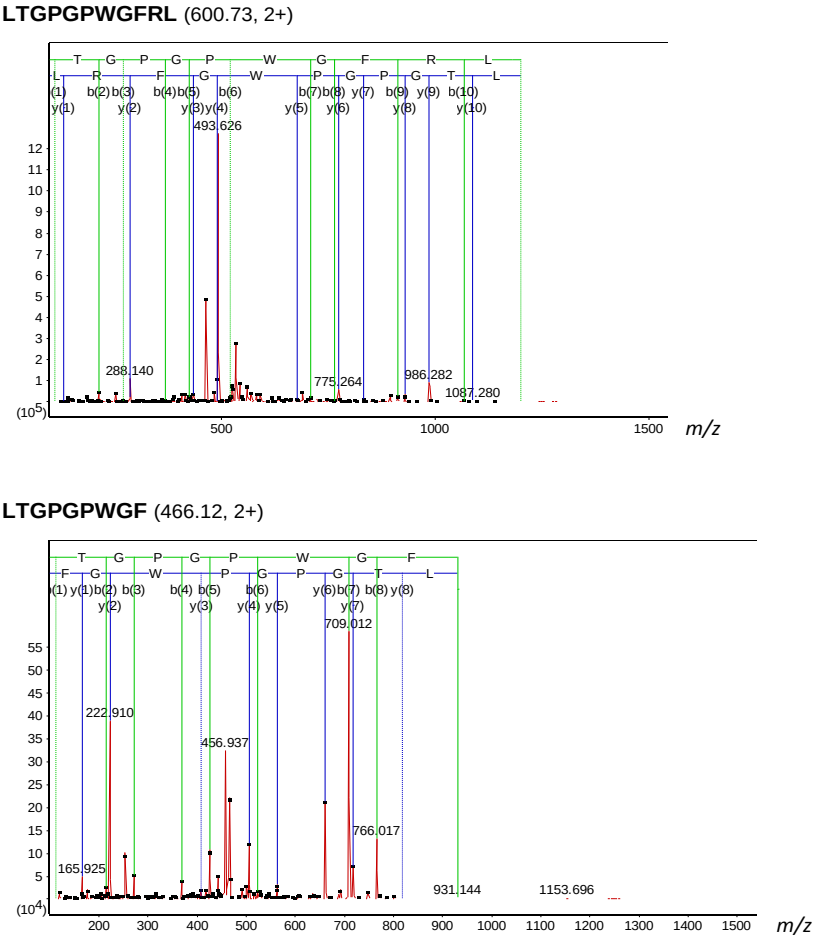
CONCLUSIONS

Proteolysis is a complex and dynamic process which takes place throughout the whole process of dry-curing. The peptides generated through the degradation of LIM domain-binding protein 3 has been observed at different times of processing

providing an evidence of the action of cathepsins, but more remarkable is the action of aminopeptidases, carboxypeptidases, di- and tripeptidyl peptidases to the release of peptides and free amino acids during dry-cured ham processing. Additional studies are required to asses these peptides as biological markers of dry-curing time.

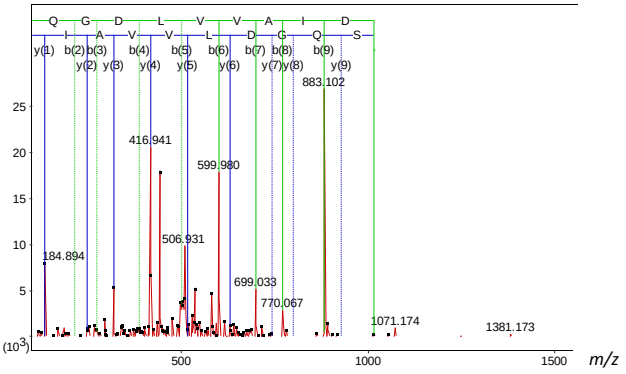
SUPPLEMENTARY DATA

Supplementary Figure 1. MS/MS spectra of peptides 6 and 7 respectively, obtained from the analysis of Spanish dry-cured ham at 3.5 months of processing. The spectra are presented with their corresponding *b* and *y* ions matched by Mascot search engine.

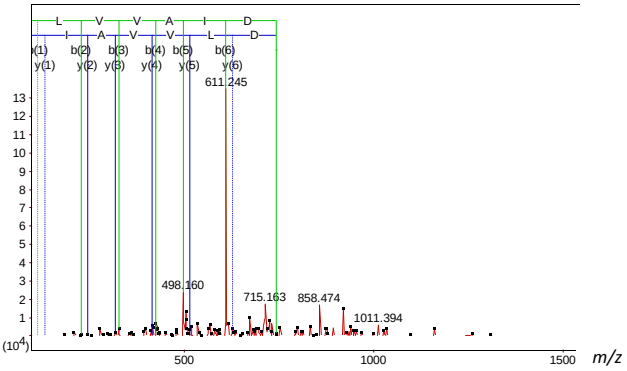


Supplementary Figure 2. MS/MS spectra of peptides 20, 21 and 22 respectively, obtained from the analysis of Spanish dry-cured ham at 9 months of processing. The spectra are presented with their corresponding *b* and *y* ions matched by Mascot search engine.

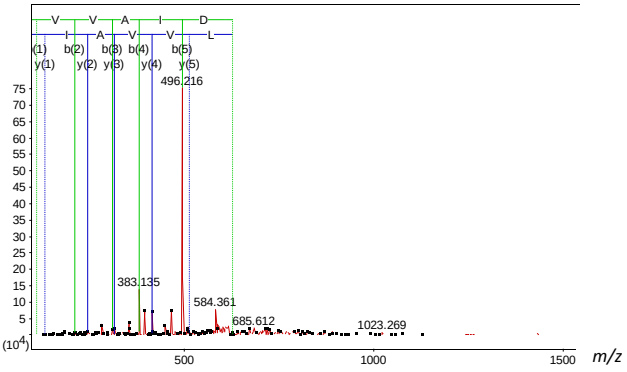
SQGDLVVAID (1016.27+)



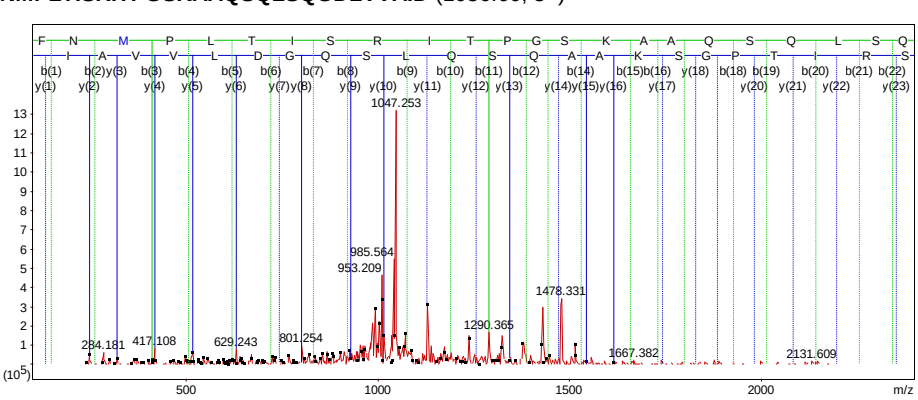
DLVVAID (744.23+)



LVVAID (629.26+)



Supplementary Figure 3. MS/MS spectra of peptide 11 obtained from the analysis of Spanish dry-cured ham at 3.5 months of processing. The spectra are presented with their corresponding *b* and *y* ions matched by Mascot search engine.



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Capítulo 2

Peptides naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of dry-cured ham processing time

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Peptides naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of dry-cured ham processing time

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Abstract

Proteolysis is a complex and dynamic process which takes place throughout the whole dry-cured processing due to the action of endogenous muscle peptidases, and results in the generation of a high number of small peptides and free amino acids responsible for the final quality of dry-cured ham. In this study, a total of sixty-eight peptides derived from the ubiquitin-60S ribosomal protein have been identified in dry-cured ham at 2, 3.5, 5, 6.5, and 9 months of processing using various chromatographic separations and a quadrupole/time-of-flight mass spectrometer in tandem. Some of the identified peptides have been detected during the whole process, whereas a total of fourteen of them were exclusively identified at 9 months of curing. The presence of any of these peptides could be a good indicative that dry-cured ham pieces have reached a minimum curing process of 9 months. The study of the generated peptides has contributed both to a better knowledge of proteolysis evolution and the endogenous enzymes participating, and to determine their potential to be used as quality markers to monitor the processing time.

Keywords: Dry-cured ham; Peptides; Proteolysis; Mass spectrometry; Biomarker; Ubiquitin protein.

INTRODUCTION

Protein degradation is a phenomena that occurs both *in vivo*, as one of the tactics employed by the cell for irreversible inactivating the proteins, and post-mortem, which has been widely reported during the last decades due to its importance in the final quality characteristics of meat and meat-derived products. There are two major pathways to control the destruction of intracellular proteins *in vivo*, the ubiquitin proteasome system (UPS), and the autophagy lysosome system (Wustrow, Zhou, & Rolfe, 2013). In the UPS, the proteins that are destined for degradation are targeted to the 26S proteasome by covalent attachment of a multi-ubiquitin chain. As a result, an enzymatic cascade that includes two ubiquitin-activating enzymes (E1s), approximately forty ubiquitin conjugating enzymes (E2s), and more than five hundred ubiquitin ligases (E3s) occurs. In the final step of this cascade, is the ubiquitin ligase the enzyme that controls the specificity of the ubiquitination reaction (Golab, Bauer, Daniel, & Naujokat, 2004). Different studies suggested that the UPS plays an important role in the degradation of muscle proteins under various catabolic conditions, and the degradation of ubiquitin in *quadriceps femoris* beef muscle, that almost disappear at 10 days post-mortem, has been previously reported (Sekikawa, Seno, & Mikami, 1998).

The natural protein degradation occurred in the post-mortem period during the maturation or curing processes of meat has been widely reported during the last decade. In this respect, fragments of peptides and amino acids are generated in large amounts from the progressive enzymatic degradation of sarcoplasmic and myofibrillar proteins, contributing to flavour, texture, and final quality of meat-derived products such as dry-cured ham (Aristoy & Toldrá, 1995; Toldrá & Flores, 1998; Lametsch, Karlsson, Rosenvold, Andersen, Roepstorff & Bendixen, 2003).

The processing of dry-cured ham is very long, lasting up to 24 months in the highest quality pieces, and its final economic value is mainly given according to the porcine breed and the time of curing. Analytical methods to control the time of curing and the final quality of dry-cured ham would be very useful to assure that fraudulent or accidental mislabelling does not arise, especially when sold as

sliced ham packages. The advances carried out during the last years in the techniques used in the separation of small peptides as well as the experimental design used in proteomic researches, have allowed an increase in the knowledge of the specific peptide sequences generated as well as its tracking during the whole curing process. This fact could permit the control of the dry-cured processing through the study of some of the identified peptide sequences as markers of the time of curing. Recently, a total of 107 sequences of peptides naturally generated from the degradation of the LIM domain-binding protein 3 at different times of curing during the processing of dry-cured ham have been identified using an ion trap mass spectrometer (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014) and its potential as markers of time of processing discussed.

In the present study, ubiquitin-60S ribosomal protein degradation has been studied at different times during the dry-cured ham processing (2, 3.5, 5, 6.5, and 9 months). The sequences of the identified peptides will allow to study its evolution through the process as well as to elucidate the possible proteolytic enzymes that are responsible for its generation. Finally, the potential of some of the identified peptides to be used as markers to control the processing time is discussed.

MATERIALS AND METHODS

Spanish dry-cured ham processing

This study was done by triplicate using raw hams from 6 months old pig (Landrace x Large White) for the production of Spanish dry-cured ham according to the methodology described by Gallego et al. (2014). Briefly, hams were bled and prepared according to traditional procedures consisting on the pre-salting stage for 30 min; the salting period where hams were entirely buried in salt and placed in a cold room for 10-12 days at 2-4 ºC and 90-95% relative humidity; the post-salting stage where hams were kept for 60 days at 4-5 ºC and 75-85% relative humidity; and the ripening-drying period at temperatures increasing from 5 ºC to 14-20 ºC and relative humidity decreasing to 70%. The total length of the dry-curing process was 9 months. Samples were taken at 2 months, 3.5

months, 5 months, 6.5 months, and 9 months of processing. The weight of the hams during processing were comprised between 8.8 and 9.6 kg at 2 months, 8.2-8.6 kg at 3.5 months, 7.5-8.6 at 5 months, 6.8-7.1 kg at 6.5 months, and 5.8-6.3 kg at 9 months.

Extraction of peptides and deproteinisation

Fifty grams of samples of *Biceps femoris* muscle coming from each sampling time of the processed Spanish dry-cured hams were minced and homogenised with 200 mL of 0.01 N HCl for 8 min in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged at 4 ºC and 12000 *g* for 20 min. The resulting supernatant was filtered through glass wool and deproteinised by adding 3 volumes of ethanol maintaining the sample at 4 ºC for 20 h. Samples were deproteinised as the aim of the study is the identification of the generated peptides so the biggest fragments of proteins as well as oligopeptides were precipitated to avoid interferences. Finally, the sample was centrifuged again at 4 ºC and 12000 *g* for 10 min and the supernatant was dried in a rotatory evaporator. The dried extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA) and stored at -20 ºC until use.

Size-exclusion chromatography

Size-exclusion chromatography was used to fractionate cured ham extracts according to molecular mass. A 5 mL aliquot of each extract filtered through a 0.45 µm nylon membrane filter (Millipore) was injected in a Sephadex G25 column (2.5 x 65 cm, Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 0.01 N HCl. The separation was performed using 0.01 N HCl as mobile phase, at a flow rate of 15 mL/h and 4 ºC, and fractions of 5 mL were collected using an automatic fraction collector and further monitored by ultraviolet absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England). Fractions corresponding to elution volumes from 125 to 160 mL were pooled together and aliquots of 100 µL were lyophilised for the mass spectrometry analysis.

Peptide identification by mass spectrometry in tandem (nESI-LC-MS/MS)

The nanoLC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole-time-of-flight (Q-TOF) TripleTOF® 5600+ system from AB Sciex Instruments (Framingham, MA, USA) that is equipped with a nanoelectrospray ionisation source.

Lyophilised samples were resuspended in 100 µL of H_2O with 0.1% of trifluoroacetic acid (TFA). Twenty microlitres of each sample at different times of processing were cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) according to manufacturer's instructions and kept at -20ºC until analysis. Five microlitres of the supernatant were injected into the LC-MS system through the autosampler.

Samples were then preconcentrated on an Eksigent C18 trap column (3µ, 350µm x 0.5mm) (Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min and using 0.1% v/v TFA as mobile phase. After 5 min of preconcentration, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3 cm, C18) (Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A, containing 0.1% v/v FA in water, and solvent B, containing 0.1% v/v FA in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 µL/min and running temperature of 30 ºC.

The outlet of the capillary column was directly coupled to a nano-electrospray ionisation system (nano-ESI). The Q/TOF was operated in positive polarity and information-dependent acquisition mode, in which a 0.25-s TOF MS scan from *m/z* of 300 to 1250 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to 1500 on the 50 most intense 1-5 charged ions.

Data analysis

Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com). The identification of protein origin of peptides was done using UniProt protein database, with a significance threshold

p<0.05 and a FDR of 0.5. The tolerance on the mass measurement was 0.3 Da in MS mode and 0.3 Da for MS/MS ions.

RESULTS AND DISCUSSION

Sample extracts were deproteinised by precipitation with ethanol and fractionated by size-exclusion chromatography. Those fractions corresponding to the first eluted peak (a total of 7 fractions of 5 mL) were pooled together and lyophilised for nESI-LC-MS/MS mass spectrometry identification of their peptidic content.

A total of sixty-eight peptides generated from ubiquitin-60S ribosomal protein have been identified at five different times of processing. The sequences of the identified peptides together with the *m/z* obtained in the detector as well as the molecular mass calculated according to the charge of ionisation are reported in **Table 1**. The time of processing when the peptides have been identified is also indicated in order to facilitate the tracking of the sequences.

Figure 1 shows the sequence of the ubiquitin-60S ribosomal protein, and peptides identified are indicated with black arrows. The identified peptides cover a 56% of the studied sequence with accession number P63053 according to UniProtKB/Swiss-Prot database. All of them have been identified between positions 3 and 74 of this sequence. Although the ubiquitin system of cellular protein degradation has been widely investigated in the fields of clinical medicine and cellular biology, there are not many studies about the state of this protein in post-mortem muscle (Sekikawa et al., 1998), and to our knowledge, no reports regarding naturally generated ubiquitin peptides have been previously published.

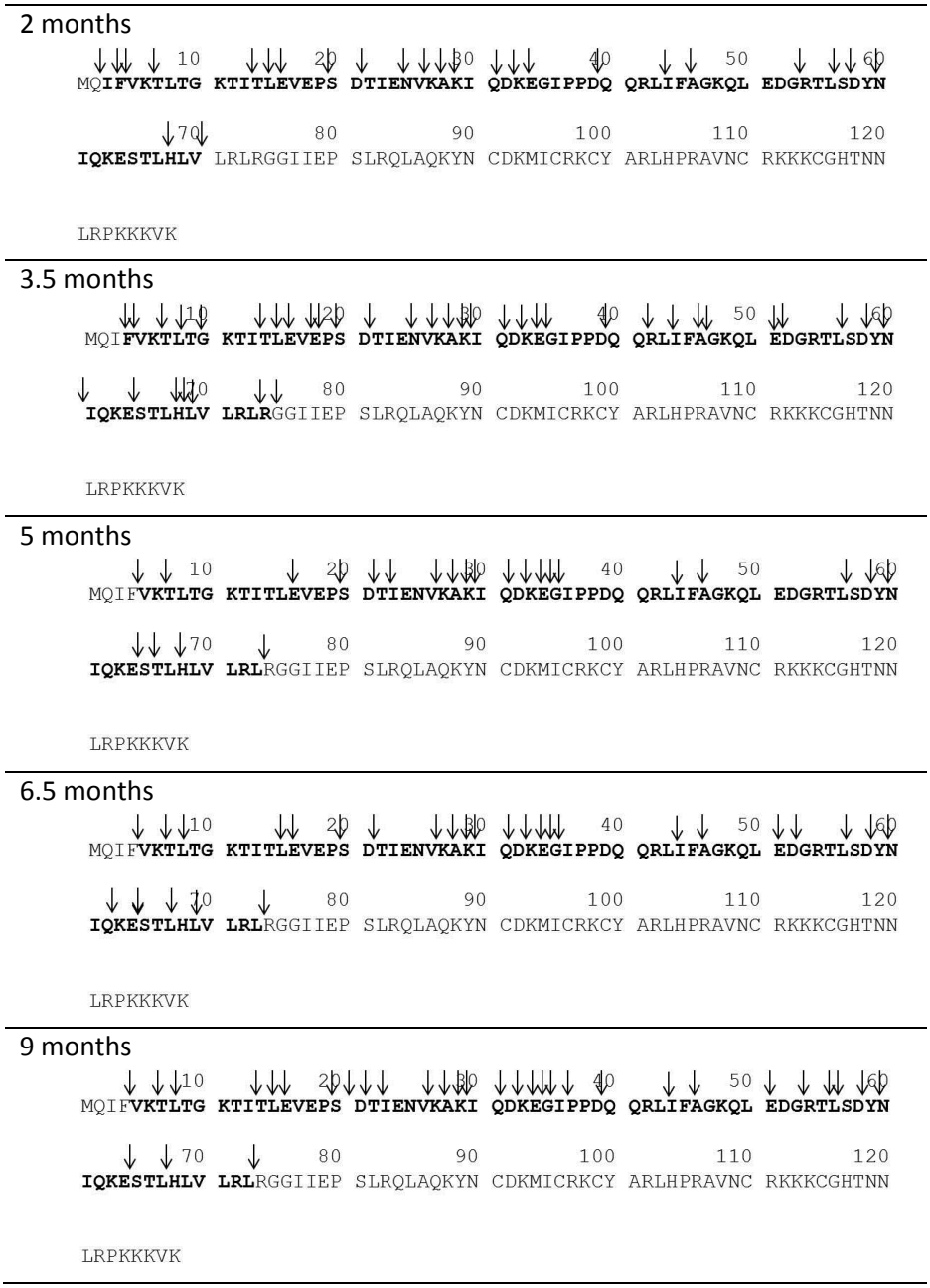


Figure 1. Primary sequence of ubiquitin-60S ribosomal protein from *Sus scrofa* (UniprotKB/Swiss-Prot protein database accession number P63053). Cleavage sites of the identified peptides at each time of processing are indicated with black arrows.

Table 1. Peptides identified by nESI-LC-MS/MS derived from ubiquitin-60S ribosomal protein at different times of dry-cured ham processing.

	Obtained	Charge	Calculated				Time of processing (months)[c]				
Pept.	(*m/z*)[a]	(+)	(*Mr*)[b]	P_0	Sequence	P_f	2	3.5	5	6.5	9
				3'	**IFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLR**	74'					
1	423.77	2	845.52	T	LTGKTITL	E					
2	587.87	2	1173.73	F	VKTLTGKTITL	E					
3	900.46	2	1798.93	L	EVEPSDTIENVKAKIQ	D					
4	957.98	2	1913.96	L	EVEPSDTIENVKAKIQD	K					
5	511.52	4	2042.05	L	EVEPSDTIENVKAKIQDK	E					
6	680.33	2	1358.66	L	EVEPSDTIENVK	A					
7	715.85	2	1429.69	L	EVEPSDTIENVKA	K					
8	487.59	3	1459.75	P	SDTIENVKAKIQD	K					
9	491.77	4	1963.09	V	KAKIQDKEGIPPDQQRL	I					
10	612.67	3	1834.99	K	AKIQDKEGIPPDQQRL	I					
11	514.28	3	1539.84	D	KEGIPPDQQRLIF	A					
12	576.80	2	1151.59	K	EGIPPDQQRL	I					
13	640.85	2	1279.69	D	KEGIPPDQQRL	I					
14	698.36	2	1394.72	Q	DKEGIPPDQQRL	I					
15	649.31	2	1296.62	L	SDYNIQKESTL	H					
16	548.29	2	1094.56	D	YNIQKESTL	H					
17	483.27	3	1446.78	L	IFAGKQLEDGRTL	S					
18	604.97	3	1811.91	L	IFAGKQLEDGRTLSDY	N					
19	636.13	5	3175.64	L	EVEPSDTIENVKAKIQDKEGIPPDQQRL	I					
20	661.40	2	1320.80	I	FVKTLTGKTITL	E					
21	726.41	3	2176.20	E	NVKAKIQDKEGIPPDQQRL	I					
22	367.27	2	732.44	K	TLTGKTI	T					
23	531.33	2	1060.65	F	VKTLTGKTIT	L					
24	661.45	2	1320.80	Q	IFVKTLTGKTIT	L					
25	719.78	2	1437.78	V	KAKIQDKEGIPPD	Q					
26	404.25	5	2016.06	G	RTLSDYNIQKESTLHLV	L					
27	321.24	3	960.55	L	TGKTITLEV	E					
28	495.19	2	988.54	T	GKTITLEVE	P					
29	588.99	3	1763.95	A	KIQDKEGIPPDQQRL	I					
30	587.74	2	1173.69	Q	RLIFAGKQLE	D					

31	767.91	2	1533.82	L	IFAGKQLEDGRTLS	D						
32	659.83	2	1317.65	A	GKQLEDGRTLSD	Y						
33	449.24	3	1344.70	D	YNIQKESTLHL	V						
34	616.82	2	1231.62	D	YNIQKESTLH	L						
35	302.87	3	905.59	L	HLVLRLR	G						
36	375.75	2	749.49	L	HLVLRL	R						
37	397.71	2	793.40	D	YNIQKE	S						
38	779.90	2	1557.79	L	EVEPSDTIENVKAK	I						
39	512.28	2	1022.55	E	GIPPDQQRL	I						
40	483.77	2	965.53	G	IPPDQQRL	I						
41	449.25	3	1344.72	P	SDTIENVKAKIQ	D						
42	381.89	3	1142.67	D	TIENVKAKIQ	D						
43	629.85	2	1257.69	D	TIENVKAKIQD	K						
44	474.29	2	946.57	K	LTGKTITL	E						
45	736.87	2	1471.74	T	LEVEPSDTIENVK	A						
46	528.23	2	1054.46	L	EDGRTLSDY	N						
47	550.62	3	1648.84	L	IFAGKQLEDGRTLSD	Y						
48	466.75	2	931.50	Y	NIQKESTL	H						
49	348.21	3	1041.62	T	IENVKAKIQ	D						
50	462.93	3	1385.79	D	TIENVKAKIQDK	E						
51	367.22	2	732.44	L	TGKTITL	E						
52	616.29	2	1230.56	L	EVEPSDTIENV	K						
53	387.72	2	773.43	D	TIENVKA	K						
54	420.24	3	1257.69	S	DTIENVKAKIQ	D						
55	427.23	2	852.45	I	PPDQQRL	I						
56	613.85	2	1225.68	G	IPPDQQRLIF	A						
57	642.35	2	1282.70	E	GIPPDQQRLIF	A						
58	552.63	3	1654.87	Q	DKEGIPPDQQRLIF	A						
59	1020.00	2	2038.08	K	EGIPPDQQRLIFAGKQLE	D						
60	706.88	2	1411.75	K	EGIPPDQQRLIF	A						
61	584.31	2	1166.60	D	KEGIPPDQQR	L						
62	879.95	2	1757.95	D	QQRLIFAGKQLEDGR	T						
63	518.27	3	1551.75	F	AGKQLEDGRTLSDY	N						
64	555.27	2	1108.54	T	LSDYNIQKE	S						
65	377.69	2	753.37	G	RTLSDY	N						
66	406.20	2	810.39	D	GRTLSDY	N						
67	414.23	2	826.45	Q	KESTLHL	V						
68	441.22	2	880.43	D	YNIQKES	T						

a. Molecular ion mass observed in the nESI-LC-MS/MS analysis; b. Calculated molecular mass in Da of the matched peptide; c. Peptides identified at each time of curing.

Enzymes involved in the extense proteolysis occurred during the processing of dry-cured ham are muscle endopeptidases such as cathepsins and calpains, which hydrolyse proteins giving rise to large polypeptides; and exopeptidases like aminopeptidases, carboxypeptidases, peptidyl peptidases and peptidases, which degrade polypeptides to small peptides and free amino acids (Toldrá, Aristoy & Flores, 2000; Toldrá & Flores, 1998). Cathepsins are stable during the full process (Toldrá, Rico & Flores, 1993), although cathepsin D activity disappears after 6 months of processing (Toldrá et al., 1993). Calpains are inactivated after the salting stage (Rosell & Toldrá, 1996), and regarding exopeptidases, only the activity of aminopeptidases and dipeptidyl peptidases have been studied along the curing process, showing good stability during this period of time (Sentandreu & Toldrá, 2001; Toldrá et al., 2000; Toldrá, Flores & Sanz, 1997).

The degradation of muscle proteins among time have been described in different studies and related with pork meat quality traits. Pas, Jansen, Broekman, Reimert, and Heuven (2009) investigated meat quality traits and proteolysis profiles of the *longissimus dorsi* proteome at 1, 2, 3, 7, and 10 days of ageing after slaughter, obtaining four types of temporal expression profiles and suggesting potential protein biomarkers for dip loss and shear force. The protein degradation profile at 9 months of processing is more intense than that obtained at 10 days of ageing due to time is crucial in the proteolysis phenomena occurring during dry-curing. In fact, despite after 10 days of ageing the integrity of many proteins still remains stable, after 9 months of processing most of the muscle proteins have been degraded at some extent.

Evidences about the activity of exopeptidases in the generation of the peptides identified from position 3 to position 74 of the ubiquitin-60S ribosomal protein sequence are shown in **Figure 1**, with the consecutive loss of amino acids, dipeptides, and tripeptides, from both terminal sites. These results are better appreciated in **Table 1**, where the sequences of the identified peptides have been aligned according to the sequence. In this sense, previous studies discuss the role of these exopeptidases in the generation of small peptides and amino acids at the end of the dry-curing process in proteins such as actin (Sentandreu

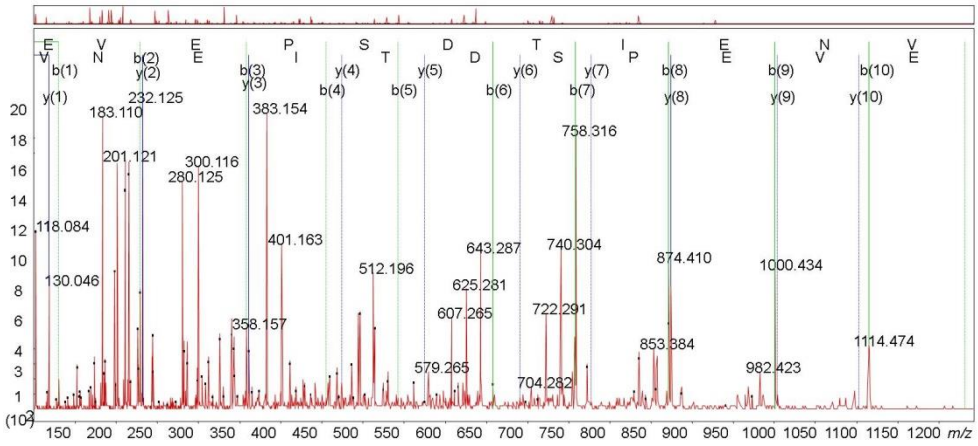
et al., 2007), myosin light chain and titin (Mora et al., 2009), troponin T (Mora, Sentandreu & Toldrá, 2010), sarcoplasmic proteins like creatine kinase (Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009), myoglobin (Mora & Toldrá, 2012) or glycolytic enzymes (Mora, Valero, Sánchez del Pino, Sentandreu & Toldrá, 2011). In this respect, the identification of the sequences of these small naturally generated peptides has become only possible through the use of advanced proteomic techniques such as tandem mass spectrometry like Time-of-Flight (TOF-TOF), ion trap (Q-Trap), and Quadrupole/Time-of-Flight mass spectrometers.

Table 1 shows the time of processing, in months, when the different peptide sequences have been detected. This information reveals the complexity, dynamism, and variability of proteolysis phenomena as a result of the random action of muscle peptidases, that result in the generation of a complex mixture of peptides with unspecified cleavage sites (Gallego et al., 2014). The smallest peptides such as dipeptides or tripeptides that result from the degradation of ubiquitin protein are not able to be identified under the conditions specified in the MS analysis as it would be difficult to relate their sequence to a specific protein origin due to the high amount of possible matches when considering the whole pig proteome. This fact would explain the unidentification of the peptides MQ, MQI or MQIF from position 1 to 2, 1 to 3, and 1 to 4 of ubiquitin sequence, respectively, although it would be expected to have them in the extract of dry-cured ham.

A total of sixteen of the identified peptides remain stable during the 9 months of the studied processing period (**Table 1**). However, other peptides remain intact only during the first 5 months of processing and disappear at 6.5 and 9 months (peptides 17 and 18), whereas fourteen of the peptides (from peptide 52 to peptide 65) appear only at 9 months of processing. As an example, **Figure 2** shows the MS/MS spectra of peptides 52 and 53, where it is shown the loss of the dipeptide KA due to the action of exopeptidases in the C-terminal site. Moreover, these peptides could be a good indicative for a minimum curing time of 9 months. This fact suggests the potential of some of the identified peptides,

derived from the natural degradation of ubiquitin protein, to be used as biomarkers in this type of product with the aim to give a good estimation and control of the time of processing.

Peptide 52: **EVEPSDTIENV** (616.29, 2+)



Peptide 53: **TIENVKA** (387.72, 2+)

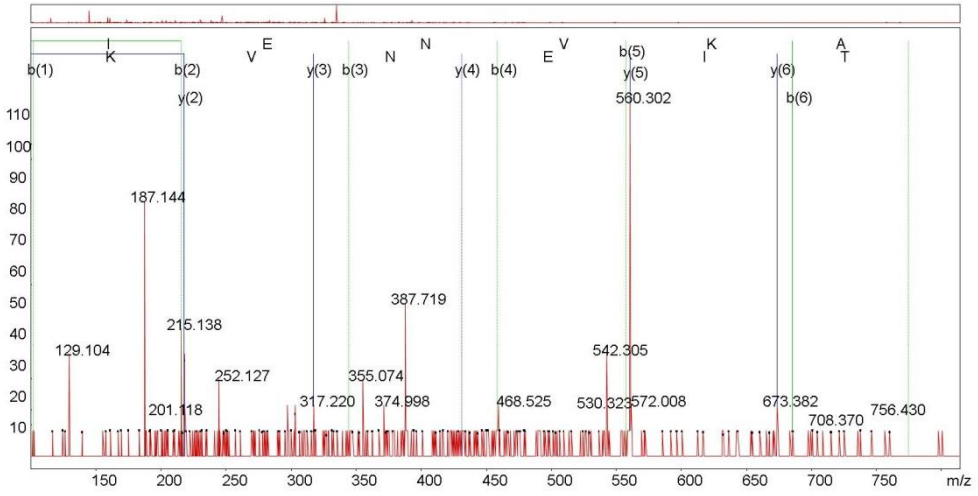


Figure 2. MS/MS spectra of ions 616.29^{2+} and 387.72^{2+} that correspond to peptides 52 and 53, respectively, detected at 9 months of dry-cured ham processing.

CONCLUSIONS

An increase in the knowledge of the specific peptide sequences generated from muscle proteolysis has been achieved during the last years. This situation has been possible through the use of modern advances carried out in separation techniques for small peptides, and proteomic experimental design, which have been used to identify natural peptides generated during the curing process. This fact could permit the control of the dry-cured processing through the study of some of the identified peptide sequences as markers of the time of curing. In fact, a better control of the processing chain of dry-cured ham would guarantee its safety and quality, making them more suitable for export trade, betting for the consumer protection. The ubiquitin peptides identified in this study provide an evidence of the action of aminopeptidases, carboxypeptidases, and di- and tripeptidyl peptidases to the release of peptides and free amino acids during the processing of dry-cured ham, and some of the identified sequences have resulted to be detected only at 9 months of processing, which indicates the potential of the peptides generated during the dry-curing to be used as markers of the time of processing.

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Capítulo 3

Titin-derived peptides as processing time markers in dry-cured ham

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Titin-derived peptides as processing time markers in dry-cured ham

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Abstract

The complex proteolysis in Spanish dry-cured ham processing generates large amounts of small peptides and free amino acids which are responsible for the characteristic texture and flavour of this traditional product. The aim of this work was to study the degradation of the giant protein titin throughout the dry-curing process (2, 3.5, 5, 6.5, and 9 months) through the use of proteomic tools. A total of 320 peptides have been identified by nanoliquid chromatography coupled to tandem mass spectrometry, being some of them identified only at 9 months of processing. In order to confirm the absence of these peptides at other times of processing, MALDI-TOF MS was also employed as a fast and easier technique. Only four peptides, KDEAAKPKGPIKGVAKK, KKLRPGSGGEK, KNTDKWSECAR and ISIDEGKVL, were exclusively identified at 9 months of curing by using both methodologies so that these peptides could be used as potential biomarkers of dry-cured ham processing time.

Keywords: Dry-cured ham; Titin; Connectin; Proteolysis; Peptides; Mass spectrometry; Biomarker.

INTRODUCTION

Numerous and complex biochemical reactions take place in the processing of dry-cured hams, which are responsible for the characteristic texture, flavor and final quality of this typical Spanish product. Proteolysis is the main reaction that occurs during the dry-curing process of ham, where the proteins responsible for muscle structure are broken down by muscle peptidases and release a lot of small peptides and free amino acids (Toldrá, 1998; Toldrá & Flores, 1998; Lametsch, Karlsson, Rosenvold, Andersen, Roepstorff, & Bendixen, 2003). Previous studies have described the role of peptidases in the generation of peptides and amino acids from sarcoplasmic and myofibrillar proteins at the end of dry-cured ham processing (Sentandreu, Armenteros, Calvete, Ouali, Aristoy, & Toldrá, 2007; Mora, Sentandreu, Koistinen, Fraser, Toldrá, & Bramley, 2009; Mora, Sentandreu, & Toldrá, 2010; Mora, Valero, Sánchez del Pino, Sentandreu, & Toldrá, 2011), and even recently throughout the whole dry-curing process (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014; Mora, Gallego, Aristoy, Fraser, & Toldrá, 2014).

Titin, also called connectin, is a giant cytoskeletal protein of vertebrate striated muscles. The molecule is formed by a single polypeptide with a molecular weight of approximately 3 MDa and 2-2.5 µm long, and it consists mainly of multiple domains similar to immunoglobulins and fibronectins. Furthermore, titin is the third most abundant protein of striated muscle, only after myosin and actin (Tskhovrebova & Trinick, 2003; Huff Lonergan, Zhang, & Lonergan, 2010; Tskhovrebova & Trinick, 2010).

Titin protein extends half the sarcomere, with the N-terminus in the Z-disc and the C-terminus in the M-line, and it has an important role in controlling extensibility and structure of sarcomeres during muscle contraction and relaxation (Sorimachi et al., 1997; Lametsch et al., 2003). Thus, it is involved in the assembly and interaction between the contractile proteins like actin and myosin and the sarcomere, contributing to mechanisms that control elasticity during cycles of contraction and extension, and in tension-related biochemical processes (Labeit & Kolmerer, 1995; Tskhovrebova & Trinick, 2003).

Several studies have been done about the degradation of titin in meat and how this process contributes to postmortem tenderization (Fritz, Mitchell, Marsh & Greaser, 1993; Lametsch, et al., 2003; Huff Lonergan et al., 2010). Moreover, these works have shown that the calpain system, and mostly the μ-calpain, plays a central role in postmortem proteolysis and tenderisation. The main ultrastructural change occurred is the break at the junction of the I band and Z-disk, but both the Z- and M-line attachments to the sarcolemma, and the myofibrillar and cytoskeletal proteins, are also degraded during the tenderisation process, including titin protein (Koohmaraie & Geesink, 2006). Nevertheless, to our knowledge there are no studies in-depth about the degradation of titin protein along dry-cured ham processing.

In order to study the degradation of proteins and specially to identify the sequences of naturally generated peptides is necessary the use of advanced proteomic techniques due to their unique capabilities such as high accuracy, molecular specificity, detection sensitivity, and versatility (Léonil, Gagnaire, Mollé, Pezennec, & Bouhallab, 2000).

The main purpose of this study was the proteomic identification of those peptides naturally generated from titin protein along the processing of Spanish dry-cured ham by using nESI-LC-MS/MS as well as the discussion of their potential use as possible biomarkers of dry-cured ham processing time. The use of MALDI-TOF MS methodology as an additional tool to check the presence of these peptides of interest has also been reported.

MATERIALS AND METHODS

Dry-cured ham processing

The study was done by triplicate using Spanish dry-cured hams, which were prepared from raw hams coming from 6 months old pigs (Landrace x Large White). In our pilot plant, the 15 hams were bled and prepared according to the traditional process consisting on the pre-salting stage (using salt, nitrate and nitrite for 30 min), the salting period (hams were buried in salt and piled up at 2-4 ºC and 90-95% relative humidity for 10-12 days), the post-salting stage

(at 4-5 ºC and 75-85% relative humidity for 60 days), and finally the ripening-drying period (at temperatures increasing from 5 ºC to 14-20 ºC and relative humidity decreasing to 70%) until a total length of processing of 9 months.

For the study, samples were selected at five different times of processing, comprising the end of the post-salting stage (2 months), three times during the ripening-drying period (3.5, 5 and 6.5 months), and the end of the dry-cured process (9 months).

Dry-cured ham extraction and deproteinisation

A 50 g sample of *Biceps femoris* muscle coming from each time of processing were minced and homogenised in a stomacher (IUL Instrument, Barcelona, Spain) with 200 mL of 0.01 M HCl for 8 min. The homogenate was centrifuged at 4 ºC and 12,000 *g* for 20 min, filtered through glass wool, and the resulting solution was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 ºC for 20 h. Then, the sample was centrifuged at 4 ºC and 12,000 *g* for 10 min and the supernatant was dried in a rotatory evaporator. Finally, the dried deproteinised extract was dissolved in 25 mL of 0.01 M HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA), and stored at -20 ºC until use.

Size-exclusion chromatography

A 5 mL aliquot of each extract was subjected to size-exclusion chromatography in order to fractionate deproteinised ham extracts according to their molecular mass. A Sephadex G25 column (2.5 x 65 cm; Amersham Biosciences, Uppsala, Sweden), equilibrated with 0.01 M HCl and filtered through a 0.45 µm nylon membrane filter (Millipore), was employed for the separation, using 0.01 M HCl as mobile phase at a flow rate of 15 mL/h and 4 ºC. Fractions of 5 mL were automatically collected and further monitored by ultraviolet absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England). Lastly, fractions corresponding to elution volumes from

125 to 160 mL were pooled together and aliquots of 100 µL were lyophilised for the following analysis.

Peptide identification by nESI-LC-MS/MS

The identification of the peptides was done by nanoliquid chromatography-tandem mass spectrometry using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole/time-of-flight (Q-TOF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (ESI).

Lyophilised samples were resuspended in 100 µL of H_2O with 0.1% of TFA, and 20 µL of each sample at different times of processing were cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) according to manufacturer's guidelines, and kept at -20ºC until analysis. Then, 5 µL of the supernatant were injected into the nESI-LC-MS/MS system.

Samples were then preconcentrated on an Eksigent C18 trap column (3µ, 350µm x 0.5mm; Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min for 5 min and using 0.1% v/v TFA as mobile phase. Then, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3 cm, C18) (Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A, containing 0.1% v/v formic acid in water, and solvent B, containing 0.1% v/v formic acid in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 µL/min and running temperature of 30 ºC.

The outlet of the capillary column was directly coupled to a nano-electrospray ionisation system (nano-ESI). The Q/TOF was operated in positive polarity and information-dependent acquisition mode, in which a 0.25-s TOF MS scan from *m/z* of 300 to 1250 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to 1500 on the 50 most intense 1 - 5 charged ions.

Peptide-mass mapping by MALDI-TOF MS

To determine the molecular mass of peptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, the analysis was performed using a 5800 MALDI-TOF/TOF (AB Sciex, MA, USA).

Lyophilised samples were resuspended in 100 µL acetonitrile:H_2O (2:98, v/v) with 0.1% (v/v) of trifluoroacetic acid (TFA), and 1 µL of the peptide mixture was spotted onto the MALDI target plate. After the droplets were air-dried at room temperature, 1 µL of matrix (which contains 5 mg/mL of saturated α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics, Germany)) in acetonitrile:H_2O (1:1, v/v) with 0.1% TFA was added and air-dried at room temperature. Then, the plate was introduced in the MALDI-TOF/TOF instrument, which was used in automatic positive-ion reflector mode for mass analysis between 600 and 3500 Da. Spectra were obtained from 3000 shots in every position with a laser intensity of 3500. Previously, the plate and the acquisition method were calibrated with 0.5 µL of CM5 calibration mixture (AB Sciex, MA, USA) in 13 positions.

Data analysis

Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com). The identification of protein origin of the peptides was done using the tool BLAST (Basic Local Alignment Search Tool) from UniProt protein database, with a FDR of 0.5 and a significance threshold $p<0.05$. The tolerance on the mass measurement was 0.3 Da in MS mode and 0.3 Da for MS/MS ions. The alignment of protein sequences was also carried out using the Uniprot protein resource (http://www.uniprot.org). The spectra obtained by MALDI-TOF MS were analysed using mMass - Open Source Mass Spectrometry Tool software v5.5 (Strohalm, Kavan, Novák, Volný, & Havlíček, 2010; Niedermeyer & Strohalm, 2012).

RESULTS AND DISCUSSION

Samples at different times of dry-cured ham processing were fractionated by size-exclusion chromatography. Then, seven fractions of 5 mL, corresponding to the first peak eluted which contained the highest size peptides, were pooled together and lyophilised. Subsequently, samples were analysed by nESI-LC-MS/MS to identify the sequences of the peptides contained in these fractions, and moreover samples were submitted to MALDI-TOF MS analysis. Following this procedure at each of the five different processing times, a total of 320 peptides generated from the degradation of titin protein have been identified as reported in **Tables 1-4**. These tables list the position of the identified peptides in the protein origin and their sequences, the observed and calculated masses together with the charge states, and the amino acids residues preceding and following the identified sequence. Furthermore, the time of processing (2, 3.5, 5, 6.5, and 9 months) when the peptides have been identified by using only the nESI-LC-MS/MS technique is indicated in grey, whereas peptides detected by both nESI-LC-MS/MS and MALDI-TOF MS techniques are shown in black.

Table 1. Peptides identified by nESI-LC-MS/MS and detected by MALDI-TOF MS from position 12704 to 12923 of titin protein in dry-cured ham (UniprotKB/TrEMBL protein database accession number A2ASS6 of *Mus musculus* and corresponding to accession number O97771 of *Sus scrofa*).

Peptide number	Position[a]	Observed (m/z)[b]	Charge (+)	Calculated (Da)[c]	P_o[d]	Sequence	P_f[e]	nESI-LC-MS/MS Processing times (months)[f]				
								2	3.5	5	6.5	9
1	12704-12714	605.29	2	1208.67	P	**EVVEKVEPAPL**	K					
2	12707-12723	932.11	2	1862.09	V	**EKVEPAPLKVPTAEKKV**	R					
3	12708-12715	441.25	2	880.54	E	**KVEPAPLK**	V					
4	12712-12719	398.88	2	795.49	P	**APLKVPTA**	E					
5	12717-12724	310.22	3	927.55	V	**PTAEKKVR**	K					
6	12729-12746	702.07	3	2103.24	P	**EPKPQPKEEVVLKSVLRK**	K					
7	12731-12747	669.35	3	2005.24	P	**KPQPKEEVVLKSVLRKK**	P					
8	12732-12740	519.65	2	1037.58	K	**PQPKEEVVL**	K					
9	12733-12741	535.30	2	1068.62	P	**QPKEEVVLK**	S					
10	12733-12743	419.26	3	1254.72	P	**QPKEEVVLKSV**	L					
11	12733-12744	456.98	3	1367.80	P	**QPKEEVVLKSVL**	R					
12	12733-12747	594.31	3	1780.09	P	**QPKEEVVLKSVLRKK**	P					
13	12733-12748	470.26	4	1877.15	P	**QPKEEVVLKSVLRKKP**	E					
14	12733-12752	599.33	4	2393.32	P	**QPKEEVVLKSVLRKKPEEEE**	P					
15	12734-12754	623.61	4	2490.41	Q	**PKEEVVLKSVLRKKPEEEEPK**	V					
16	12737-12749	762.90	2	1523.94	E	**EVVLKSVLRKKPE**	E					
17	12753-12762	591.31	2	1180.72	E	**PKVEPKKVEK**	A					
18	12757-12764	464.27	2	926.59	E	**PKKVEKAK**	K					
19	12757-12765	528.28	2	1054.69	E	**PKKVEKAKK**	P					
20	12757-12766	576.84	2	1151.74	E	**PKKVEKAKKP**	E					
21	12760-12770	641.86	2	1281.69	K	**VEKAKKPEEPQ**	P					
22	12762-12770	352.21	3	1053.58	E	**KAKKPEEPQ**	P					
23	12764-12775	673.37	2	1344.74	A	**KKPEEPQPPPKA**	V					
24	12765-12775	609.30	2	1216.65	K	**KPEEPQPPPKA**	V					
25	12765-12784	722.38	3	2164.11	K	**KPEEPQPPPKAVEVEAPPEP**	T					
26	12765-12787	623.61	4	2490.30	K	**KPEEPQPPPKAVEVEAPPEPTPK**	E					
27	12767-12773	397.28	2	792.37	P	**EEPQPPP**	K					
28	12767-12776	546.33	2	1090.57	P	**EEPQPPPKAV**	E					
29	12769-12782	728.39	2	1454.78	E	**PQPPPKAVEVEAPP**	E					
30	12769-12784	561.32	3	1680.87	E	**PQPPPKAVEVEAPPEP**	T					
31	12770-12785	422.25	4	1684.87	P	**QPPPKAVEVEAPPEPT**	P					
32	12770-12793	530.68	5	2648.42	P	**QPPPKAVEVEAPPEPTPKERKVPE**	P					
33	12779-12795	624.57	3	1870.98	V	**EAPPEPTPKERKVPEPA**	K					
34	12785-12808	864.45	3	2590.52	P	**TPKERKVPEPAKVPEIKPAIPLPG**	P					
35	12786-12801	615.69	3	1844.09	T	**PKERKVPEPAKVPEIK**	P					
36	12787-12797	427.57	3	1279.76	P	**KERKVPEPAKV**	P					
37	12787-12798	689.36	2	1376.81	P	**KERKVPEPAKVP**	E					
38	12787-12800	405.73	4	1618.94	P	**KERKVPEPAKVPEI**	K					
39	12787-12805	532.31	4	2125.26	P	**KERKVPEPAKVPEIKPAIP**	L					
40	12790-12797	434.24	2	866.52	R	**KVPEPAKV**	P					
41	12790-12810	736.05	3	2205.28	R	**KVPEPAKVPEIKPAIPLPGPE**	P					
42	12790-12816	577.34	5	2881.67	R	**KVPEPAKVPEIKPAIPLPGPEPKPKPE**	P					
43	12796-12807	651.33	2	1300.81	A	**KVPEIKPAIPLP**	G					
44	12796-12811	561.30	3	1680.98	A	**KVPEIKPAIPLPGPEP**	K					
45	12796-12817	590.35	4	2357.37	A	**KVPEIKPAIPLPGPEPKPKPEP**	E					
46	12796-12818	622.61	4	2486.41	A	**KVPEIKPAIPLPGPEPKPKPEPE**	V					
47	12796-12819	862.83	3	2585.48	A	**KVPEIKPAIPLPGPEPKPKPEPEV**	K					
48	12796-12820	679.39	4	2713.58	A	**KVPEIKPAIPLPGPEPKPKPEPEVK**	T					
49	12797-12819	615.35	4	2457.39	K	**VPEIKPAIPLPGPEPKPKPEPEV**	K					
50	12797-12820	647.37	4	2585.48	K	**VPEIKPAIPLPGPEPKPKPEPEVK**	T					

Table 1 (continued)

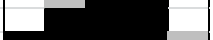
51	12798-12817	533.56	4	2130.21	V	**PEIKPAIPLPGPEPKPKPEP**	E	
52	12798-12818	565.82	4	2259.25	V	**PEIKPAIPLPGPEPKPKPEPE**	V	
53	12798-12819	590.58	4	2358.32	V	**PEIKPAIPLPGPEPKPKPEPEV**	K	
54	12798-12820	498.29	5	2486.41	V	**PEIKPAIPLPGPEPKPKPEPEVK**	T	
55	12798-12830	705.41	5	3521.96	V	**PEIKPAIPLPGPEPKPKPEPEVKTMKAPPIEPA**	P	
56	12799-12818	541.55	4	2162.20	P	**EIKPAIPLPGPEPKPKPEPE**	V	
57	12800-12816	603.29	3	1807.06	E	**IKPAIPLPGPEPKPKPE**	P	
58	12800-12818	509.29	4	2033.16	E	**IKPAIPLPGPEPKPKPEPE**	V	
59	12800-12819	534.06	4	2132.22	E	**IKPAIPLPGPEPKPKPEPEV**	K	
60	12801-12810	509.73	2	1017.59	I	**KPAIPLPGPE**	P	
61	12801-12811	558.32	2	1114.64	I	**KPAIPLPGPEP**	K	
62	12801-12813	670.86	2	1339.79	I	**KPAIPLPGPEPKP**	K	
63	12801-12815	783.61	2	1564.93	I	**KPAIPLPGPEPKPKP**	E	
64	12801-12819	505.79	4	2019.14	I	**KPAIPLPGPEPKPKPEPEV**	K	
65	12802-12818	598.33	3	1791.98	K	**PAIPLPGPEPKPKPEPE**	V	
66	12802-12819	631.35	3	1891.05	K	**PAIPLPGPEPKPKPEPEV**	K	
67	12802-12820	505.79	4	2019.14	K	**PAIPLPGPEPKPKPEPEVK**	T	
68	12803-12812	509.80	2	1017.59	P	**AIPLPGPEPK**	P	
69	12803-12817	522.97	3	1565.88	P	**AIPLPGPEPKPKPEP**	E	
70	12803-12818	565.98	3	1694.92	P	**AIPLPGPEPKPKPEPE**	V	
71	12803-12819	599.00	3	1793.99	P	**AIPLPGPEPKPKPEPEV**	K	
72	12803-12820	481.53	4	1922.09	P	**AIPLPGPEPKPKPEPEVK**	T	
73	12804-12818	542.30	3	1623.89	A	**IPLPGPEPKPKPEPE**	V	
74	12804-12819	575.32	3	1722.96	A	**IPLPGPEPKPKPEPEV**	K	
75	12804-12820	463.77	4	1851.05	A	**IPLPGPEPKPKPEPEVK**	T	
76	12805-12816	643.37	2	1284.71	I	**PLPGPEPKPKPE**	P	
77	12805-12818	756.41	2	1510.80	I	**PLPGPEPKPKPEPE**	V	
78	12806-12818	472.26	3	1413.75	P	**LPGPEPKPKPEPE**	V	
79	12806-12819	505.28	3	1512.82	P	**LPGPEPKPKPEPEV**	K	
80	12806-12820	411.23	4	1640.91	P	**LPGPEPKPKPEPEVK**	T	
81	12807-12820	382.96	4	1527.83	L	**PGPEPKPKPEPEVK**	T	
82	12808-12818	602.94	2	1203.61	P	**GPEPKPKPEPE**	V	
83	12808-12820	477.93	3	1430.78	P	**GPEPKPKPEPEVK**	T	
84	12809-12815	396.80	2	791.45	G	**PEPKPKP**	E	
85	12809-12820	458.92	3	1373.76	G	**PEPKPKPEPEVK**	T	
86	12810-12820	426.57	3	1276.70	P	**EPKPKPEPEVK**	T	
87	12812-12819	462.27	2	922.51	P	**KPKPEPEV**	K	
88	12812-12824	494.95	3	1481.83	P	**KPKPEPEVKTMKA**	P	
89	12812-12827	597.36	3	1789.02	P	**KPKPEPEVKTMKAPPI**	E	
90	12812-12828	640.35	3	1918.06	P	**KPKPEPEVKTMKAPPIE**	P	
91	12812-12835	642.35	4	2565.42	P	**KPKPEPEVKTMKAPPIEPAPTPIA**	A	
92	12813-12820	462.37	2	922.51	K	**PKPEPEVK**	T	
93	12814-12828	565.31	3	1692.91	P	**KPEPEVKTMKAPPIE**	P	
94	12815-12824	377.28	3	1128.58	K	**PEPEVKTMKA**	P	
95	12819-12850	642.33	5	3206.85	E	**VKTMKAPPIEPAPTPIAAPVTAPVVGKKAEAK**	P	
96	12820-12830	394.88	3	1181.65	V	**KTMKAPPIEPA**	P	
97	12820-12833	739.42	2	1476.80	V	**KTMKAPPIEPAPTP**	I	
98	12823-12841	613.30	3	1837.03	M	**KAPPIEPAPTPIAAPVTAP**	V	
99	12825-12851	882.77	3	2645.52	A	**PPIEPAPTPIAAPVTAPVVGKKAEAKP**	K	
100	12827-12836	327.25	3	978.54	P	**IEPAPTPIAA**	P	
101	12829-12850	705.16	3	2112.23	E	**PAPTPIAAPVTAPVVGKKAEAK**	P	
102	12830-12841	553.33	2	1104.62	P	**APTPIAAPVTAP**	V	
103	12830-12847	844.44	2	1687.00	P	**APTPIAAPVTAPVVGKKA**	E	
104	12837-12859	590.54	4	2358.36	A	**PVTAPVVGKKAEAKPKDEAAKPK**	G	
105	12845-12854	572.31	2	1142.63	G	**KKAEAKPKDE**	A	

Table 1 (continued)

106	12845-12859	547.05	3	1637.95	G	**KKAEAKPKDEAAKPK**	G	
107	12846-12856	579.32	2	1156.61	K	**KAEAKPKDEAA**	K	
108	12846-12857	643.49	2	1284.70	K	**KAEAKPKDEAAK**	P	
109	12848-12861	489.30	3	1464.79	A	**EAKPKDEAAKPKGP**	I	
110	12850-12861	633.34	2	1264.71	A	**KPKDEAAKPKGP**	I	
111	12852-12861	520.80	2	1039.57	P	**KDEAAKPKGP**	I	
112	12852-12864	447.02	3	1337.77	P	**KDEAAKPKGPIKG**	V	
113	12852-12868	588.98	3	1764.06	P	**KDEAAKPKGPIKGVAKK**	T	
114	12855-12865	533.28	2	1064.67	E	**AAKPKGPIKGV**	A	
115	12855-12866	379.57	3	1135.71	E	**AAKPKGPIKGVA**	K	
116	12855-12868	464.97	3	1391.90	E	**AAKPKGPIKGVAKK**	T	
117	12855-12874	673.08	3	2016.21	E	**AAKPKGPIKGVAKKTPSPIE**	A	
118	12855-12875	696.76	3	2087.25	E	**AAKPKGPIKGVAKKTPSPIEA**	E	
119	12856-12874	649.40	3	1945.17	A	**AKPKGPIKGVAKKTPSPIE**	A	
120	12857-12867	562.00	2	1121.73	A	**KPKGPIKGVAK**	K	
121	12857-12869	676.40	2	1350.87	A	**KPKGPIKGVAKKT**	P	
122	12857-12872	545.07	3	1632.01	A	**KPKGPIKGVAKKTPSP**	I	
123	12857-12879	498.27	5	2486.51	A	**KPKGPIKGVAKKTPSPIEAERKK**	L	
124	12859-12877	669.34	3	2005.17	P	**KGPIKGVAKKTPSPIEAER**	K	
125	12859-12894	750.84	5	3749.10	P	**KGPIKGVAKKTPSPIEAERKKLRPGSGGEKPPDEAP**	F	
126	12864-12875	399.90	3	1196.68	K	**GVAKKTPSPIEA**	E	
127	12866-12889	854.45	3	2560.44	V	**AKKTPSPIEAERKKLRPGSGGEKP**	P	
128	12867-12877	419.24	3	1254.69	A	**KKTPSPIEAER**	K	
129	12868-12896	624.76	5	3118.64	K	**KTPSPIEAERKKLRPGSGGEKPPDEAPFT**	Y	
130	12875-12892	651.02	3	1950.03	E	**AERKKLRPGSGGEKPPDE**	A	
131	12875-12895	567.30	4	2265.19	E	**AERKKLRPGSGGEKPPDEAPF**	T	
132	12875-12897	633.33	4	2529.30	E	**AERKKLRPGSGGEKPPDEAPFTY**	Q	
133	12876-12892	470.76	4	1878.99	A	**ERKKLRPGSGGEKPPDE**	A	
134	12876-12895	549.54	4	2194.15	A	**ERKKLRPGSGGEKPPDEAPF**	T	
135	12876-12896	574.81	4	2295.20	A	**ERKKLRPGSGGEKPPDEAPFT**	Y	
136	12876-12897	492.66	5	2458.26	A	**ERKKLRPGSGGEKPPDEAPFTY**	Q	
137	12877-12895	517.28	4	2065.11	E	**RKKLRPGSGGEKPPDEAPF**	T	
138	12878-12886	450.27	2	898.53	R	**KKLRPGSGG**	E	
139	12878-12888	578.97	2	1155.67	R	**KKLRPGSGGEK**	P	
140	12878-12892	532.28	3	1593.85	R	**KKLRPGSGGEKPPDE**	A	
141	12878-12896	1006.03	2	2010.05	R	**KKLRPGSGGEKPPDEAPFT**	Y	
142	12879-12890	408.23	3	1221.68	K	**KLRPGSGGEKPP**	D	
143	12880-12896	585.63	3	1753.86	K	**LRPGSGGEKPPDEAPFT**	Y	
144	12886-12896	594.14	2	1186.55	G	**GEKPPDEAPFT**	Y	
145	12887-12896	377.57	3	1129.53	G	**EKPPDEAPFT**	Y	
146	12888-12905	511.28	4	2041.12	E	**KPPDEAPFTYQLKAVPLK**	F	
147	12888-12906	730.41	3	2188.19	E	**KPPDEAPFTYQLKAVPLKF**	V	
148	12900-12913	543.28	3	1627.01	L	**KAVPLKFVKEIKDI**	V	
149	12903-12910	325.29	3	972.60	V	**PLKFVKEI**	K	
150	12907-12916	579.51	2	1156.71	F	**VKEIKDIVLT**	E	
151	12911-12923	674.36	2	1346.69	I	**KDIVLTEAESVGS**	S	

[a]Position of the identified peptides inside the titin sequence. [b]Molecular ion mass observed in the ESI-LC-MS/MS analysis calculated in Daltons. [c]Calculated relative molecular mass of the matched peptide. [d]Position of the amino acid residue preceding the peptide sequence. [e]Position of the amino acid residue following the peptide sequence. [f]Peptides identified at each time of ham processing using nESI-LC-MS/MS are indicated in grey, and those detected by both nESI-LC-MS/MS and MALDI-TOF MS are shown in black.

Table 2. Peptides identified by nESI-LC-MS/MS and detected by MALDI-TOF MS from position 24600 to 25126 of the titin protein in dry-cured ham (UniprotKB/TrEMBL protein database accession number A2ASS6 of *Mus musculus* and corresponding to accession number Q29117 of *Sus scrofa*).

Peptide number	Position[a]	Observed (m/z)[b]	Charge (+)	Calculated (Da)[c]	P_o[d]	Sequence	P_f[e]	nESI-LC-MS/MS Processing times (months)[f]				
								2	3.5	5	6.5	9
152	24600-24611	382.24	3	1143.54	R	VSAENAAGLSEP	S					
153	24604-24614	520.14	2	1038.50	E	NAAGLSEPSPP	S					
154	24605-24614	309.26	3	924.46	N	AAGLSEPSPP	S					
155	24606-24619	477.82	3	1430.70	A	AGLSEPSPPSAYQK	A					
156	24608-24624	902.08	2	1801.86	G	LSEPSPPSAYQKACDPI	Y					
157	24609-24620	631.38	2	1260.60	L	SEPSPPSAYQKA	C					
158	24612-24621	351.21	3	1050.48	P	SPPSAYQKAC	D					
159	24614-24632	687.33	3	2058.98	P	PSAYQKACDPIYKPGPPNN	P					
160	24618-24633	580.30	3	1737.85	Y	QKACDPIYKPGPPNNP	K					
161	24619-24628	546.31	2	1090.55	Q	KACDPIYKPG	P					
162	24619-24629	594.86	2	1187.60	Q	KACDPIYKPGP	P					
163	24620-24631	636.40	2	1270.60	K	ACDPIYKPGPPN	N					
164	24620-24637	652.56	3	1954.93	K	ACDPIYKPGPPNNPKVMD	V					
165	24621-24639	695.59	3	2084.01	A	CDPIYKPGPPNNPKVMDVT	R					
166	24622-24640	713.37	3	2137.10	C	DPIYKPGPPNNPKVMDVTR	S					
167	24626-24632	362.22	2	722.37	Y	KPGPPNN	P					
168	24626-24643	641.70	3	1922.00	Y	KPGPPNNPKVMDVTRSSV	F					
169	24649-24661	481.46	3	1441.65	T	KPIYDGGCEIQGY	I					
170	24657-24676	763.61	3	2288.02	C	EIQGYIVEKCDVSVGEWTMC	T					
171	24663-24679	627.61	3	1879.82	I	VEKCDVSVGEWTMCTPP	T					
172	24664-24670	390.25	2	778.35	V	EKCDVSV	G					
173	24667-24684	645.63	3	1933.89	C	DVSVGEWTMCTPPTGINK	T					
174	24673-24689	635.57	3	1903.92	E	WTMCTPPTGINKTNLEV	E					
175	24677-24685	310.22	3	927.50	C	TPPTGINKT	N					
176	24679-24687	479.28	2	956.53	P	PTGINKTNL	E					
177	24682-24695	835.93	2	1669.96	G	INKTNLEVEKLLEK	H					
178	24687-24696	619.36	2	1236.71	N	LEVEKLLEKH	E					
179	24695-24707	546.05	3	1634.84	E	KHEYNFRICAINK	A					
180	24696-24713	686.57	3	2056.99	K	HEYNFRICAINKAGVGEH	A					
181	24711-24731	744.74	3	2231.08	V	GEHADVPGPVMVEEKLEAPDI	D					
182	24712-24726	413.26	4	1648.81	G	EHADVPGPVMVEEKL	E					
183	24715-24726	656.84	2	1311.67	A	DVPGPVMVEEKL	E					
184	24728-24736	500.68	2	999.48	E	APDIDLDLE	L					
185	24739-24755	618.31	3	1852.14	R	KVINIRAGGSLRLFVPI	K					
186	24746-24757	415.20	3	1242.74	A	GGSLRLFVPIKG	R					
187	24764-24779	591.29	3	1770.93	V	KWGKVDGDIRDAAIID	V					
188	24765-24771	388.73	2	775.35	K	WGKVDGD	I					
189	24775-24796	483.46	5	2412.22	D	AAIIDVTSSFTSLVLDNVNRYD	S					
190	24779-24794	589.55	3	1765.88	I	DVTSSFTSLVLDNVNR	Y					
191	24785-24796	470.31	3	1407.70	F	TSLVLDNVNRYD	S					
192	24791-24796	390.75	2	779.36	D	NVNRYD	S					
193	24799-24806	491.27	2	980.52	G	KYTLTLEN	S					
194	24799-24807	534.92	2	1067.55	G	KYTLTLENS	S					
195	24800-24811	657.34	2	1312.65	K	YTLTLENSSGTK	S					

Table 2 (continued)

196	24806-24818	677.48	2	1352.70	E	NSSGTKSAFVTVR	V	
197	24819-24835	608.39	3	1822.01	R	VLDTPSPPVNLKVTEIT	K	
198	24823-24838	862.99	2	1723.94	T	PSPPVNLKVTEITKDS	V	
199	24825-24852	998.28	3	2991.58	S	PPVNLKVTEITKDSVSITWEPPLLDGGS	K	
200	24826-24840	815.48	2	1628.90	P	PVNLKVTEITKDSVS	I	
201	24859-24877	541.31	4	2161.11	I	VEKREATRKSYAAVVTNCH	K	
202	24860-24871	705.37	2	1408.74	V	EKREATRKSYAA	V	
203	24868-24888	487.69	5	2433.17	K	SYAAVVTNCHKNSWKIDQLQE	G	
204	24885-24900	954.50	2	1906.85	D	QLQEGCSYYFRVTAEN	E	
205	24890-24904	605.69	3	1813.80	G	CSYYFRVTAENEYGI	G	
206	24906-24912	372.34	2	742.40	G	LPARTAD	P	
207	24907-24930	629.37	4	2513.35	L	PARTADPIKVAEVPQPPGKITVDD	V	
208	24909-24922	507.70	3	1519.84	A	RTADPIKVAEVPQP	P	
209	24912-24920	323.19	3	966.54	A	DPIKVAEVP	Q	
210	24919-24939	442.67	5	2208.18	E	VPQPPGKITVDDVTRNSVSLS	W	
211	24921-24939	671.68	3	2012.05	P	QPPGKITVDDVTRNSVSLS	W	
212	24924-24932	474.27	2	946.50	P	GKITVDDVT	R	
213	24934-24945	462.14	3	1383.68	R	NSVSLSWTKPEH	D	
214	24941-24953	705.49	2	1408.73	W	TKPEHDGGSKIIQ	Y	
215	24943-24951	313.90	3	938.45	K	PEHDGGSKI	I	
216	24955-24970	618.35	3	1851.85	Y	IVEMQAKNTDKWSECA	R	
217	24961-24971	669.17	2	1336.62	A	KNTDKWSECAR	V	
218	24963-24973	661.88	2	1321.64	N	TDKWSECARVK	S	
219	24965-24975	653.85	2	1305.69	D	KWSECARVKSL	D	
220	24965-24978	796.44	2	1590.82	D	KWSECARVKSLDAV	I	
221	24970-24982	700.34	2	1398.82	C	ARVKSLDAVITNL	T	
222	24972-24981	530.29	2	1058.60	R	VKSLDAVITN	L	
223	24972-24983	425.31	3	1272.73	R	VKSLDAVITNLT	Q	
224	24973-24983	587.85	2	1173.66	V	KSLDAVITNLT	Q	
225	24982-24990	367.23	3	1098.52	N	LTQGEEYLF	R	
226	24998-25006	508.25	2	1014.56	E	KGRSDPRSL	A	
227	24998-25007	362.88	3	1085.59	E	KGRSDPRSLA	V	
228	25002-25010	323.22	3	966.55	S	DPRSLAVPI	I	
229	25003-25012	346.20	3	1035.64	D	PRSLAVPIIA	K	
230	25011-25022	684.35	2	1366.78	I	IAKDLVIEPDVR	P	
231	25015-25027	715.54	2	1428.76	D	LVIEPDVRPAFSS	Y	
232	25016-25029	783.91	2	1565.77	L	VIEPDVRPAFSSYS	V	
233	25018-25024	392.25	2	782.39	I	EPDVRPA	F	
234	25018-25028	634.30	2	1266.59	I	EPDVRPAFSSY	S	
235	25021-25042	608.85	4	2431.31	D	VRPAFSSYSVQVGQDLKIEVPI	S	
236	25026-25034	477.82	2	953.45	F	SSYSVQVGQ	D	
237	25026-25038	712.35	2	1422.74	F	SSYSVQVGQDLKI	E	
238	25029-25037	487.27	2	972.52	Y	SVQVGQDLK	I	
239	25029-25039	608.33	2	1214.65	Y	SVQVGQDLKIE	V	
240	25029-25051	487.69	5	2433.36	Y	SVQVGQDLKIEVPISGRPKPSIS	W	
241	25033-25046	503.62	3	1507.84	V	GQDLKIEVPISGRP	K	
242	25034-25043	381.23	3	1140.64	G	QDLKIEVPIS	G	
243	25037-25046	548.33	2	1094.64	L	KIEVPISGRP	K	
244	25037-25051	804.41	2	1606.94	L	KIEVPISGRPKPSIS	W	
245	25037-25053	632.33	3	1894.07	L	KIEVPISGRPKPSISWT	K	

Table 2 (continued)

246	25050-25062	752.87	2	1503.78	S	ISWTKDGMPLKQT	T					
247	25053-25063	610.18	2	1218.63	W	TKDGMPLKQTT	R					
248	25055-25066	687.36	2	1372.71	K	DGMPLKQTTRIN	V					
249	25060-25074	568.94	3	1703.91	L	KQTTRINVTDSLDLT	T					
250	25069-25086	663.38	3	1986.97	T	DSLDLTTLSIKETHKDDG	G					
251	25076-25094	683.37	3	2047.02	T	LSIKETHKDDGGQYGITVS	N					
252	25081-25092	431.26	3	1290.58	E	THKDDGGQYGIT	V					
253	25083-25090	420.23	2	838.35	H	KDDGGQYG	I					
254	25083-25091	476.59	2	951.43	H	KDDGGQYGI	T					
255	25099-25110	439.25	3	1314.74	G	QKTAAIEIITLD	K					
256	25099-25112	514.28	3	1539.89	G	QKTAAIEIITLDKP	D					
257	25100-25109	536.80	2	1071.65	Q	KTAAIEIITL	D					
258	25111-25118	418.24	2	834.46	D	KPDPPKGP	V					
259	25111-25121	605.35	2	1208.69	D	KPDPPKGPVKF	D					
260	25111-25124	522.99	3	1565.85	D	KPDPPKGPVKFDEI	S					
261	25114-25126	692.87	2	1383.74	D	PPKGPVKFDEISA	E					

[a]Position of the identified peptides inside the titin sequence. [b]Molecular ion mass observed in the ESI-LC-MS/MS analysis calculated in Daltons. [c]Calculated relative molecular mass of the matched peptide. [d]Position of the amino acid residue preceding the peptide sequence. [e]Position of the amino acid residue following the peptide sequence. [f]Peptides identified at each time of ham processing using nESI-LC-MS/MS are indicated in grey, and those detected by both nESI-LC-MS/MS and MALDI-TOF MS are shown in black.

Table 3. Peptides identified by nESI-LC-MS/MS and detected by MALDI-TOF MS from position 34807 to 34953 of the titin protein in dry-cured ham (UniprotKB/TrEMBL protein database accession number A2ASS6 of *Mus musculus* and corresponding to accession number Q9N251 of *Sus scrofa*).

Peptide number	Position[a]	Observed (m/z)[b]	Charge (+)	Calculated (Da)[c]	P_o[d]	Sequence	P_f[e]	nESI-LC-MS/MS Processing times (months)[f]				
								2	3.5	5	6.5	9
262	34807-34815	521.28	2	1040.55	E	KSIVHEEVT	K					
263	34807-34824	667.71	3	2000.01	E	KSIVHEEVTKTSQASEEV	K					
264	34807-34834	1037.27	3	3108.63	E	KSIVHEEVTKTSQASEEVKTHAEIKTLS	T					
265	34811-34821	406.25	3	1215.57	V	HEEVTKTSQAS	E					
266	34821-34831	424.22	3	1269.66	A	SEEVKTHAEIK	T					
267	34822-34831	395.24	3	1182.62	S	EEVKTHAEIK	T					
268	34827-34833	406.24	2	810.46	T	HAEIKTL	S					
269	34837-34853	596.32	3	1785.99	Q	MNITKGQRATLKANIAG	A					
270	34846-34858	651.52	2	1300.74	A	TLKANIAGATDVK	W					
271	34852-34866	787.40	2	1572.81	I	AGATDVKWVLNGTEL	P					
272	34859-34870	453.47	3	1357.65	K	WVLNGTELPNSE	E					
273	34868-34888	782.64	3	2345.11	P	NSEEYRYGVSGSDQTLTIKQA	S					
274	34874-34885	620.87	2	1239.60	R	YGVSGSDQTLTI	K					
275	34888-34910	810.70	3	2429.22	Q	ASHREEGILSCIGKTSQGVVKCQ	F					
276	34901-34917	628.35	3	1881.95	G	KTSQGVVKCQFDLTLSE	E					
277	34903-34914	442.24	3	1323.65	T	SQGVVKCQFDLT	L					
278	34908-34923	599.34	3	1794.83	V	KCQFDLTLSEELSDAP	S					
279	34916-34930	559.54	3	1675.81	L	SEELSDAPSFITQPR	S					
280	34922-34928	382.22	2	762.39	D	APSFITQ	P					
281	34925-34936	723.90	2	1445.73	S	FITQPRSQNINE	G					
282	34926-34945	759.61	3	2276.09	F	ITQPRSQNINEGQNVLFSCE	V					
283	34936-34962	610.74	5	3048.42	N	EGQNVLFSCEVSGEPSPEIEWFKNNLP	I					
284	34938-34948	591.82	2	1181.54	G	QNVLFSCEVSG	E					
285	34939-34950	640.84	2	1279.58	Q	NVLFSCEVSGEP	S					
286	34940-34951	627.27	2	1252.56	N	VLFSCEVSGEPS	P					
287	34941-34953	690.71	2	1379.59	V	LFSCEVSGEPSPE	I					

[a]Position of the identified peptides inside the titin sequence. [b]Molecular ion mass observed in the ESI-LC-MS/MS analysis calculated in Daltons. [c]Calculated relative molecular mass of the matched peptide. [d]Position of the amino acid residue preceding the peptide sequence. [e]Position of the amino acid residue following the peptide sequence. [f]Peptides identified at each time of ham processing using nESI-LC-MS/MS are indicated in grey, and those detected by both nESI-LC-MS/MS and MALDI-TOF MS are shown in black.

Table 4. Peptides identified by nESI-LC-MS/MS and detected by MALDI-TOF MS from position 35016 to 35167 of the titin protein in dry-cured ham (UniprotKB/TrEMBL protein database accession number A2ASS6 of *Mus musculus* and corresponding to accession number Q9N250 of *Sus scrofa*).

Peptide number	Position[a]	Observed (m/z)[b]	Charge (+)	Calculated (Da)[c]	P_o[d]	Sequence	P_f[e]	nESI-LC-MS/MS Processing times (months)[f] 2	3.5	5	6.5	9
288	35016-35035	726.62	3	2177.13	L	VEEPPREVVLKTSSDVSLHG	S					
289	35017-35028	692.36	2	1382.74	V	EEPPREVVLKTS	S					
290	35023-35032	517.81	2	1033.57	E	VVLKTSSDVS	L					
291	35032-35039	387.20	2	772.37	V	SLHGSVSS	Q					
292	35032-35049	924.48	2	1846.88	V	SLHGSVSSQSVQMSASKQ	E					
293	35038-35074	766.99	5	3829.64	V	SSQSVQMSASKQEASFSSFSSSSASSMTEMKFASMSA	Q					
294	35040-35049	547.29	2	1092.52	S	QSVQMSASKQ	E					
295	35043-35053	607.31	2	1212.54	V	QMSASKQEASF	S					
296	35049-35063	499.26	3	1494.61	K	QEASFSSFSSSSASS	M					
297	35060-35080	739.61	3	2215.89	S	SASSMTEMKFASMSAQSMSSM	Q					
298	35080-35097	662.60	3	1984.82	S	MQESFVEMSSSSFMGKSS	M					
299	35082-35093	684.13	2	1366.54	Q	ESFVEMSSSSFM	G					
300	35087-35100	499.31	3	1494.62	E	MSSSSFMGKSSMTQ	L					
301	35095-35103	505.80	2	1009.48	G	KSSMTQLES	S					
302	35095-35106	643.37	2	1284.59	G	KSSMTQLESSTS	R					
303	35095-35107	481.28	3	1440.69	G	KSSMTQLESSTSR	M					
304	35095-35110	605.27	3	1812.91	G	KSSMTQLESSTSRMLK	A					
305	35095-35117	808.15	3	2421.25	G	KSSMTQLESSTSRMLKAGGRGIP	P					
306	35101-35138	788.06	5	3935.14	Q	LESSTSRMLKAGGRGIPPKIEALPSDISIDEGKVLTVA	C					
307	35106-35119	734.43	2	1466.85	T	SRMLKAGGRGIPPK	I					
308	35109-35118	483.44	2	964.58	M	LKAGGRGIPP	K					
309	35110-35122	647.36	2	1292.76	L	KAGGRGIPPKIEA	L					
310	35110-35124	501.95	3	1502.89	L	KAGGRGIPPKIEALP	S					
311	35115-35123	313.29	3	936.56	R	GIPPKIEAL	P					
312	35127-35135	325.29	3	972.55	D	ISIDEGKVL	T					
313	35128-35145	919.02	2	1835.90	I	SIDEGKVLTVACAFTGEP	T					
314	35129-35139	383.20	3	1146.60	S	IDEGKVLTVAC	A					
315	35133-35145	668.35	2	1334.69	G	KVLTVACAFTGEP	T					
316	35135-35142	413.33	2	824.41	V	LTVACAFT	G					
317	35140-35160	764.64	3	2291.10	C	AFTGEPTPEITWSCGGRKIQN	Q					
318	35145-35155	574.33	2	1146.50	E	PTPEITWSCGG	R					
319	35146-35156	603.93	2	1205.55	P	TPEITWSCGGR	K					
320	35157-35167	459.28	3	1374.70	R	KIQNQEQQGRF	H					

[a]Position of the identified peptides inside the titin sequence. [b]Molecular ion mass observed in the ESI-LC-MS/MS analysis calculated in Daltons. [c]Calculated relative molecular mass of the matched peptide. [d]Position of the amino acid residue preceding the peptide sequence. [e]Position of the amino acid residue following the peptide sequence. [f]Peptides identified at each time of ham processing using nESI-LC-MS/MS are indicated in grey, and those detected by both nESI-LC-MS/MS and MALDI-TOF MS are shown in black.

All the peptide sequences identified in this study came from titin protein of mouse (*Mus musculus*) species according to UniProt protein database (accession number A2ASS6). In porcine species titin has not been completely sequenced and only four fragments of the whole protein sequence have been characterised (UniProt database with accession numbers O97771, Q29117, Q9N251, and Q9N250), which have been searched in BLAST to compare the sequence similarity with titin protein of *Mus musculus*. The titin fragment with accession number O97771 characterised in *Sus scrofa* species showed alignment to the sequence of *Mus musculus* titin protein in the fragment comprised from position 12704 to 12923, the Q29117 *Sus scrofa* fragment was aligned to *Mus musculus* titin protein from position 24600 to 25126, the Q9N251 *Sus scrofa* fragment from position 34807 to 34953 of *Mus musculus* titin sequence, and the Q9N250 fragment from position 35016 to 35167. Thus, BLAST revealed 82.9%, 93.7%, 82.4%, and 93.8% of homology between the corresponding sequence fragments in mouse species and the fragments of titin sequenced in *Sus scrofa* with accession numbers O97771, Q29117, Q9N251, and Q9N250, respectively. **Figure 1** shows these alignments, as well as the peptides identified due to the enzymatic action in each sequence fragment, which are indicated with black arrows.

An extensive proteolysis takes place during the processing of dry-cured ham, in which the degradation of proteins due to the action of endogenous muscle peptidases releases a large number of peptides and free amino acids. These enzymes are endopeptidases, like cathepsins and calpains, and exopeptidases, such as aminopeptidases, carboxipeptidases, peptidyl peptidases and peptidases (Toldrá & Flores, 1998). Calpains are widely distributed in the cytosol and the Z-disc region, and titin protein spans from Z-line to M-line region of sarcomeres. Thus, calpains have an important role in the initial breakdown of Z-discs together with the hydrolysis of titin (Koohmaraie & Geesink, 2006), but only during the early steps of dry-cured processing because they are inactivated after the salting stage (Rosell & Toldrá, 1996). Besides, endopeptidases cathepsins and subsequently exopeptidases would be responsible for titin degradation along the dry-curing ham process.

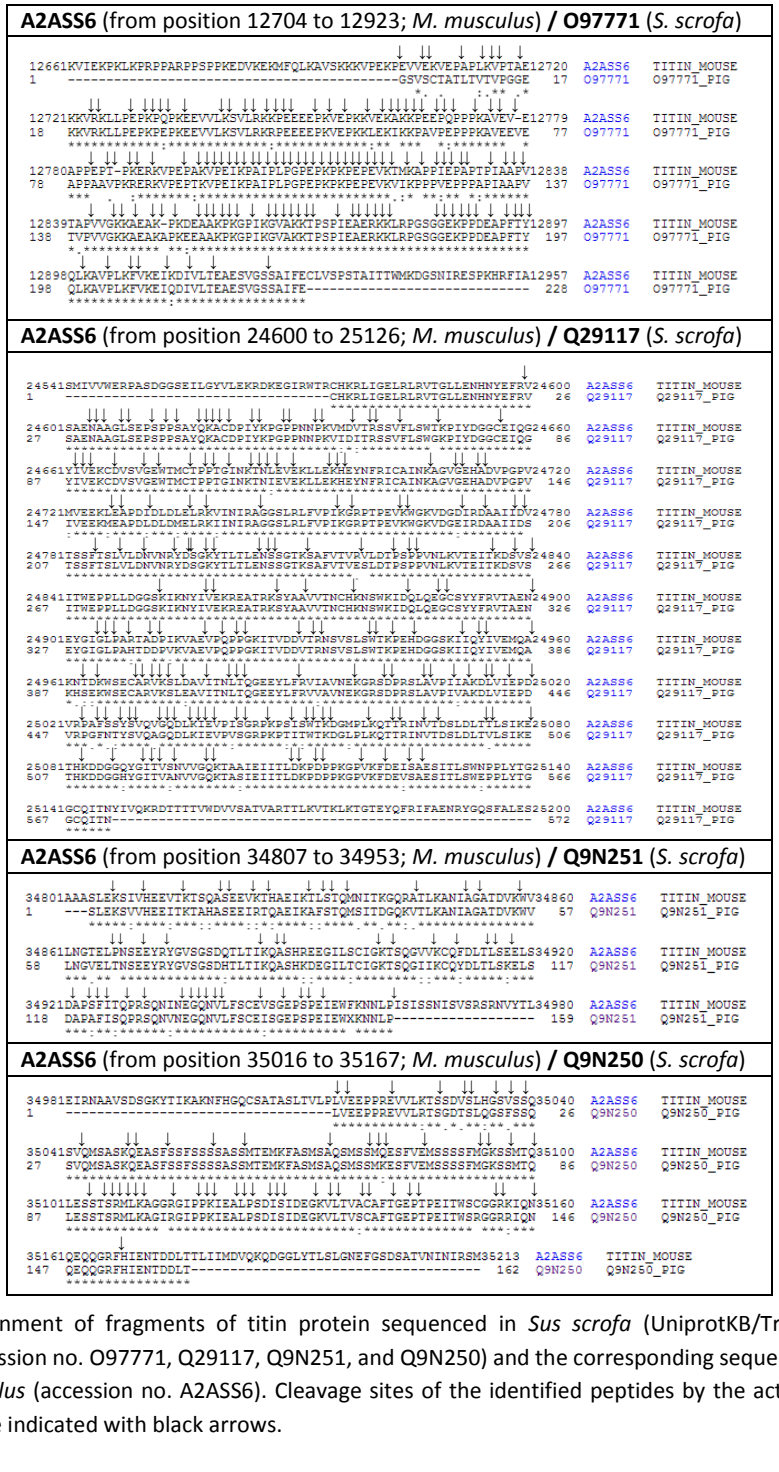


Figure 1. Alignment of fragments of titin protein sequenced in *Sus scrofa* (UniprotKB/TrEMBL protein database accession no. O97771, Q29117, Q9N251, and Q9N250) and the corresponding sequence fragments in *Mus musculus* (accession no. A2ASS6). Cleavage sites of the identified peptides by the action of muscle peptidases are indicated with black arrows.

Previous studies have shown that cathepsins hydrolyse proteins into large polypeptides, and they remain stable during the full process, except cathepsin D which is active up to 6 months after processing (Toldrá, Rico, & Flores, 1993) and quite inhibited by sodium chloride present in the ham (Rico, Flores & Toldrá, 1990). On the other hand, exopeptidases are involved in the latter stages of proteolysis, degrading polypeptides to small peptides and free amino acids. Only aminopeptidases and dipeptidyl peptidases have been studied throughout the processing of dry-cured ham, showing good activity along the full process (Toldrá, Flores, & Sanz, 1997; Sentandreu & Toldrá, 2001).

As can be observed in **Tables 1 to 4**, from the total of 320 peptides only 57 have been detected solely at 9 months of dry-cured processing by nESI-LC-MS/MS. These naturally generated peptides could be used as biomarkers to estimate the time of processing of hams, indicating a minimum curing time of 9 months. Samples of dry-cured ham at different times of processing were also analysed by using MALDI-TOF MS technique, which allows determining the molecular masses of the peptides. So, these masses were compared which those of peptides obtained by using nESI-LC-MS/MS in order to check the presence of the possible biomarkers. Thus, the molecular masses of the peptides obtained with MALDI-TOF MS were compared with those of peptides identified by nESI-LC-MS/MS in order to both confirm their exclusive presence at 9 months of curing discarding those peptides appearing at different times, and test the absence of any interfering ions nearby. A previous study done by Mora et al. (2009) showed evidence of a high degree of ionisation of titin protein peptides by MALDI-TOF MS methodology. In that work, five peptides generated from titin in a dry-cured ham of 9 months of curing were identified by nESI-LC-MS/MS, and four of them were also detected by MALDI-TOF MS. In our study, the evolution of the degradation of titin has been analysed throughout the whole dry-curing process, and the spectra obtained by MALDI-TOF MS at each time of processing are shown in **Figure 2**.

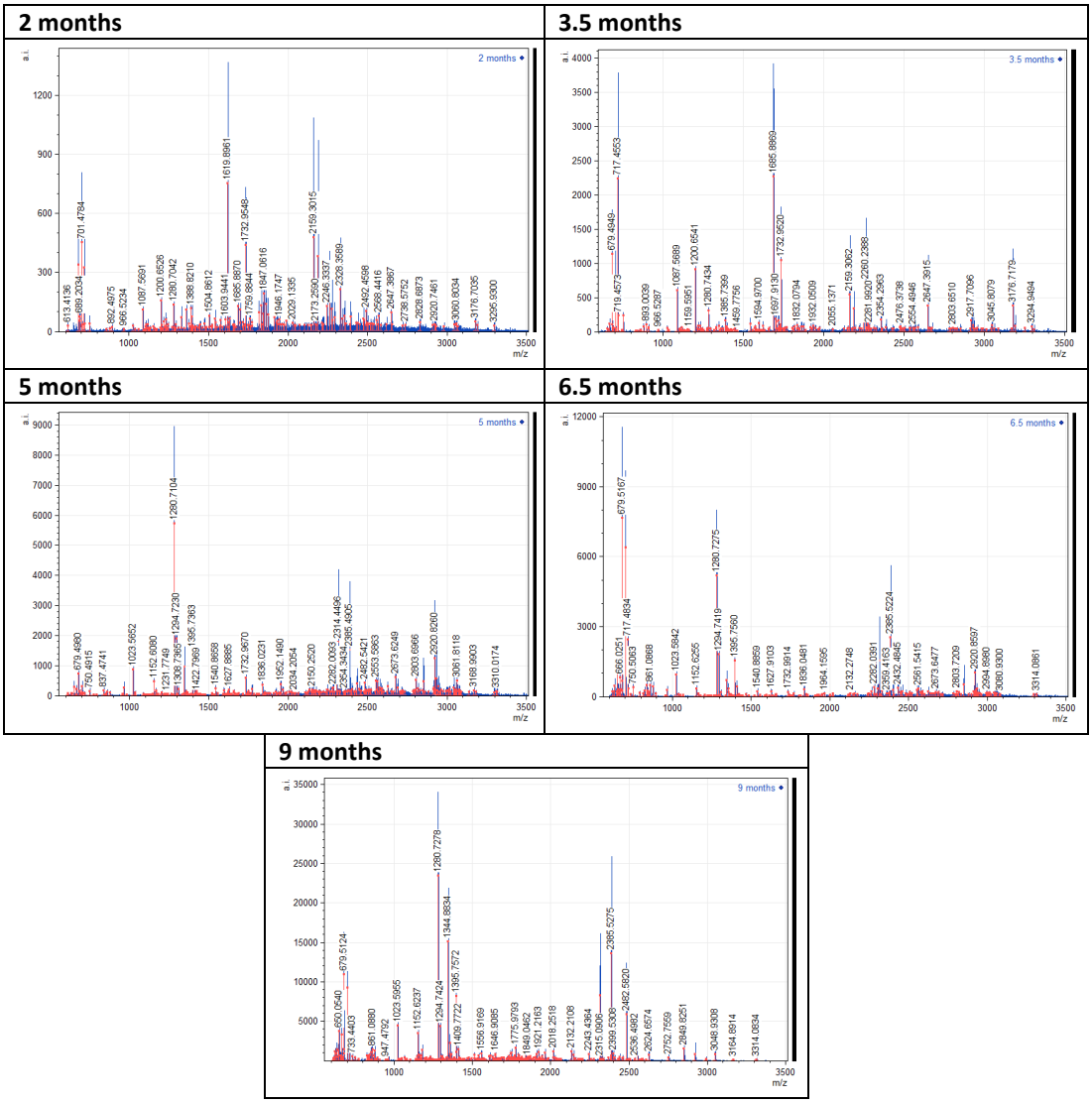


Figure 2. MS spectra obtained at different times of dry-cured ham processing by using MALDI-TOF MS. Numbers indicate *m/z* ([MH$^+$]) of the peptides derived from titin protein.

The analysis by MALDI-TOF MS system is highly effective in determining the molecular masses of peptides, being an economically accessible and fast method, with high mass resolution and accuracy, as well as no need of specialised personnel. On the other hand, nESI-LC-MS/MS system is a powerful tool for peptide identification, characterisation and quantification but the equipment is more expensive and complex to use than MALDI-TOF MS instrument, even though it provides very high resolution, accuracy and detection sensitivity (Careri, Bianchi, & Corradini, 2002; Careri & Mangia, 2003; Contreras, López-Expósito, Hernández-Ledesma, Ramos, & Recio, 2008).

Among the 57 peptides identified at 9 months of processing by nESI-LC/MS-MS, 16 of them have been also detected at this time using MALDI-TOF MS technique. However, most of these sixteen peptides have been detected by MALDI-TOF MS also at other previous times of curing, and only four of them have been solely detected at 9 months. Therefore, only peptides number 113 (KDEAAKPKGPIKGVAKK), 139 (KKLRPGSGGEK), 217 (KNTDKWSECAR) and 312 (ISIDEGKVL), identified by using nESI-LC-MS/MS, and also detected by MALDI-TOF MS can be considered to appear exclusively at 9 months of curing.

These differences in detection by using both techniques could be due to ESI which is a very different ionisation process than MALDI since it is a liquid technique that may produce multiple charged ions, extending the mass range use of the analyser. Many authors during the last decade have studied the optimal choice of the ionisation technique (ESI or MALDI) depending on the mass spectrometry needs enhancing their complementarity, as results obtained when compared are different depending on the characteristics of the studied peptides (Bodnar, Blackburn, Krise, & Moseley, 2003; Yang et al., 2007; Zaia, 2010). Moreover, amino acid composition of peptides could result in different behaviour in their ionisation, so that MALDI preferentially ionises peptides that contain basic and aromatic amino acids, and ESI tends to ionise those with hydrophobic amino acids (Stapels & Barofsky, 2004).

In this case, the detection of peptides KDEAAKPKGPIKGVAKK, KKLRPGSGGEK, KNTDKWSECAR, and ISIDEGKVL identified and confirmed by using nESI-LC-MS/MS and MALDI-TOF MS, could be a good indicative for a minimum curing time of 9 months. Moreover, MALDI-TOF MS could be used as an accessible, fast, and sensitive technique to check the presence of these potential biomarkers, which could be used to control the time of curing in dry-cured hams and thus prevent possible economic frauds and ensure the safety and quality of this type of product.

CONCLUSIONS

The use of modern proteomic techniques is a powerful tool to study the peptides naturally generated due to the intense proteolysis that occurs along the processing of dry-cured ham. In this work, nESI-LC-MS/MS was used to analyse the degradation of titin protein at different times of processing, whereas MALDI-TOF MS was subsequently employed as a fast and easier technique to check the presence of those peptides only identified at 9 months of time of processing that could be used as potential biological markers of dry-curing time. So, peptides KDEAAKPKGPIKGVAKK, KKLRPGSGGEK, KNTDKWSECAR and ISIDEGKVL have been identified and confirmed by using nESI-LC-MS/MS and MALDI-TOF MS, confirming them as good markers for a minimum curing time of 9 months.

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Capítulo 4

Evidence of peptide oxidation from major myofibrillar proteins in dry-cured ham

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Evidence of peptide oxidation from major myofibrillar proteins in dry-cured ham

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Abstract

In this study, a peptidomic approach has been used in the identification of naturally generated peptides during a dry-curing process, showing methionine (Met) oxidation in their sequence. A total of 656 peptides derived from major myofibrillar proteins in Protected Designation of Origin (PDO) Teruel dry-cured ham have been identified by nanoliquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS), including 120 peptides showing methionine oxidation. The percentage of oxidised peptides in the studied proteins ranged from 6 to 35%, being peptides derived from nebulin, titin, myosin heavy chains, and troponin I proteins, those showing the highest number of oxidised methionine. The identification of the peptide sequence incorporating the oxidised amino acid provides valuable information of neighbouring amino acids, degree of hydrolysis of the sample, and characteristics of the peptide, which might be very useful for a future better understanding of the oxidation mechanisms occurring in dry-curing processing.

Keywords: Methionine; Oxidation; Peptides; Dry-cured ham; Proteomics; Mass spectrometry.

INTRODUCTION

Teruel dry-cured ham was the first Spanish meat product with accepted Protected Designation of Origin (PDO). This denomination was created to control and guarantee the traditional manufacture that yields in high quality dry-cured ham with typical organoleptic and nutritional characteristics. The final product is the result of numerous and complex biochemical reactions which take place along the fourteen months of Teruel dry-cured ham processing. Proteolysis is the most important reaction that generates large amounts of peptides and free amino acids from the degradation of myofibrillar and sarcoplasmic proteins by the action of endogenous muscle peptidases (Toldrá, Rico, & Flores, 1993; Aristoy, & Toldrá, 1995; Toldrá, & Flores, 1998; Lametsch et al., 2003).

Oxidation of lipids and proteins is the main cause for quality deterioration during processing and storage of meat products, causing changes in nutritional value and sensory traits (Ladikos, & Lougovois, 1990; Lund, Heinonen, Baron, & Estévez, 2011). However, lipid oxidation is also essential in the maturation process and the desirable flavour of dry-cured hams, being this process extensively studied (Antequera, et al., 1992; Toldrá, & Flores, 1998; Ruiz, Ventanas, Cava, Andrés, & García, 1999; Gandemer, 2002). On the other hand, protein oxidation has been less studied in dry-cured hams, even though certain increase from the salting stage, tending to stabilise towards the ham maturation period, have been reported (Armenteros, Heinonen, Ollilainen, Toldrá, & Estévez, 2009; Estévez, 2011; Wang et al., 2011; Koutina, Jongberg, & Skibsted, 2012). In fact, the oxidation of muscle proteins comprises colour, flavour and texture deterioration as well as the loss of essential amino acids, and changes in conformation and functionality of proteins due to oxidative changes, that includes (i) formation of covalent intermolecular cross-linked protein derivatives, (ii) cleavage of peptide bonds, and (iii) modification of amino acid side chains (Estévez, Ventanas, & Cava, 2005; Estévez, 2011; Lund, et al., 2011; Zhang, Xiao, & Ahn, 2013).

During the last few decades, most of the studies about protein oxidation have been focused on the role played by oxidised proteins in different human

diseases, whereas the mechanisms of protein oxidation in food systems are largely unknown (Elias, Kellerby, & Decker, 2008). The oxidation of essential amino acids has been a concern, not only because of its effect on meat quality, but also on its nutritive value due to the decreased availability of essential amino acids and poorer digestibility of oxidized proteins (Lund, et al., 2011). However, although the oxidation of proteins has been described, no information about the identification of oxidised peptides generated during dry-cured ham processing has been reported to date.

Methionine is one of the most susceptible amino acids to oxidation by almost all forms of reactive oxygen species because of its reactive sulfur atom, leading to modifications in structure of proteins and loss of enzyme activity (Zhang, et al., 2013). The first and the main oxidation product formed is methionine sulfoxide, which can be further oxidised to methionine sulfone although in a lesser amount (Vogt, 1995; Shacter, 2000). However, the oxidation can be reversible due to the action of methionine sulfoxide reductases, which reduce methionine sulfoxides to methionine residues. Moreover, this cyclic oxidation-reduction process could act in antioxidant mechanisms and in regulation of cellular processes (Levine, Mosoni, Berlett, & Stadtman, 1996; Ghesquière & Gevaert, 2014; Kim, Weiss, & Levine, 2014).

In the present work, a peptidomic strategy has been used to identify and sequence those naturally generated peptides showing methionine oxidation in PDO Teruel dry-cured ham, focusing on those peptides derived from the degradation of the major myofibrillar proteins along the dry-curing process.

MATERIALS AND METHODS

Materials

Dry-cured hams manufactured according to the specifications of PDO Teruel (Spain) were used in this study. Hams were obtained from white-breed pigs (maternal line Landrace x Large White, and paternal line Duroc) and prepared according to the traditional process, for a total time of processing of fourteen months.

Extraction of peptides

A total of 100 g of *Biceps femoris* muscle from the dry-cured hams were minced and homogenised with 500 mL of 0.01 N HCl for 5 min using an Ultra-Turrax® T-25 (IKA®-Werke, Germany). The homogenate was kept at 4 ºC overnight for decanting, and the supernatant was filtered to retain the large pieces, firstly through a plastic mesh, and secondly through a qualitative filter paper (Whatman™, UK). Afterwards, the sample was freeze-dried and desalted by solid phase extraction using an Oasis® HLB cartridge (35 cc, Waters, Ireland) in which peptides were retained and then eluted with methanol:water (95:5, v/v). Finally, the sample was lyophilised for the following analysis by tandem mass spectrometry.

Peptide identification by nESI-LC-MS/MS

The identification of the peptides was performed by nanoliquid chromatography-tandem mass spectrometry using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) and a quadrupole/time-of-flight (Q/TOF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (nESI).

Desalted and lyophilised samples were resuspended to a concentration of 10 mg/mL with 0.1% of TFA and centrifuged in cold conditions at 200*g* for 3 min. Then, 15 µL of the sample were cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) according to the manufacturer recommendations. Peptides eluted from Zip-Tip were dried and then resuspended in 20 µL of 0.1% of TFA.

The mass spectrometry analysis was done according to the methodology described by Gallego, Mora, Aristoy, and Toldrá (2015). Five microliters of the sample were injected into the nESI-LC-MS/MS system, and then preconcentrated on an Eksigent C18 trap column (3 µm, 350 µm x 0.5 mm; Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min for 5 min, using 0.1% v/v TFA as mobile phase. The trap column was automatically switched in-line onto a nano-HPLC capillary column (3 µm, 75 µm x 12.3 cm, C18; Nikkyo Technos Co, Ltd. Japan).

Mobile phases consisted of solvent A, containing 0.1% v/v formic acid in water, and solvent B, containing 0.1% v/v formic acid in 100% acetonitrile. The HPLC conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 µL/min and temperature of 30 ºC.

The column outlet was directly coupled to a nanoelectrospray ionisation system (nESI). The Q/TOF was operated in positive polarity and information-dependent acquisition mode, in which a 0.25-s TOF MS scan from *m/z* of 100 to 1250 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to 1500 on the 50 most intense 1 - 5 charged ions.

Data analysis

Mascot Distiller v2.5.1. software (Matrix Science, Inc., Boston, MA, USA; http://www.matrixscience.com) has been used for automated spectral processing, peak list generation, and database search. The identification of peptides has been performed using UniProt database. The taxonomy parameter was designated as Mammalia, and oxidation of methionine (M) was selected as variable modification. Other amino acid oxidations such as cysteine (C), histidine-tryptophan (HW), and proline (P) were also tested, with non-significant results. Generated MS/MS spectra were searched using a significant threshold of $p < 0.1$, a FDR of 1.5%, and a peptide tolerance on the mass measurements of 0.3 Da in MS mode and MS/MS ions.

RESULTS AND DISCUSSION

Teruel dry-cured ham peptide extracts have been analysed by nESI-LC-MS/MS to identify those peptides showing oxidation in their methionine residues. As a result of the intense proteolysis which takes place along the dry-curing process of ham, approximately 62,000 peptides have been identified, including more than 17,700 which contain oxidations of methionine. In this study, only peptides derived from the degradation of major myofibrillar proteins were selected, comprising 120 peptides with methionine oxidation, from a total of 656 peptides,

resulting from the degradation of α-actin (ACTS), nebulin (NEBU), titin (TITIN), troponin I fast skeletal muscle (TNNI2), two types of myosin light chain proteins (myosin regulatory light chain 2 (MLRS) and myosin light chain (MYL1)), and two types of myosin heavy chain proteins (myosin-1 (MYH1) and myosin-7 (MYH7)).

Tables 1 and 2 show the sequences of the oxidised peptides, the protein of origin and the species in which it has been described, the observed and calculated masses together with the charge states, amino acid residues preceding and following the sequence, and the number of methionine oxidations detected in the peptide. Some of the identified peptides have been detected both with and without methionine oxidations (see those marked with an asterisk in **Tables 1 and 2**). This differs from results of an earlier elution of the oxidised peptides from reversed-phase columns and a molecular mass increase of 16 Da for the respective non-oxidised peptides (Liu, Ponniah, Neill, Patel, & Andrien, 2013). Three, two, four, and eight peptides derived from ACTS, MLRS, MYH1, and MYL1 proteins, respectively, were present in both oxidised and non-oxidised forms. Differences on both types of spectrums are described in **Figure 1**, where the MS/MS spectrums of the peptide DKEGNGTVMGAELR with and without methionine oxidation from MYL1 protein are shown.

Cysteine and methionine are the most susceptible amino acids to oxidative changes (Zhang, et al., 2013). Nevertheless, Cys, His-Trp and Pro oxidations were also included in the search, with the result that only Met oxidations were detected.

Table 1. Oxidised peptides identified by nESI-LC-MS/MS from actin, titin and troponin I proteins in dry-cured ham.

Protein name[a]	Species	Observed (m/z)[b]	Calculated (mr)[c]	Charge (+)	P_o[d]	Sequence	Pf[e]	No ox[f]
ACTS	BOVIN	293.21	876.45	3	G	IADRMQK	E	1
		530.25	1058.47	2	F	IGMESAGIHE*	T	1
		589.31	1176.61	2	K	EITALAPSTMK*	I	1
		608.31	1214.56	2	P	RHQGVMVGMGQ	K	1
		636.29	1270.55	2	M	VGMGQKDSYVGD*	E	1
		671.32	1340.60	2	M	SGGTTMYPGIADR	M	1
		464.24	1389.59	3	D	LYANNVMSGGTTM	Y	2
		477.82	1430.58	3	Y	ANNVMSGGTTMYPG	I	2
		615.32	1842.93	3	L	TEAPLNPKANREKMTQ	I	1
		716.33	2145.98	3	D	LYANNVMSGGTTMYPGIADR	M	1
		856.54	2566.35	3	H	AIMRLDLAGRDLTDYLMKILTE	R	1
TITIN	HUMAN	561.26	1120.51	2	A	RMSPARMSPG	R	2
		355.07	708.35	2	E	VKSQMT	E	1
		253.18	756.28	3	A	SMSAQSM	S	1
		405.26	808.37	2	K	ASDRLTM	K	1
		419.31	836.41	2	K	LTVLNMM	K	1
		432.24	862.47	2	P	VVTNRKM	C	1
		439.24	876.45	2	D	HGLYMIK	V	1
		318.52	952.41	3	S	VEVMETET	A	1
		325.20	972.49	3	G	RPVPAMTW	F	1
		327.22	978.51	3	R	IDLSVAMKS	L	1
		338.15	1011.46	3	R	TYIPVMSGE	N	1
		514.28	1026.48	2	I	DDYLAMKR	T	1
		531.26	1060.43	2	H	GERYQMDF	L	1
		359.19	1074.61	3	S	ASIMVKAINI	A	1
		575.29	1148.57	2	I	DLSTMPQKTI	H	1
		384.16	1149.59	3	R	VDKYKHMLT	I	1
		393.23	1176.57	3	P	IRMSPARMSP	A	2
		598.31	1194.54	2	N	ADISMGDVATLS	V	1
		399.22	1194.58	3	T	FQVMAVNSAGR	S	1
		629.44	1256.67	2	K	EMRLNVLGRPG	P	1
		650.68	1299.49	2	T	CQATNDVGKDMC	S	1
		441.30	1320.64	3	R	IAAENSIGMGPFV	E	1
		669.37	1336.65	2	S	LEVTNIAKDSMT	V	1
		465.77	1394.55	3	T	EMKFASMSAQSM	S	3
		466.24	1395.63	3	S	SQSVQMSASKQEA	S	1
		469.25	1404.70	3	S	FVQKPDPMDVLT	G	1
		490.26	1467.81	3	R	LMVSVSGRPPPVIT	W	1
		746.85	1491.74	2	L	MRKTQASTVATGPE	V	1
		500.84	1499.66	3	L	ISQSERCSITMTE	K	1
		504.28	1509.72	3	A	RLMIREAFAEDSG	R	1
		507.94	1520.82	3	T	LTVKNASGTKAVSVM	V	1
		510.31	1527.73	3	D	YELLMKSQQEML	Y	1
		523.34	1566.72	3	I	KHGERYQMDFLQ	D	1
		530.28	1587.78	3	F	QVMAVNSAGRSAPRE	S	1
		533.27	1596.83	3	K	VPVTMTRYRSTGLT	E	1
		543.28	1626.77	3	E	PPEIDMKNFPSHTV	Y	1
		545.30	1632.75	3	K	GSMLVSWTPPLDNGGS	P	1
		563.31	1686.78	3	E	SYVIEMLKTGTDEW	V	1
		564.29	1689.77	3	Q	WTKPVYDGGSMITGY	I	1
		567.53	1699.71	3	S	SFSSSSASSMTEMKFA	S	1
		871.45	1740.90	2	K	AMTLGVSYKVTGLIEGS	D	1
		590.49	1768.71	3	S	MTEMKFASMSAQSMSS	M	1
		607.59	1819.91	3	P	AVIVEKAGPMTVTVGETC	T	1
		611.61	1831.82	3	G	LYICKAENMLGESTCAA	E	1
		616.33	1845.97	3	G	FIIERKDAKMHTWR	Q	1
		985.06	1967.95	2	E	NLYGISDPLVSDSMKAKD	R	1
		657.37	1968.84	3	D	GMTLTWYPPEDDGGSQVT	G	1
		988.49	1974.77	2	S	ASSMTEMKFASMSAQSMS	S	4
		718.35	2152.09	3	A	NLKMELRDALCAIIYEEI	D	1
		719.37	2155.18	3	D	KSAAVATVVAAVDMARVREPV	I	1
		778.47	2332.14	3	P	IRMSPARMSPARMSPARMSPA	R	2
		908.31	2722.09	3	S	SMTEMKFASMSAQSMSSMQESFVE	M	2
TNNI2	HUMAN	424.73	847.45	2	K	SVMLQIAA	T	1
		327.22	978.40	3	I	PGSMSEVQE	L	1

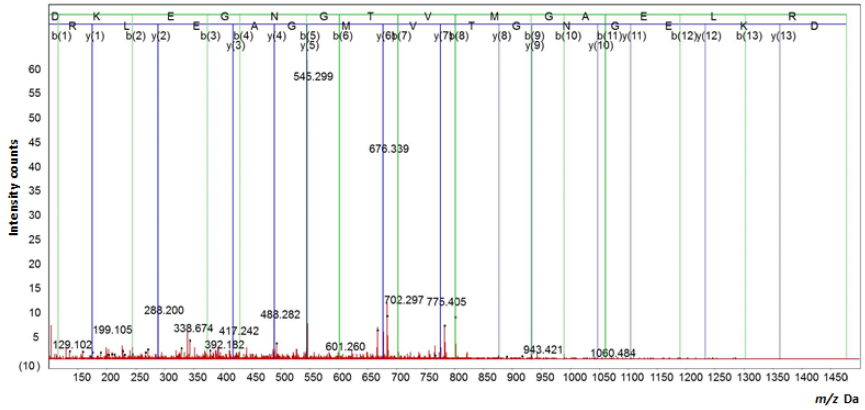
[a]Protein name according to Uniprot database. ACTS: Actin, alpha skeletal muscle; TITIN: Titin; TNNI2: Troponin I, fast skeletal muscle.[b] Molecular ion mass observed in the nESI-LC-MS/MS analysis calculated in Daltons. [c]Calculated relative molecular mass of the matched peptide. [d]Position of the amino acid residue preceding the peptide sequence. [e]Position of the amino acid residue following the peptide sequence. [f]Number of methionine oxidations in the identified peptide. *Sequences identified with and without methione oxidation.

Table 2. Oxidised peptides identified by nESI-LC-MS/MS from myosin and nebulin proteins in dry-cured ham.

Protein name[a]	Species	Observed (m/z)[b]	Calculated (mr)[c]	Charge (+)	P_o[d]	Sequence	Pf[e]	No ox[f]
MLRS	BOVIN	590.77	1179.53	2	D	AMMKEASGPIN	F	2
		605.81	1209.57	2	M	MKEASGPINFT*	V	1
		536.25	1605.66	3	R	AAAEGGSSSVFSMFDQ	T	1
		669.98	2006.93	3	N	VKNEELDAMMKEASGPIN*	F	2
MYH1	PIG	413.27	824.37	2	P	TSGKMQGT	L	1
		511.43	1020.57	2	E	KPMGIFSIL	E	1
		520.14	1038.47	2	R	VVESMQSML	D	1
		550.26	1098.50	2	K	TPGAMEHELV	L	1
		646.33	1290.67	2	Y	QKMVERRESI	F	1
		652.32	1302.61	2	I	IPNETKTPGAME*	H	1
		668.34	1334.63	2	E	DQVFPMNPPKF*	D	1
		759.37	1516.72	2	L	TVKEDQVFPMNPP*	K	1
		554.29	1659.85	3	K	IEDEQALAMQLQKK	I	1
		636.65	1906.91	3	L	TVKEDQVFPMNPPKFD*	K	1
		488.76	1950.87	4	N	EVEDLMIDVERSNAACAA	L	1
MYH7	PIG	537.28	1072.46	2	C	MFPKATDMT	F	2
		404.26	1209.58	3	T	KGQNVQQVMY	A	1
		407.86	1220.63	3	R	KELEEKMVSL	L	1
		656.35	1310.63	2	V	TKGQNVQQVMY	A	1
		665.42	1328.68	2	A	LAKAVYEKMFN	W	1
		522.28	1563.71	3	R	LEEAGGATSVQIEMN	K	1
		524.27	1569.77	3	I	QLSHANRMAAEAQK	Q	1
MYL1	BOVIN	557.25	1112.48	2	V	FDKEGNGTVM*	G	1
		585.76	1169.50	2	V	FDKEGNGTVMG*	A	1
		606.78	1211.55	2	R	VFDKEGNGTVM*	G	1
		635.29	1268.57	2	R	VFDKEGNGTVMG*	A	1
		670.81	1339.61	2	R	VFDKEGNGTVMGA*	E	1
		498.24	1491.70	3	F	DKEGNGTVMGAELR*	H	1
		543.92	1628.76	3	F	DKEGNGTVMGAELRH*	V	1
		554.29	1659.81	3	N	AEVKKVLGNPSNEEM	N	1
		592.29	1773.86	3	N	AEVKKVLGNPSNEEMN*	A	1
		615.94	1844.89	3	N	AEVKKVLGNPSNEEMNA	K	1
		696.54	2086.85	3	E	EVEALMAGQEDSNGCINYE	A	1
		818.40	2452.19	3	K	VLGNPSNEEMNAKKIEFEQFL	P	1
NEBU	HUMAN	401.22	800.39	2	V	KHAMEVA	K	1
		402.22	802.40	2	N	ALTMSKH	L	1
		452.23	902.48	2	S	PVDMLSIL	L	1
		302.20	903.45	3	L	SMLGRPDI	E	1
		911.62	910.41	1	K	FSSPVDML	G	1
		498.28	994.43	2	R	GKGLTEMED	T	1
		525.38	1048.51	2	H	TPLDMVSVTA	A	1
		351.22	1050.47	3	Y	TMSPDLPQF	L	1
		357.20	1068.48	3	K	SNYSIMLEP	P	1
		363.19	1086.50	3	Y	HTPADMLSVT	A	1
		551.30	1100.54	2	K	AHMLKTRND	Y	1
		408.90	1223.50	3	I	HTYNMLPDAM	S	2
		423.24	1266.68	3	G	KGKMVGFRSLE	D	1
		427.27	1278.62	3	L	LSRVNQITMSD	K	1
		714.42	1426.68	2	K	DSQLYKVMKDAN	N	1
		775.38	1548.70	2	E	KSMSYYETVLDTP	E	1
		535.62	1603.77	3	A	KMQSEREYKKDF	E	1
		821.49	1640.79	2	A	DYADFMKGIGWLPL	G	1
		555.37	1662.82	3	D	MLSVTAAKDAQANITN	T	1
		779.41	2335.20	3	L	EQVLAKNNALNMNKRLYTEA	W	1
		797.50	2389.29	3	V	IRKKVDPSKFMTPYIAHSQK	M	1

[a]Protein name according to Uniprot database. MLRS: Myosin regulatory light chain 2, skeletal muscle; MYH1: Myosin-1; MYH7: Myosin-7; MYL1: Myosin light chain 1/3, skeletal muscle isoform; NEBU: Nebulin. [b] Molecular ion mass observed in the nESI-LC-MS/MS analysis calculated in Daltons. [c]Calculated relative molecular mass of the matched peptide. [d]Position of the amino acid residue preceding the peptide sequence. [e]Position of the amino acid residue following the peptide sequence. [f]Number of methionine oxidations in the identified peptide. *Sequences identified with and without methione oxidation.

A) DKEGNGTVMGAELR (3+)



B) DKEGNGTV**M**GAELR (3+)

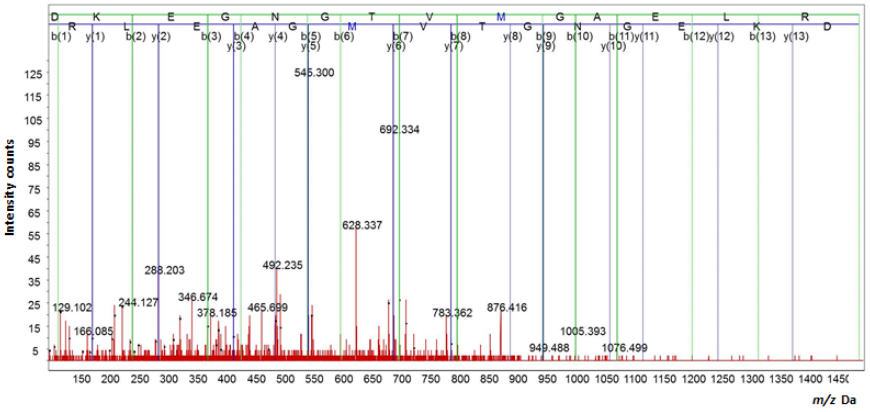


Figure 1. A) MS/MS spectrum of peptide DKEGNGTVMGAELR from myosin light chain protein (MYL1_BOVIN). B) Spectrum of same peptide showing the oxidised methionine in blue.

Table 3 lists the number of oxidised peptides compared to the total number of peptides identified for each protein as well as the total percentage of methionine amino acid residues on the studied sequences. Although theoretically, a high presence of Met residues on a sequence could indicate a higher predisposition for oxidation, not all methionine amino acid residues in a protein are susceptible in the same way, because it depends on their location within the protein structure and their accessibility to oxidants. In fact, the highest percentages of Met are present in ACTS, TNNI2, and both myosin light chain proteins (4.5%, 5.5%, 4.1%, and 3.4%, respectively), corresponding to the lowest percentages of identified oxidised peptides (11.6%, 22.2%, 5.9%, and 7.4%, respectively). So, the smallest of the studied proteins are those showing the highest sequence coverage when identifying the sequence of the generated peptides. On the other hand, heavier proteins such as TITIN, NEBU, or MYH present more complex structures that are difficult to hydrolyse, showing lower percentages of sequence coverage. TITIN, NEBU, and MYH showed the highest percentage of oxidation, comprising between 35% and 17% of the total number of peptides identified from their sequences. The neighbouring amino acid residues also determine methionine oxidation, being those surrounded by Ala, Thr, Ser, and acidic amino acids such as Asp and Glu, who are more susceptible to oxidation than Met located close to basic amino acids (Levine, et al., 1996; Ghesquière, & Gevaert, 2014). In this study, a total of 61 of the 120 oxidised peptides identified in dry-cured ham extract show Asp, Glu, Ala, Thr and Ser residues next to the oxidised Met. However, only 31 of the identified peptides showing oxidation had any of the basic amino acids Arg, Lys, and His next to the oxidised Met.

Table 3. Characteristics of studied proteins in relation to the Met oxidation identified from the dry-cured ham generated peptides.

Protein name[a]	Molecular mass (kDa)	Protein lenght (aa)	Number of Methionine	Percentage of Methionine (%)	Oxidised peptides[b]	Non-oxidised peptides[c]	Total number of peptides[d]	Percentage oxidation (%)	Sequence coverage[e] (%)
TITIN	3816.03	34350	398	1.15	52	240	292	17.81	30
NEBU	772.914	6669	185	2.77	21	39	60	35.00	23
MYH1	223.173	1939	52	2.68	11	51	62	17.74	30
MYH7	223.298	1935	52	2.68	7	22	29	24.14	32
ACTS	42.051	377	17	4.5	11	84	95	11.58	66
TNNI2	21.339	182	10	5.49	2	7	9	22.22	21
MYL1	19.505	176	6	3.4	12	150	162	7.41	80
MLRS	19.013	170	17	4.11	4	63	67	5.97	73

[a]Protein name according to Uniprot database. [b]Number of peptides showing Met oxidation identified in the protein. [c]Number of non-oxidised peptides identified in the protein. [d]Total number of peptides identified. [e]Percentage coverage of the protein sequence.

Methionine oxidation can be caused by different factors, being influenced by the oxidative stress suffered by the animal during slaughtering and by meat industry processing, sample preparation and analysis. In fact, technological operations such as pre-freezing, pre-mincing or packaging have been described to have a great impact on the oxidation of muscle proteins (Utrera & Estévez, 2013; Utrera, Parra & Estévez, 2014). On the other hand, methionine amino acids have also been described to be spontaneously oxidised during sample preparation, chromatography separation and ionisation of MS analysis (Liu, et al., 2013; Drazic, & Winter, 2014), although it has been established that these factors, when they occur, do not represent more than 2% of total amount of Met oxidations.

Recently, it has been shown that oxidative protein modifications in dry-cured products are accelerated during the first steps of processing and stabilised towards the final stages of maturation (Koutina, et al., 2012). The same authors also showed that oxidation may change the susceptibility of proteins to proteolytic enzymes in Parma ham, influencing the proteolysis reactions (Koutina, et al., 2012). Proteolysis, protein oxidation and peptidases activity have also been studied in Xuanwei ham, concluding that although oxidation occurs during salting stage, it is deccelerated due to the low temperatures employed (Wang, et al., 2011). Finally, Gallego, Mora, Fraser, Aristoy, and Toldrá (2014) reported the identification by LC-MS/MS of some peptides derived from LIM domain-binding protein, showing methionine oxidation in dry-cured hams at different times of ripening, identifying most of them at the end of the processing.

CONCLUSIONS

This study evidences the importance of methionine oxidation in dry-cured ham processing by identifying a total of 120 sequences of peptides, including a minimum of one oxidised Met in their sequence. The described peptidomic approach gives a novel point of view in the detection and identification of those peptides responsible for oxidation. Moreover, it provides valuable information of neighbouring amino acids, the degree of hydrolysis of the sample, the origins of

proteins affected, and characteristics of the peptide, which might be very useful to better understand and characterise the complex oxidation mechanisms occurring in dry-cured processing, as well as to detect possible changes in nutritional and sensory characteristics of the product.

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Capítulo 5

Optimisation of a simple and reliable label-free methodology for the relative quantitation of raw pork meat proteins

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Optimisation of a simple and reliable label-free methodology for the relative quantitation of raw pork meat proteins

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Abstract

Recent advances in proteomics have become an indispensable tool for a fast, precise and sensitive analysis of proteins in complex biological samples at both, qualitative and quantitative level. In this study, a label-free quantitative proteomic methodology has been optimised for the relative quantitation of proteins extracted from raw pork meat. So, after the separation of proteins by one-dimensional gel electrophoresis and trypsin digestion, their identification and quantitation have been done using nanoliquid chromatography coupled to a quadrupole/time-of-flight (Q/TOF) mass spectrometer. Relative quantitation has been based on the measurement of mass spectral peak intensities, which have been described that are correlated with protein abundances. The results obtained regarding linearity, robustness, repeatability and accuracy show that this procedure could be used as a fast, simple, and reliable method to quantify changes in protein abundance in meat samples.

Keywords: Label-free; Quantitation; Proteins; Proteomic; Peak intensity; Mass spectrometry; Raw pork meat.

INTRODUCTION

Mass spectrometry has become a fundamental tool among proteomic techniques to identify and precisely quantify proteins of complex biological samples such as meat and meat products (Aebersold & Mann, 2003; Cravatt, Simon, & Yates III, 2007).

Classical methodologies using one-dimensional (1D) or two-dimensional (2D) gel electrophoresis with different detection methods such as dyes, fluorophores or radioactivity have allowed the separation and quantitation of proteins through the measurement of stained spot intensities, providing good sensibility and linearity. However, the applicability of these methods is limited to abundant and soluble proteins when the aim is to achieve high-resolution protein separation, as well as they do not reveal the identity of the underlying proteins, and neither provide accurate results on changes of protein expression levels, especially in the case of overlapping proteins (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007; Szabo, Szomor, Foeldi, & Janaky, 2012). These difficulties are overcome by modern mass-spectrometry-based quantitation techniques, which can be separated into two categories: (i) the use of labelling methodologies that involve stable isotopes, and (ii) the use of label-free techniques. Labelling techniques are considered to be the most accurate in quantitating protein abundances, but they present some limitations as well as require expensive isotope labels, a large amount of starting material, and an increased complexity of experimental protocols. Moreover, some of the labelling techniques cannot be used in all types of samples due to the restricted number of available labels, which is deficient for the simultaneous study of multiple samples (Aebersold & Mann, 2003; Bantscheff et al., 2007; Schulze & Usadel, 2010; Neilson et al., 2011). On the other hand, label-free methods are considered to be less accurate, but they are a simple, reliable, versatile, and cost-effective alternative to labelled quantitation.

There are currently two strategies extensively implemented as label-free approaches: (1) quantitation based on the signal intensity measurement based on precursor ion spectra; and (2) spectral counting (Zhu, Smith, & Huang, 2010;

Neilson et al., 2011). Focusing quantitation on the basis of peak intensity, it has been demonstrated that ion amount and signal are linearly correlated within the dynamic range of a mass spectrometer. In fact, despite spectral counting such as Exponentially Modified Protein Abundance Index (emPAI) or Absolute Protein Expression (APEX) techniques are very useful in the estimation of the relative amounts of proteins in a single sample, MS1 quantitation results more precise and accurate when aim is to estimate changes in protein from sample to sample (Wang et al., 2003; Levin, Hradetzky, & Bahn, 2011).

Numerous recent studies describe quantitative proteomic analysis in plants (Schaff, Mbeunkui, Blackburn, Bird, & Goshe, 2008; Stevenson, Chu, Ozias-Akins, & Thelen, 2009; Mora, Bramley, & Fraser, 2013), but to the best of our knowledge, there are not many studies in meat or meat products. Thus, the purpose of the present study is the optimisation of a label-free procedure, using ion peak intensity-based comparative nLC-MS/MS, for the relative quantitation of proteins extracted from raw pork meat.

MATERIALS AND METHODS

Preparation of a mixture of protein standards for the optimisation of the methodology

The viability and practicability of the methodology were proved using a mixture of six standard proteins typically found in muscle and meat with a wide range of molecular weights, containing myoglobin (MYG, 17 kDa), tropomyosin (TPM, 33 kDa), actin (ACT, 43 kDa), troponin (TNN, 52 kDa), and alpha-actinin (ACTN, 103 kDa). Beta-lactoglobulin protein (LACB, 19 kDa) was also included in the mixture as normaliser of data as is not naturally present in meat. All protein standards were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Working solutions of 5 nmol for each protein were prepared with 50 mM ammonium bicarbonate (ABC) at pH 8, and subsequently an in-solution digestion was carried out using trypsin enzyme (Sequencing grade modified trypsin; Promega Corp., Madison, WI, USA). Samples were reduced with dithiothreitol (DTT) and cysteines were

alkylated by using iodoacetamide (IAA). Finally, the digestion was started by adding 0.125 µg/µL trypsin to obtain a final enzyme:substrate ratio of 1:50 (w/w), and the sample was incubated overnight at 37 ºC. After incubation, 10% formic acid (FA; v/v) was added to stop the digestion. The digested proteins were used to prepare standard proteins mixtures at different proportions as indicate the ratios shown in **Table 1**. The concentration of beta-lactoglobulin was kept constant for the normalisation of quantitative data. Moreover, working solutions at concentrations of 100, 50, 20, 10, 5, 2, and 1 fmol/µL of the digested LACB were prepared to test the linearity under the experimental conditions.

Table 1. Composition of each protein mixture containing six standard proteins.

Protein name	Control	Set 1	Set 2	Set 3
LACB	1	1	1	1
ACT	1	0.5	0.5	1.5
TPM	1	1	0.5	1
MYG	1	1.5	1	0.5
TNN	1	0.5	1.5	1.5
ACTN	1	1	1.5	0.5

LACB, beta-lactoglobulin; ACT, actin; TPM, tropomyosin; MYG, myoglobin; TNN, troponin C; ACTN, alpha-actinin.

Preparation of raw meat samples and extraction of proteins

Optimised methodology for protein quantitation was carried out using raw meat from 6 months old pig (Landrace x Large White) at 24 h post-mortem. Extraction of sarcoplasmic and myofibrillar proteins was done in triplicate according to Sentandreu, Fraser, Halket, Patel, and Bramley (2010), and protein concentrations were determined by using the Bradford protein assay (Bradford, 1976).

Separation of raw meat myofibrillar and sarcoplasmic proteins by 1D-SDS-PAGE

Solutions with sarcoplasmic and myofibrillar proteins were diluted at concentrations of N, N/2, and N/4 (N = 2 mg/mL) with regard to the concentration values obtained by the Bradford assay. A total of 100 µL of each dilution was mixed with 100 µL of sample buffer (containing 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) and the homogenate was heat denatured at 95 ºC for 4 min. Then, 10 µL of each sample was loaded onto the gel, and the electrophoresis was carried out at 120 V and 50 W, using a separation gel (12% acrylamide) and a stacking gel (4% acrylamide) (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) were used to assess them molecular weights of the proteins.

In-gel digestion of raw meat myofibrillar and sarcoplasmic proteins

After the separation by SDS-PAGE, one section from the gel of sarcoplasmic proteins and another section from the gel of myofibrillar proteins at the three concentrations assayed (N, N/2, and N/4) were selected for in-gel digestion and the posterior quantitation, as can be seen in **Figure 1**.

The stained bands were excised into small pieces, and then reduced and alkylated by using DTT and IAA, respectively. Gel pieces were dried three times for 10 min with 100 µL of ACN. Once the gel fragments became dry and opaque, they were placed in ice for 10 min, and 1 µL of freshly prepared LACB protein solution of 500 fmol/µL was added. The digestion was started by adding 12.5 ng/µL of trypsin enzyme dissolved in 50 mM ABC pH 8, in order to obtain an enzyme:substrate ratio of 1:50 (w/w), and maintaining the samples in ice for 30 min to allow the enzyme to come into the gel. Samples were incubated at 37 ºC overnight, and then 10% (v/v) FA was added to stop the enzyme activity. Peptides were extracted from the gel pieces after sonication for 10 min with 50 µL of 0.1% v/v TFA in ACN:H_2O (50:50, v/v), and the extract was evaporated using a vacuum concentrator. Once the samples were dried, the remaining residue was

reconstituted in 30 µL of loading buffer containing 0.1% v/v TFA, for further MS/MS analysis.

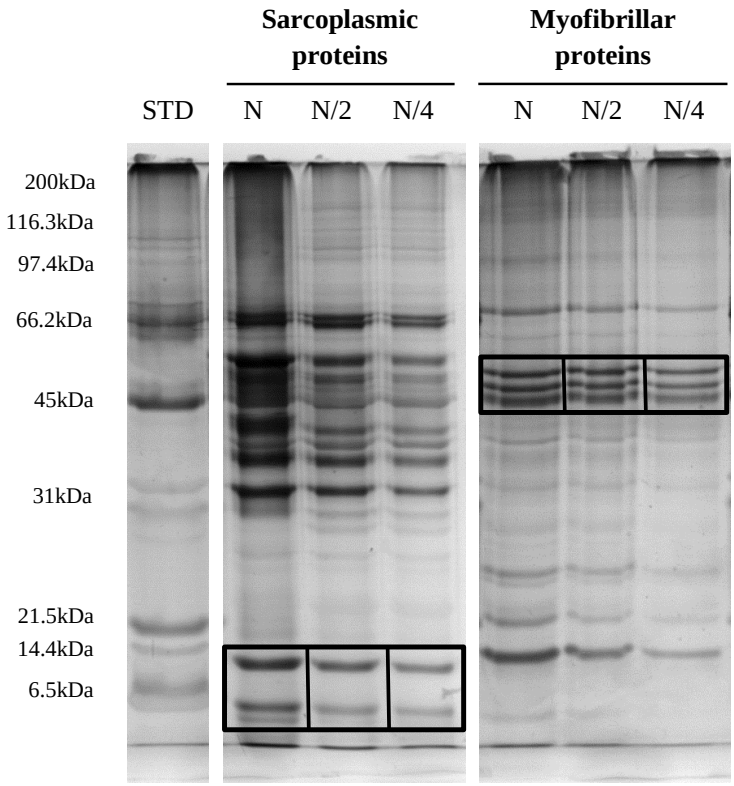


Figure 1. Separation of sarcoplasmic and myofibrillar proteins from raw pork meat using SDS-PAGE at three different concentrations (N, N/2, N/4). Sections indicated in rectangles contain those bands selected for the label-free quantitation of each group of proteins. Molecular weights of the standards (STD) are indicated.

Analysis of trypsin digested samples by nLC-MS/MS

The analysis by nanoliquid chromatography-tandem mass spectrometry (nLC-MS/MS) was done using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole/time-of-flight (Q/TOF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source.

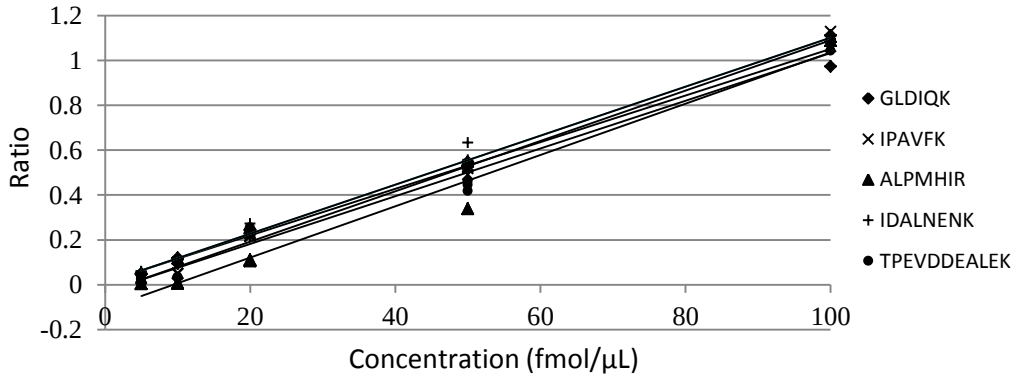
A total of 5 µL of each sample were injected through an autosampler, and preconcentrated on an Eksigent C18 trap column (3µ, 350 µm x 0.5mm; Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min for 5 min and using 0.1% v/v TFA as mobile phase. Then, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3 cm, C18; Nikkyo Technos Co, Ltd. Japan). The mobile phases were solvent A, containing 0.1% v/v FA, and solvent B, containing 0.1% v/v FA in 100% ACN. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at 30 ºC and a flow rate of 0.30 µL/min. The column outlet was directly coupled to a nanoelectrospray ionisation system (nano-ESI). The Q/TOF was used in positive polarity and information-dependent acquisition mode, in which a 0.25-s TOF MS scan in the range from *m/z* 300 to 1250 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to 1500 on the 50 most intense 1 - 5 charged ions.

Data analysis

Automated spectral processing, peak list generation, database search, normalisation and quantitative comparisons were performed using Mascot Distiller v2.4.3.3 software (Matrix Science, Inc., Boston, MA, USA; http://www.matrixscience.com).

The methodology used in this study for label-free quantitation is based on replicates of the relative intensities of extracted ion chromatograms (XICs) for precursors aligned using mass and elution time (Silva et al., 2005; Wang, Wu, Zeng, Chou, & Shen, 2006). The label-free quantitation methodology used in this study requires robust search parameters because it is based on the identification at peptide level. The identification of the protein origin of peptides was done using SwissProt database, and the taxonomy parameter was designated as Mammalia. Moreover, the selected search parameters include the use of trypsin as the enzyme, allowing up to two missed cleavage site, and oxidation of methionine (M), carbamidomethyl (C), and deamidated (NQ) as variable modifications.

Generated MS/MS spectra were searched using a significance threshold $p<0.05$, a FDR of 0.5, and a tolerance on the mass measurement of 0.3 Da in MS mode and 0.3 Da for MS/MS ions. Quantitation parameters were selected using the label-free option provided in the Mascot search engine. Quality criteria to determine peptide ratios used to quantify were established to effectively eliminate outlier points. In this sense, the method of integration was optimised and a standard error of 0.2 and a correlation coefficient of 0.95 with a fraction threshold value of 0.5, that is the fraction of the peak area in the precursor region accounted for by the components. LACB protein was added to the sample and a median of five peptides described in **Figure 2**, was used to normalise data.



Peptide number	Mass (Da)	Peptide sequence	Equation	R-squared value	LOD (fmol/µL)
1	673.388	GLDIQK	y = 0.0104x + 0.0121	R^2 = 0.99	5
2	674.423	IPAVFK	y = 0.0112x - 0.0315	R^2 = 0.99	5
3	837.476	ALPMHIR	y = 0.0114x - 0.1076	R^2 = 0.97	5
4	916.473	IDALNENK	y = 0.0109x + 0.0091	R^2 = 0.99	5
5	1245.584	TPEVDDEALEK	y = 0.0106x + 0.0304	R^2 = 0.98	5

Figure 2. Representation of the linearity range and regression coefficients of LACB protein (n=3), showing the five peptides obtained after trypsin digestion of this protein which were used to normalise the datasets. A digested control sample at a concentration of 100 fmol/µL was used to calculate the ratio. The limits of detection (LOD) were determined for the five peptides.

Principal component analysis (PCA) and loading plot statistical analysis for the control and sets of standard proteins mixture (results not shown) were performed using Simca-P+ 13.0 (Umetrics AB, Sweden). Results were exported from Mascot Distiller into Excel in order to perform statistical analyses of the data.

RESULTS

Normalisation of the data

The digested protein LACB was added to the samples, and five of the peptides generated were used to normalise data (see **Figure 2**) allowing a more robust analysis and quantitation. A good linearity was established in a range of masses from 670 to 1250 Daltons corresponding to the five peptides selected from the trypsin digestion of beta-lactoglobulin protein. So, a plot of the LACB concentration against the mean of each peptide ratio calculated with 100 fmol/µL value as reference is shown in **Figure 2**. Regression coefficients obtained for all the peptides were between 0.99 and 0.97, and the limit of detection (LOD) and identification was determined as 5 fmol/µL for the five peptides used in the normalization after testing samples at lower concentrations such as 3 fmol/µL and 1 fmol/µL.

Repeatability of the procedure

The repeatability of the digestion with trypsin enzyme and analysis by using nLC-MS/MS was evaluated in triplicate for each protein mixture. So, the ratios of LACB, ACT, TPM, MYG, TNN, and ACTN, together with their standard deviations and coefficients of variation were estimated, as can be observed in **Table 2**. Very good repeatability was obtained with percentages of coefficients of variation smaller than 11.5% for all proteins in all samples tested. The different measurements were done by the same analyst, and the instrument worked with the same procedure and under the same experimental conditions to test the repeatability of the methodology.

Table 2. Repeatibility of the method in each sample of proteins mixture (n=3).

Sample	Protein[a] name	Average[b] ratio	SD[c]	CV[d] (%)
Control	LACB	1.00	0.00	0.00
	ACT	1.06	0.02	2.27
	TPM	1.05	0.04	3.69
	MYG	1.01	0.01	1.23
	TNN	1.02	0.03	2.65
	ACTN	1.08	0.04	4.12
Set 1	LACB	1.00	0.00	0.00
	ACT	0.56	0.03	4.97
	TPM	1.06	0.04	3.49
	MYG	1.50	0.02	1.12
	TNN	0.55	0.02	3.66
	ACTN	1.05	0.03	2.88
Set 2	LACB	1.00	0.00	0.30
	ACT	0.54	0.01	1.83
	TPM	0.51	0.01	1.74
	MYG	1.01	0.02	1.81
	TNN	1.51	0.01	0.65
	ACTN	1.49	0.03	2.26
Set 3	LACB	1.00	0.00	0.00
	ACT	1.81	0.10	5.50
	TPM	1.00	0.01	0.55
	MYG	0.55	0.06	11.48
	TNN	1.46	0.10	7.09
	ACTN	0.53	0.05	9.95

[a]See Table 1 for the complete name of the protein. [b]Calculated value obtained from the replicates (n=3). [c]Standard Deviation. [d]Coefficient of Variation, expressed as percentage.

Recovery of the method

The percentage of recovery was evaluated in order to compare the agreement between the values obtained by the method and the theoretical values of the protein mixtures. **Table 3** shows the theoretical and calculated ratios, accuracy of the method, standard deviations and coefficients of variation obtained from the three replicates. The accuracy values obtained comprised between 97% and 120% for all the proteins in the four samples, and the percentages of coefficients

of variation were smaller than 11.5% in all instances. The mean of the median pair of 11, 9, 9, 6, 3, and 3 peptides of ACT, LACB, TPM, MYG, TNN, and ACTN proteins, respectively, have been used to calculate the ratio of the respective proteins. Same peptides were used in repeatability and recovery studies.

Table 3. Recovery (%) of the method in each protein mixture (n=3).

Sample	Protein[a] name	Theoretical[b] value	Calculated[c] value	Accuracy[d] (%)	SD[e]	CV[f] (%)
Control	LACB	1	1.00	100.00	0.00	0.00
	ACT	1	1.06	105.54	2.40	2.27
	TPM	1	1.05	105.05	3.88	3.69
	MYG	1	1.01	101.34	1.24	1.23
	TNN	1	1.02	101.64	2.69	2.65
	ACTN	1	1.08	107.88	4.45	4.12
Set 1	LACB	1	1.00	100.00	0.00	0.00
	ACT	0.5	0.56	111.98	5.57	4.97
	TPM	1	1.06	105.99	3.70	3.49
	MYG	1.5	1.50	99.94	1.12	1.12
	TNN	0.5	0.55	110.20	4.04	3.66
	ACTN	1	1.05	105.14	3.02	2.88
Set 2	LACB	1	1.00	100.18	0.30	0.30
	ACT	0.5	0.54	108.51	1.98	1.83
	TPM	0.5	0.51	102.66	1.78	1.74
	MYG	1	1.01	100.96	1.83	1.81
	TNN	1.5	1.51	100.34	0.65	0.65
	ACTN	1.5	1.49	99.34	2.24	2.26
Set 3	LACB	1	1.00	100.00	0.00	0.00
	ACT	1.5	1.81	120.34	6.62	5.50
	TPM	1	1.00	99.92	0.55	0.55
	MYG	0.5	0.55	109.14	12.53	11.48
	TNN	1.5	1.46	97.26	6.90	7.09
	ACTN	0.5	0.53	106.66	10.62	9.95

[a]See Table 1 for the complete name of the protein. [b]Theoretical ratio of each protein in each sample. [c]Average ratio obtained from the replicates (n=3). [d]Accuracy obtained from the theoretical and calculated values, expressed as percentage. [e]Standard Deviation. [f]Coefficient of Variation, expressed as percentage.

Identification and quantitation of meat proteins

Sarcoplasmic and myofibrillar protein extracts from raw ham meat were separated by SDS-PAGE electrophoresis at three different concentrations (N, N/2, and N/4). The selected bands (see **Figure 1**) were subjected to in-gel trypsin digestion, followed by nLC-MS/MS analysis in order to identify the proteins present and to assess the differences in protein concentrations.

Regarding sarcoplasmic proteins, some proteins such as beta-hemoglobin, myoglobin, and fatty acid-binding protein (accession numbers P02067, P02189, and O02772, respectively, according to Uniprot protein database) were identified from the selected bands in the SDS-PAGE gel after searching MS/MS spectra against the protein database.

On the other hand, the analysis of the selected bands of myofibrillar proteins by nLC-MS/MS allowed to identify several proteins such as α-actin-1, actin cytoplasmic 1, β-actin-like protein 2, desmin, and troponin T, with Uniprot protein database accession numbers P68138, P60712, Q562R1, O62654, and P02641, respectively (see **Table 4**).

The recovery of protein in raw meat extracts was calculated by comparing the theoretical ratio with the calculated value for each identified protein as it is also shown in **Table 4**. Thus, the accuracy percentage obtained was between 99% and 123% in sarcoplasmic proteins, with coefficients of variation smaller than 10%. In myofibrillar proteins the accuracy values were between 100% and 113%, with percentages of coefficients of variation smaller than 9%.

Table 4. Label-free quantitation obtained using SDS-PAGE separation at different dilutions of the raw pork meat extracts and accuracy of the method.

	Protein[a] name	Molecular mass (Da)		Theoretical[b] value	Calculated[c] value	Accuracy[d] (%)	SD[e]	CV[f] (%)	No[g] peptides
Sarcoplasmic	HBB	16155	N	1	1.08	108.23	2.25	2.08	5
			N/2	0.5	0.58	115.57	8.96	7.75	8
			N/4	0.25	0.31	122.84	3.21	2.57	2
Sarcoplasmic	MYG	17074	N	1	1.04	104.01	4.61	4.44	6
			N/2	0.5	0.51	102.37	5.91	5.77	5
			N/4	0.25	0.25	101.82	2.14	2.10	4
Sarcoplasmic	FABPH	14740	N	1	1.08	107.83	2.25	2.09	4
			N/2	0.5	0.50	99.36	7.00	7.05	3
			N/4	0.25	0.26	102.34	10.02	9.79	3
Myofibrillar	ACTS	42024	N	1	1.04	104.01	4.41	4.24	36
			N/2	0.5	0.51	101.20	2.09	2.06	20
			N/4	0.25	0.25	101.53	2.10	2.07	31
Myofibrillar	ACTB	41710	N	1	1.04	104.21	4.59	4.41	21
			N/2	0.5	0.50	100.40	3.35	3.34	12
			N/4	0.25	0.26	102.20	0.68	0.67	16
Myofibrillar	ACTBL	41976	N	1	1.03	103.33	4.81	4.65	10
			N/2	0.5	0.54	107.17	6.67	6.22	6
			N/4	0.25	0.26	105.43	1.12	1.07	7
Myofibrillar	DESM	53499	N	1	1.04	103.53	5.76	5.56	5
			N/2	0.5	0.51	101.65	1.54	1.52	5
			N/4	0.25	0.26	103.34	0.91	0.88	4
Myofibrillar	TNNT3	33014	N	1	1.00	100.26	1.57	1.56	2
			N/2	0.5	0.56	112.88	1.41	1.25	2
			N/4	0.25	0.27	108.88	9.52	8.74	3
Myofibrillar	TPM2	32817	N	1	1.08	107.68	0.06	0.06	4
			N/2	0.5	0.55	109.59	3.39	3.09	8
			N/4	0.25	0.26	104.74	3.73	3.56	4

HBB, beta-hemoglobin; MYG, myoglobin; FABPH, fatty acid-binding protein; ACTS, alpha-actin-1; ACTB, actin cytoplasmic 1; ACTBL, beta-actin like protein 2; DESM, desmin; TNNT3, troponin T; TPM2, beta-tropomyosin. [a]Protein name according to Uniprot database. [b]Theoretical ratio of each protein in each sample. [c]Average ratio obtained from the replicates (n=3). [d]Accuracy obtained from the theoretical and calculated values, expressed as percentage. [e]Standard Deviation. [f]Coefficient of Variation, expressed as percentage. [g]Number of peptides used to calculate the ratios of each identified protein.

DISCUSSION

The use of the latest generation proteomic techniques for label-free quantitation like tandem mass spectrometry with electrospray ionisation (ESI), provides high resolution, mass precision, reproducibility and linearity, together with accuracy and reliability of the obtained data for complex proteomes (Wang et al., 2003; Wang et al., 2006; Zhu et al., 2010).

Traditionally, quantitation of proteins extracted from meat and meat products has been based on the measurement of electrophoretic bands density by using densitometric scanning (Giulian, Moss, & Greaser, 1983; Claeys, Uytterhaegen, Buts, & Demeyer, 1995), spectrophotometric measurements (Everitt & Maksimova, 1984), fluorescent scanning procedures (Goldberg & Fuller, 1978), or computer image analysis (Fritz, Mitchell, Marsh & Greaser, 1993; Morzel, Chambon, Hamelin, Santé-Lhoutellier, Sayd, & Monin, 2004). Quantitation using gel electrophoresis shows some limitations besides its limited dynamic range and poor specificity when extracted proteins from meat samples are analysed, showing problems with very hydrophobic proteins, those with very high or low molecular weight or proteins less abundant in unfractionated samples (Bendixen, 2005; Hollung, Veiseth, Jia, Færgestad, & Hildrum, 2007).

Recent advances in mass spectrometry have allowed protein map identification through a combination of 2D electrophoresis gel followed by peptide mass fingerprint MS (Bouley, Chambon, & Picard, 2004; Doherty et al., 2004; Bendixen, 2005), and improved quantitation of meat proteins in terms of robustness, sensitivity and dynamic range by using isotope labeling techniques and MS/MS analysis to quantify changes in protein abundance between samples (Doherty et al., 2004; Bjarnadóttir, Hollung, Høy, Bendixen, Codrea & Veiseth-Kent, 2012). However, the development of label-free comparative proteomics would give more simplicity in sample preparation, reliability due to the high number of replicates, as well as suitability for all kinds of samples, which provides an essential value when analysing complex matrices such as meat samples.

In the present study, a label-free methodology based on the measurements of changes in chromatographic ion intensity, has been optimised for the relative quantitation of meat proteins. It is essential that sample preparation, sample injection to LC-MS/MS system, and LC separation be highly reproducible, as well as the normalisation of the data and alignment of peaks obtained of multiple LC-MS datasets to avoid possible variations between LC and MS runs (Wang et al., 2003; Zhu et al., 2010). In fact, peptide extraction procedures and trypsin digestion are critical steps in this quantitative methodology that is based on

replicates, so it is important to avoid variance in efficiency that could lead to low proteomic quantitation or even make impossible the quantitation (Brownridge & Beynon, 2011; Szabo et al, 2012).

The peptide peak intensity methodology was used instead of other label-free methods like spectral counting because it is more accurate in reporting changes in protein abundance between samples and estimating ratios for proteins with large numbers of overlapping peptide ions. An uncertain linearity of response and relatively poor precision are obtained when spectral counting approach is used because the dynamic exclusion of ions usually employed for fragmentation is detrimental to obtain an accurate quantitation. Thus, the use of peak intensity measurements for the relative quantitation of large and global protein changes and the comparison between samples seems more advantageous and adequate than spectral counting methodology when complex mixtures of proteins are analysed (Old et al, 2005; Bantscheff et al., 2007; Chen, Ryu, Gharib, Goodlett, & Schnapp, 2008).

In this approach, four protein mixtures at different ratios were analysed by nLC-Q/TOF mass spectrometry showing the linearity, repeatability and recovery of the sample preparation and the nLC-MS/MS analysis. Regarding data processing, mass spectrometry analysis was done by triplicate. First step was the acquisition of the data; a list of survey scans for each TIC followed by groups of the MS/MS scans was created. Then, peptide peaks were detected, distinguishing from neighboring peaks and background noise by attempting to fit an ideal isotopic distribution to the experimental data. Finally, peaks from the LC-MS runs were aligned matching the retention times with the corresponding mass peaks to carry out (*i*) the identification of peptides and origin proteins by using databases, and (*ii*) the quantitation of proteins by normalising the mass spectral peak intensity preceding the statistical analysis. Normalisation with the digested LACB protein was done to eliminate the possible variability in the technical or analytical process, improving the quantitative profiling. A group of five peptides was used for normalisation, which allows a more accurate matching and quantitation than using only one peptide.

The applicability of this methodology was demonstrated using raw pork meat samples for the comparative quantitation of proteins. Thus, sarcoplasmic and myofibrillar proteins extracted from raw meat were separated using SDS-PAGE at three different concentrations. The use of 1D gel electrophoresis, instead 2D gel, simplifies the methodology for the subsequent analysis by mass spectrometry (Jafari, Primo, Smejkal, Moskovets, Kuo, & Ivanov, 2012). In fact, sample preparation influence on quantitation, as well as the variability introduced in the procedure due to uncontrolled changes during SDS-PAGE separation or trypsin digestion were also studied by analysing the results showed in **Table 4**.

In this study, protein identification was done matching spectra to peptides by database searching, and then protein quantitation was carried out according to the actual amount of protein instead than other less sensitive methods that are based on an estimation of the amount of protein calculated by using imaging densitometry. So, relative quantitation was done using a different number of peptides depending on the protein through the integration of their chromatographic peaks. Such procedure showed a high repeatability, linearity, and accuracy. Thus, this simple and reliable label-free quantitative methodology could be applied to study changes in protein abundance along processes such as fermentation, curing, and ripening in meat products.

CONCLUSIONS

A label-free methodology based on the relative intensities of extracted ion chromatograms (XICs) aligned using mass and elution time has been optimised and applied for the relative quantitation of raw pork meat proteins. After SDS-PAGE separation and in-gel digestion of proteins, nLC-MS/MS spectra were used in order to identify and quantify proteins by using ion peak intensity, as peak areas of peptides can be correlated to the concentration of the protein from which the peptide is derived. Linearity, repeatability and accuracy of the procedure have been demonstrated, and the methodology has resulted to be a simple and reliable method to quantify changes in protein abundance of meat samples. Furthermore, this procedure could be very useful in comparative

proteomics in order to evaluate changes in proteins during post-mortem meat period or along the processing of meat products.

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Capítulo 6

Relative quantitation of sarcoplasmic proteins along dry-cured ham processing by using a label-free mass spectrometry methodology

(Food Chemistry, submitted)

Relative quantitation of sarcoplasmic proteins along dry-cured ham processing by using a label-free mass spectrometry methodology

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Abstract

The aim of this work was to quantify changes in the abundance of the major sarcoplasmic proteins throughout the dry-curing ham 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 quantitation of sarcoplasmic proteins through the individual quantitation of the trypsinated peptides. A total of 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 a precise identification and quantitation of proteins compared to traditional methodologies based on gel electrophoresis, especially in the case of overlapping proteins. What is more, the label-free approach used in this study has resulted to be a simple, fast and reliable method to evaluate the degradation of sarcoplasmic proteins by proteolysis along the dry-cured ham processing.

Keywords: Label-free, quantitation; Peptides; Dry-cured ham; Sarcoplasmic proteins; Mass spectrometry; Peak intensity.

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, which results in an extensive degradation of sarcoplasmic and myofibrillar proteins by the action of endogenous muscle peptidases, generating large amounts of peptides and free amino acids (Rodríguez-Nuñez, Aristoy & Toldrá, 1995; Hansen-Møller, Hinrichsen, & Jacobsen, 1997; Toldrá & Flores, 1998; Sentandreu et al., 2003; Sforza et al., 2006).

Sarcoplasmic proteins represent the water soluble fraction that accounts for 30-35% of total muscle proteins and they are 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 color 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 gel electrophoresis described that soluble proteins are intensely hydrolysed throughout the processing of dry-cured ham (Toldrá, Rico, & Flores, 1993; Córdoba et al, 1994; Monin et al., 1997; Larrea, Hernando, Quiles, Lluch, & Pérez-Munuera, 2006; Bermúdez, Franco, Carballo, Sentandreu, & Lorenzo, 2014), whereas more recently, mass spectrometry techniques especially MALDI-TOF MS have been the method of choice to identify and follow-up the degradation profile of sarcoplasmic proteins (Di Luccia et al., 2005; Théron et al., 2011). In fact, some of these studies have quantified the relative abundance of sarcoplasmic proteins along the dry-cured ham processing using the measurement of electrophoretic bands density (Larrea et al., 2006; Bermúdez et al., 2014).

Recent advances in mass spectrometry have allowed a faster and more accurate identification and quantitation of proteins extracted from complex samples in comparison to traditional gel electrophoresis methodologies (Aebersold & Mann, 2003; Cravatt, Simon, & Yates III, 2007). The measurement of electrophoresis

band densities for quantitation provides good sensibility and linearity in protein quantitation, but presents some constraints such as low specificity and limited dynamic range. Moreover, this technique does not allow to identify underlying proteins and provides low accurate results in the case of overlapping proteins (Bendixen, 2005; Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007; Szabo, Szomor, Foeldi, & Janaky, 2012). These problems could be overcome by using modern mass-spectrometry techniques with label-free quantitation methods, which are also a simple, reliable, versatile and cost-effective alternative to labelled methodologies using stable isotopes (Bantscheff et al., 2007; Neilson et al., 2011). One of the possible strategies in the label-free MS approach is the quantitation based on the signal intensity measurements of extracted ion chromatograms, whereby the peak areas of peptides correlate to the concentration of that particular protein from which the peptides were derived. This technique allows a more precise and accurate evaluation of changes in protein abundance between samples compared to other label-free strategies such as spectral counting (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 measurements of ion peak intensities as a simple and reliable method for the relative quantitation of sarcoplasmic proteins along the processing of dry-cured ham.

MATERIALS AND METHODS

Materials

Spanish dry-cured hams coming from 6 months old pigs (Landrace x Large White) were prepared according to the traditional procedure consisting on the pre-salting stage (using a mixture of salt, nitrate and nitrite for 30 min), the salting period (hams were buried in salt and piled up at 2-4 ºC and 90-95% relative humidity for 12 days), the post-salting stage (at 4-5 ºC and 75-85% relative humidity for 60 days), and finally the ripening-drying period (at temperatures increasing from 5 ºC to 14-20 ºC and relative humidity decreasing to 70%) until a total length of processing of 9 months.

For the study, samples were taken at different times of processing: 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.

Extraction of sarcoplasmic proteins

Dry-cured hams at different times of processing were subjected to the extraction of sarcoplasmic proteins. For this purpose, two grams of *Biceps femoris* of every sample were minced and 20 mL of 50 mM Tris-HCl pH 8 were added, mixing with vortex for 2 min. The homogenate was centrifuged at 4 ºC 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.

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 was heat denatured at 95 ºC for 4 min, 10 µL of each sample was loaded onto the gel, and the 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), composed by myosin (200 kDa) β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovoalbumin (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.

In-solution digestion of sarcoplasmic proteins

Sarcoplasmic extract solutions at different times of processing were also submitted to digestion by trypsin enzyme (Sequencing grade modified trypsin; Promega Corp., Madison, WI, USA). So, the extracts were diluted at concentrations of 1 mg/mL with 50 mM ammonium bicarbonate (ABC) at pH 8. A total of 90 µL were taken and 5 µL of beta-lactoglobulin protein (5mg/mL) were added to normalise data. For in-solution digestion, 2 µL of 45 mM solution of dithiothreitol (DTT) in 50 mM ABC were added and incubated for 15 min at 50 ºC, in order to reduce disulphide bonds. Secondly, 2 µL of 100 mM solution of iodoacetamide (IAA) in 50 mM ABC was added and incubated for 15 min at room temperature in the dark for alkylation of cysteines, and then, 2 µL of the DTT solution was used to destroy the excess IAA in the protein sample solution. Finally, the digestion was started by adding 0.125µg/µL trypsin to obtain a final enzyme:substrate ratio of 1:50 (w/w), incubating the sample overnight at 37 ºC. After incubation, a small aliquot of 10% formic acid (FA; v/v) was added to each sample to stop the digestion. A total of 50 µL of each digested solution was lyophilised and resuspended in 100 µL of H_2O with 0.1% TFA for the following analysis.

Analysis of trypsin digested samples by nLC-MS/MS

The analysis by nanoliquid chromatography-tandem mass spectrometry (nLC-MS/MS) was done using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to a quadrupole/time-of-flight (Q/TOF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source, following the methodology described by Gallego, Mora, Aristoy, and Toldrá (2015). So, 5 µL of each sample were injected and preconcentrated on an Eksigent C18 trap column (3 µ, 350 µm x 0.5 mm; Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min for 5 min and 0.1% v/v TFA as mobile phase. Then, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3 cm, C18; Nikkyo Technos Co, Ltd. Japan), in which the mobile phases were solvent A, containing 0.1% v/v

FA, and solvent B, containing 0.1% v/v FA in 100% ACN. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 µL/min and temperature of 30 ºC. The column outlet was coupled to a nanoelectrospray ionisation system (nano-ESI). The Q/TOF was used in positive polarity and information-dependent acquisition mode, in which a 0.25-s TOF MS scan in the range from *m/z* 300 to 1250 was performed, followed by 0.05-s product ion scans from *m/z* of 100 to 1500 on the 50 most intense 2 - 5 charged ions.

Data analysis

Automated spectral processing, peak list generation, database search, normalisation and relative quantitation were performed using Mascot Distiller v2.4.3.3 software (Matrix Science, Inc., Boston, MA, USA; http://www.matrixscience.com).

The label-free quantitation of sarcoplasmic proteins was done by using the optimised methodology described by Gallego et al. (2015), which was based on replicates of the relative intensities of extracted ion chromatograms (XICs) for precursors aligned using mass and elution time (Silva et al., 2005; Wang et al., 2006). Briefly, the selected search parameters comprise the use of SwissProt database for protein identification, trypsin as enzyme type, allowing up to two missed cleavage sites. Generated MS/MS spectra were searched using a significance threshold $p<0.05$, a FDR of 0.5, and a tolerance on the mass measurement of 0.3 Da in MS mode and 0.3 Da for MS/MS ions.

Quantitation parameters were selected using the label-free option, establishing quality criteria to eliminate outlier points in quantitation, and using beta-lactoglobulin to normalise data. Quantitation was done at peptide level, during identification Mascot assigns peptide matches to the protein of origin and for quantitation the ratios for individual peptides, which are obtained from the integration of their XICs, are combined to determine the ratio for origin protein. In this sense, raw ham peptides (0m) were used as reference in the calculation of ratios for individual peptides, and the ratio of each protein at different times of

processing was calculated from three replicates using a different number of trypsinated peptides depending on the origin protein. Finally, results from Mascot Distiller were exported into Excel for statistical analyses.

RESULTS AND DISCUSSION

Sarcoplasmic proteins extracts from dry-cured ham at different times of processing were submitted to in-solution digestion by trypsin enzyme and analysed by nESI-LC-MS/MS for the identification and relative quantitation of proteins. In this sense, the protein quantitation was done by using a label-free methodology based on the measurement of extracted ion chromatograms (XICs) intensities of the corresponding peptides. **Table 1** shows the number of individual peptides used in the quantitation of the identified proteins. The study was focused on 20 proteins as indicated in **Table 1**, including 12 enzymes involved in the glycolytic pathway (PYGM, PFK, PK, PGM, GPI, ENO, PGK, ALDO, LDH, GAPDH, PGAM, and TPI), creatine kinase and myoglobin.

As it is shown in **Figure 1**, the most significant changes in the profile of glycolytic enzymes correspond to a sharp drop of GADPH, PYGM, and PGAM relative amounts in the first 2 months of processing of 10-, 3.3-, and 2.5-fold, respectively, as well as the decrease in the quantity of PGM after the post-salting stage (2 months), and the amount of GPI and TPI proteins after the ripening-drying stage (3.5 months). In general, calculated values for the rest of the studied glycolytic proteins show their progressively degradation during the whole process, principally along the dry-curing process (5, 6.5 and 9 months). Moreover, these results show that GAPDH is the most hydrolysed protein, which practically disappears after the post-salting stage, whereas for ALDO protein over half of its quantity is maintained at the end of the processing. On the other hand, ENO protein is the most stable up to 6.5 months of processing, showing a decrease of 2.8-fold at 9 months.

Regarding the remaining sarcoplasmic proteins evaluated in this study, **Figure 2** evidences their intense degradation occurred up to 2 months of processing, except for AATC, GPD, and MDH in which the major increase in hydrolysis takes

place from this time. Calculated values for GST and MYG indicate that they are almost completely hydrolysed after the post-salting stage, showing a 10-fold decrease, whereas the rest of proteins are degraded more progressively during the dry-curing process, reaching final calculated values lower than 0.3 in all cases.

Table 1. Sarcoplasmic proteins identified by ESI-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	Triose phosphate isomerase	26673	6	Glycolysis	r
GST	Glutathione S-transferase	23482	1	Detoxification of Reactive Oxygen Species	s
MYG	Myoglobin	17074	5	Oxygen transport (color development)	t

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

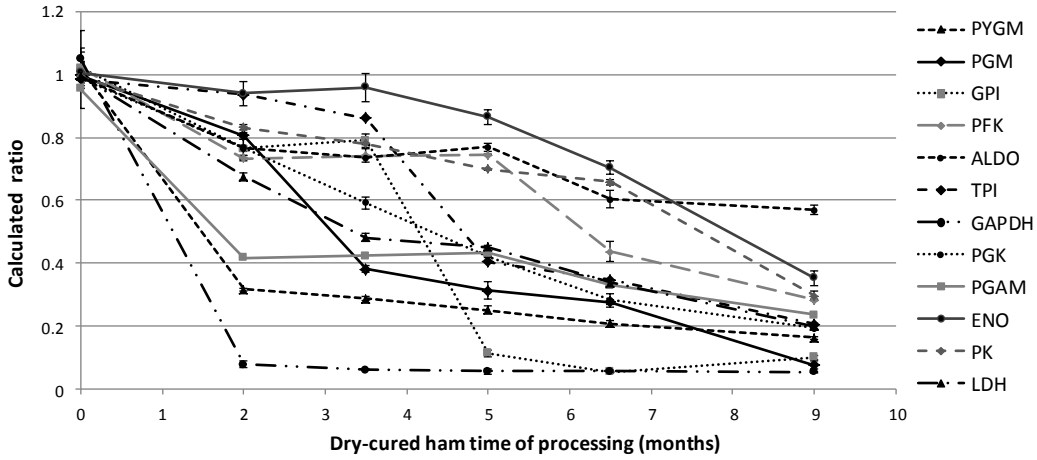

Figure 1. Relative quantitation of glycolytic enzymes (PYGM, PFK, PK, PGM, GPI, ENO, PGK, ALDO, LDH, GAPDH, PGAM, and TPI) along the dry-cured ham processing. Bars represent standard deviations from the replicates (n=3).

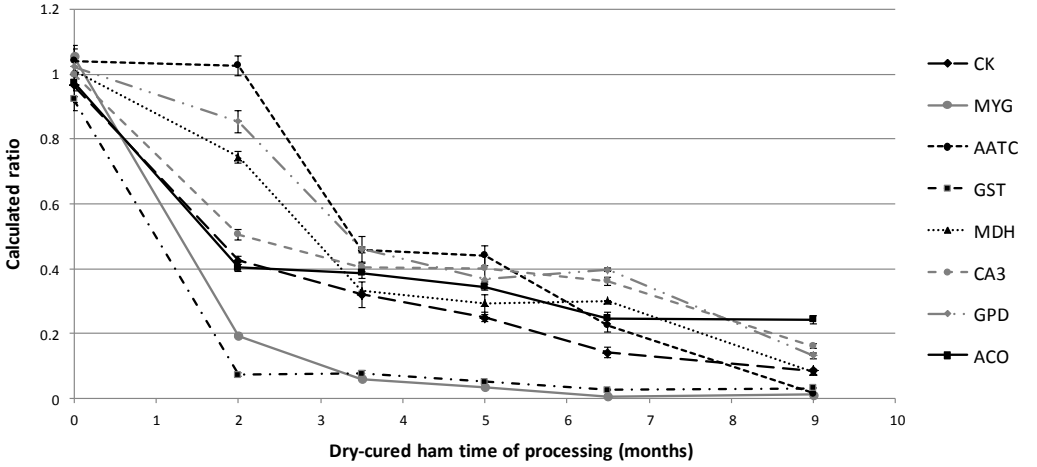

Figure 2. Relative quantitation of sarcoplasmic proteins CK, MYG, AATC, GST, MDH, CAH3, GPD, and ACON along the dry-cured ham processing. Bars represent standard deviations from the replicates (n=3).

As it is shown in **Figure 1**, the most significant changes in the profile of glycolytic enzymes correspond to a sharp drop of GADPH, PYGM, and PGAM relative amounts in the first 2 months of processing of 10-, 3.3-, and 2.5-fold, respectively, as well as the decrease in the quantity of PGM after the post-salting stage (2 months), and the amount of GPI and TPI proteins after the ripening-drying stage (3.5 months). In general, calculated values for the rest of the studied glycolytic proteins show their progressively degradation during the whole process, principally along the dry-curing process (5, 6.5 and 9 months). Moreover, these results show that GAPDH is the most hydrolysed protein, which practically disappears after the post-salting stage, whereas for ALDO protein over half of its quantity is maintained at the end of the processing. On the other hand, ENO protein is the most stable up to 6.5 months of processing, showing a decrease of 2.8-fold at 9 months.

Regarding the remaining sarcoplasmic proteins evaluated in this study, **Figure 2** evidences their intense degradation occurred up to 2 months of processing, except for AATC, GPD, and MDH in which the major increase in hydrolysis takes place from this time. Calculated values for GST and MYG indicate that they are almost completely hydrolysed after the post-salting stage, showing a 10-fold decrease, whereas the rest of proteins are degraded more progressively during the dry-curing process, reaching final calculated values lower than 0.3 in all cases.

Muscle sarcoplasmic proteins are important substrates for the proteolysis phenomena that take place throughout the dry-cured ham processing, in which endogenous enzymes such as endopeptidases and exopeptidases are responsible for the degradation of proteins. Regarding endopeptidases, cathepsins are stable during the full curing process, except cathepsin D whose activity is restricted to the first 6 months of processing (Toldrá et al., 1993; Toldrá & Flores, 1998) whereas calpains are inactivated after the salting stage (Rosell & Toldrá, 1996). On the other hand, exopeptidases enzymes degrade the polypeptides generated by endopeptidases to small peptides and free amino acids, being aminopeptidases and dipeptidyl peptidases the most studied and those showing

the best stability along the dry-curing process (Toldrá, Flores, & Sanz, 1997; Sentandreu & Toldrá, 2001).

The results of the present work are in agreement with those reported previously by Larrea et al. (2006) that evaluated the degradation of proteins along Teruel dry-cured ham processing using one-dimensional electrophoresis as well as identified eleven sarcoplasmic proteins according to their molecular weight and quantified them by doing a densitometric scanning of the gel. However, some differences were found in quantitation values especially in the case of those proteins showing low molecular weight, probably due to the quantitation of overlapping bands in the gel that corresponds to different proteins or derived fragments generated along the processing and that co-migrate during the gel separation. In this sense, the use of liquid chromatography coupled to mass spectrometry would allow not only the accurate identification of individual proteins but also the previous separation of the trypsinated peptides, which increases the total number of ionized and fragmented peptides, extending the working dynamic range in comparison to gel electrophoresis results. Additionally, differences in length and type of processing between Teruel dry-cured ham (Larrea et al., 2006) and the dry-cured ham used in the present work may lead to slight variations in the degradation profile of proteins. Thus, extracts of sarcoplasmic proteins were also analysed by one-dimensional SDS-PAGE electrophoresis as shown in **Figure 3**. Generally, the most significant changes were observed from the post-salting stage (2 months) to the end of the dry-ripening process (9 months) of dry-cured hams, which is in accordance with the results described by Larrea et al. (2006).

Moreover, it is significant the appearance of some new bands mainly at the end of the processing that could be related to any of both generated fragments from sarcoplasmic proteins as previously described or soluble peptides obtained from the hydrolysis of myofibrillar proteins that were extracted together with sarcoplasmic proteins (Córdoba et al., 1994; Toldrá & Flores, 1998). These myofibrillar fragments were previously described by other authors (Larrea et al., 2006; Bermúdez et al., 2014) although their identification was not possible by gel

electrophoresis methods used in such studies. However, the nano LC-MS/MS analysis done in this study has allowed the identification of the origin proteins of the derived products (data not shown).

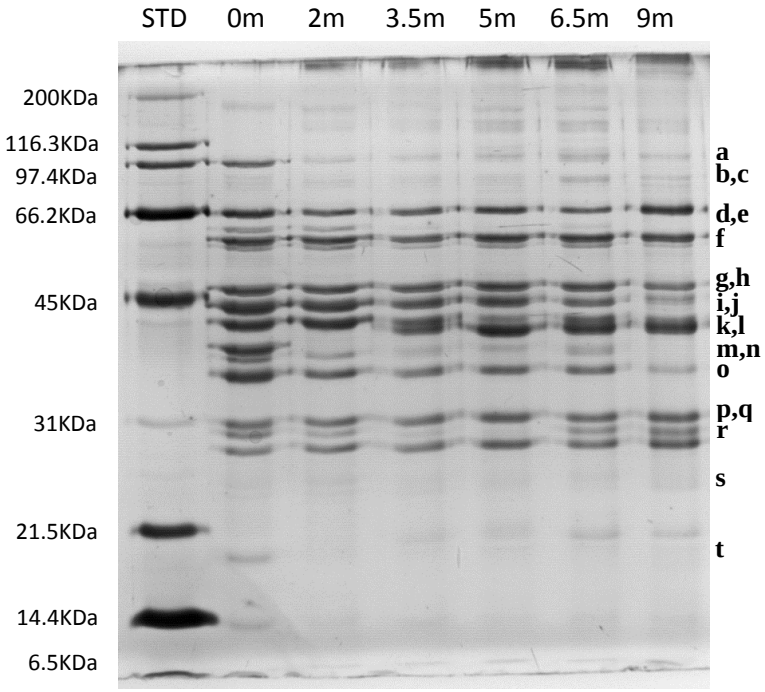

Figure 3. SDS-PAGE separation of sarcoplasmic proteins from dry-cured ham at different times of processing. Molecular weights of the standards (STD) are indicated. Bold letters designate the studied proteins: a - PYGM; b - ACON; c - PFK; d - PK; e -PGM; f - GPI; g - ENO; h - AATC; i - PGK; j - CK; k - ALDO; l - GPD; m - LDH; n - GAPDH; o - MDH; p - CAH3; q - PGAM; r - TPI; s - GST; t - MYG.

Other authors have described the protein degradation profile along dry-cured ham processing using a combination of two-dimensional electrophoresis and MALDI-TOF MS techniques for the identification of the proteins (Di Luccia et al., 2005) obtaining a higher resolution separation in comparison to one-dimensional electrophoretic techniques as well as a more precise identification of proteins by

mass spectrometry. However, this methodology involves additional steps and complexity in sample preparation because the identification of proteins by peptide mass fingerprint MS can run into difficulties when the analysis is done with complex mixtures of proteins (Baldwin, 2004; Thiede et al., 2005).

CONCLUSIONS

The label-free methodology applied in this work has resulted to be a simple and reliable alternative with regard to methods used to date for the evaluation of protein degradation throughout the dry-curing ham process, as it allows a precise identification of proteins and proteolysis fragments generated during processing as well as a more accurate quantitation of proteins changes than using gel-based methods. A total of 20 sarcoplasmic proteins including 12 enzymes involved in glycolytic phenomena, have been identified and quantified by nESI-LC-MS/MS analysis, showing an intense degradation during the processing. Moreover, the broad dynamic range showed by LC-MS/MS approaches would allow the quantitation of minor proteins which have not been deeply studied to date.

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Capítulo 7

Transepithelial transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-2 cell monolayer

(Journal of Functional Foods, submitted)

Transepithelial transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-2 cell monolayer

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Abstract

Recent studies have shown that dry-cured ham is an important source of naturally generated bioactive peptides, especially showing ACE inhibitory activity. However, due to their excessive degradation by digestive and brush-border enzymes, it is not clear whether these peptides resist intestinal absorption and reach the blood stream where they may exert their antihypertensive effect. So, dry-cured ham extracts and specific pure peptides naturally generated during the dry-curing process, showing ACE inhibitory activity, have been studied for their stability during transepithelial transport in a Caco-2 cell monolayer. The ACE inhibitory activity of transport samples was assayed, reaching the highest values in apical samples after 15 min of incubation. In basal solutions, the highest ACE inhibition was observed for peptides AAPLAP and KPVAAP after 60 min of cellular transport. However, when basal samples were four times concentrated, a considerable increased ACE inhibitory activity was observed in these peptides from 15 min of incubation. Fragments generated by cellular activity were detected by using tandem MS techniques, showing that AAATP, AAPLAP, and KPVAAP were hydrolysed during the transport, although KPVAAP was also absorbed intactly. This study highlights the potential of intact dry-cured ham peptides as well as their fragments to be absorbed across the intestinal epithelium and reach the blood stream to exert an antihypertensive action.

Keywords: Dry-cured ham; ACE inhibitory peptides; Caco-2 cell monolayer; Intestinal transport; Mass spectrometry.

INTRODUCTION

Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase which plays an essential role as a regulator of blood pressure. ACE converts angiotensin I to the potent vasoconstrictor angiotensin II, which also induces the release of aldosterone. Moreover, ACE inactivates bradykinin, which has vasodilator activity. As a result, the action of ACE on these two systems is responsible for hypertension, the most common type of cardiovascular disease (Skeggs, Kahn, & Shumway, 1956; Ondetti, Rubin, & Cushman, 1977; Unger, 2002). To exert effects on blood pressure, ACE-inhibitory compounds such as antihypertensive peptides need to resist the degradation by gastrointestinal proteases and brush border peptidases, be absorbed through the intestinal epithelium, and finally reach the bloodstream in an active form (Vermeirssen, Augustijns, Morel, Van Camp, Opsomer, & Verstraete, 2005).

Spanish dry-cured ham has recently been investigated as a natural source of antihypertensive peptides, evaluating the ACE inhibitory activity of water soluble fractions of dry-cured ham extracts (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012), and identifying some of the peptides responsible for this inhibitory effect (Escudero, Mora, Fraser, Aristoy, Arihara, & Toldrá, 2013). Moreover, Escudero, Mora, and Toldrá (2014) have recently reported that the ACE inhibitory activity of dry-cured ham peptides persists after *in vitro* digestion with gastric proteases, which may be due to both resistance of the existing antihypertensive peptides to digestion as well as generation of small fragments with ACE-inhibitory activity. Furthermore, the in vivo antihypertensive activity of dry-cured ham extracts and peptide AAATP has been studied, showing a decrease in systolic blood pressure after their oral administration to spontaneously hypertensive rats (SHR) (Escudero et al., 2012; Escudero et al., 2013).

Transport assays through Caco-2 cells, which is a cell line derived from human colon adenocarcinoma, have been established as a model for small intestinal transport of drugs and food compounds. Differentiated Caco-2 cells maintain the morphology and function of mature enterocytes and express brush border

proteases and peptidases which may affect peptide stability and transport, being therefore utilised to predict the absorption in the small intestine (Hidalgo, Raub, & Borchardt, 1989; Yee, 1997). In this sense, several studies have been focused on study the transepithelial transport of antihypertensive peptides derived from different food products such as egg or milk (Miguel, Dávalos, Manso, De la Peña, Lasunción, & López-Fandiño, 2004; Vermeirssen et al., 2005; Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008; Bejjani & Wu, 2013). However, to the best of our knowledge, there are no transport studies based on peptides showing ACE inhibitory activity derived from meat or meat products.

In this work, the Caco-2 cell line was used to study the brush border degradation and transepithelial transport of ACE inhibitory peptides derived from dry-cured ham. In addition, the ACE inhibitory activity of the transported peptides was measured to evaluate their final antihypertensive potential.

MATERIALS AND METHODS

Materials and reagents

Dulbeco's Modified Eagle's Medium (DMEM), GlutaMAX™, phosphate buffered saline (PBS), and nonessential amino acids were procured from Life Technologies (Ghent, Belgium), whereas fetal bovine serum was from Greiner Bio-One (Vilvoorde, Belgium). Angiotensin-converting enzyme (from rabbit lung) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA), and Abz-Gly-p-nitro-Phe-Pro-OH trifluoroacetate salt was from Bachem AG. (Bubendorf, Switzerland). All other chemicals and reagents used were of analytical grade.

Dry-cured ham extracts and peptides

The study was done using extracts from different types of dry-cured ham. Samples M1 and S1 were obtained from Spanish dry-cured hams with ten months of processing, which were submitted to extraction and deproteinisation according to the methodology described by Escudero et al. (2012). In the case of sample S1, the extract was fractionated by gel filtration chromatography, and fractions corresponding to elution volumes from 200 to 320 mL were pooled

together and dried. These fractions were selected because they have shown the maximum ACE inhibitory activity, reaching values of 80% of inhibition in a previous study (Escudero et al., 2012). In addition, Designation of Origin of Teruel hams with a minimum time of ripening of fourteen months were used to obtain samples M2 and M3. Peptide extraction was done according to the method described by Escudero et al. (2014) for sample M2, while sample M3 was submitted to extraction and deproteinisation following the same procedure as described above for sample M1 and S1. All dry-cured ham extracts were desalted by solid phase extraction using an Oasis® HLB cartridge (35 cc, Waters, Ireland), where peptides were retained and then eluted with 50% methanol. Finally, the eluates were lyophilised for the transport experiment across Caco-2 cells.

Since dry-cured ham extract samples contain a complex mixture of peptides that difficult a detailed study, three peptides (AAATP, AAPLAP, and KPVAAP) previously identified as ACE inhibitors (Escudero et al., 2013; Escudero et al., 2014) were selected and synthetised by GenScript Corporation (Piscataway, NJ, USA) at the highest purity certified using LC-MS analysis for subsequent transport experiments.

Caco-2 cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM containing 4.5 g/L glucose, supplemented with GlutaMAX™, 10% fetal bovine serum and 1% nonessential amino acids, at 37 ºC in a humidified atmosphere containing 10% CO_2. The passage number of the cells used in this study was between 30 and 35. For transport experiments, cells were seeded at a density of 20,000 cells/well on HTS Transwell®-24 well permeable supports (0.4 µm pore polyester membrane, 6.5 mm inserts, 0.33 cm2 cell growth area; Costar, Corning, Birmingham, UK). The culture medium was replaced every 2-3 days and cells were allowed to differentiate for at least 21 days before experiments.

Transport studies

Differentiated Caco-2 cells were gently rinsed twice with PBS and then incubated with PBS for 1 day at 37 ºC in 10% CO_2 prior to the transport assays. After removing the PBS from all wells, 1 mg/mL of the samples dissolved in PBS were added to the apical chambers (200 µL), whereas fresh PBS was added to the basolateral chambers (1 mL). A control sample, containing only PBS and no peptides, was included in the experimental setup, and samples from apical and basolateral sides were taken before (0 min) and during incubation at different time points of 15, 30, and 60 min. The action of cell proteases on samples after the transport study was immediately stopped by adding 9 volumes of methanol, centrifuged at 7500*g* for 10 min, and finally the supernatant containing the peptides was taken and dried for the following analysis. All experiments were conducted in triplicate.

ACE inhibitory activity

Apical and basal samples taken from the transepithelial transport at different times were analysed for ACE inhibitory activity. For this purpose, dried apical samples were dissolved to the original volume in bidistilled water, whereas dried basal samples were first dissolved in a volume four-fold smaller than the original one to determine the ACE inhibition in concentrated samples, and then redissolved to the original volume to test again. Additionally, the ACE inhibitory activity of several synthetised fragments derived from the degradation of AAATP, AAPLAP, and KPVAAP through the cellular transport was evaluated. In all studied cases the ACE inhibitory activity was measured according to the method developed by Sentandreu and Toldrá (2006), which is based on the ability of ACE to hydrolyse the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO2)-Pro). The assay was done in triplicate. ACE inhibition of samples is expressed as ACE inhibitory percentage and as IC_{50}, which is the peptide concentration that inhibits 50% of ACE activity in the reaction mixture.

MALDI-TOF/TOF mass spectrometry

Apical and basal samples taken after the transport of peptides AAATP, AAPLAP, and KPVAAP across Caco-2 cells were analysed using matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry to determine the molecular mass of the peptide mixture and detect those possible peptides resulting from the degradation of the precursor peptides. The analysis was done in MS mode, and a total of 1 µL of every sample was directly spotted on the MALDI plate and allowed to air dry. Then, 0.5 µL of matrix solution (which contains 5 mg/mL of α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics, Germany) in 0.1% TFA-ACN/H_2O (7:3, v/v) was spotted. The analysis was done in a 5800 MALDI TOF/TOF instrument (ABSciex, MA, USA) in positive reflectron mode (3000 shots every position) in the 150-800 Da range; the laser intensity was manually adjusted to maximize the S/N ratio. Previously, the plate model and the acquisition methods were calibrated by ABSCIEX calibration mixture (1 fmol/µL des-Arg1-Bradykinin; 2 fmol/µL Angiotensin I; 1.3 fmol/µL Glu1-Fibrinopeptide B; 2 fmol/µL ACTH (1–17 clip); 5 fmol/µL ACTH (18–39 clip); and 3 fmol/µL ACTH (7–38 clip)) in 13 positions.

The candidate precursors were then selected for every position in the MS/MS analysis in order to confirm their presence. In this sense, ten of the most intense precursors according to the threshold criteria minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, and maximum fraction gap: 4, were selected. The MS/MS method was calibrated using Angiotensin I MS/MS spectra in 13 positions, and data was acquired using the default 1kV MS/MS method.

Manual analysis of data was done using mMass – Open Source Mass Spectrometry Tool Software v.5.5 (http://www.mmass.org) (Strohalm, Kavan, Novák, Volný, & Havlíček, 2010). The search of peptide sequences previously identified showing ACE inhibitory activity was done using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep).

Statistical analysis

Statistical analysis was done using Statgraphics Centurion XVI software. One-way analysis of variance (ANOVA) was performed and the mean and standard deviation were reported. Fisher's multiple range test was carried out to analyse significant differences among mean values at $p < 0.05$.

RESULTS AND DISCUSSION

Transepithelial transport of peptides across Caco-2 cell monolayer

In order to simplify the study of dry-cured ham extracts which contain a mixture of thousands of peptides, the apical to basal flux transport in Caco-2 cell monolayers was mainly studied with the purified peptides (AAATP, AAPLAP, KPVAAP). For that, 1 mg/mL of every sample was added to the apical side, and samples were taken from both apical and basal compartments at different times: 0, 15, 30, and 60 min.

The evolution of peptides AAATP, AAPLAP, and KPVAAP during their transcellular transport was then monitored by using MALDI-TOF mass spectrometry to determine the profile of peptides according to their molecular masses. As illustrated in **Figures 1A and 1B**, there are more and smaller peptides in the samples taken from the basal sides than those of the apical ones, suggesting a partial degradation of the precursor peptides into smaller fragments by the action of cell peptidases as well as their absorption through the Caco-2 cell monolayer. The size, amino acid sequence, charge, hydrogen-bonding capacity, and hydrophobicity of peptides are critical in determining their susceptibility to peptidases and their permeability across the intestinal epithelium (Pauletti, Okumu, & Borchardt, 1997; Shimizu, Tsunogai, & Arai, 1997; Artursson, Palm, & Luthman, 2001).

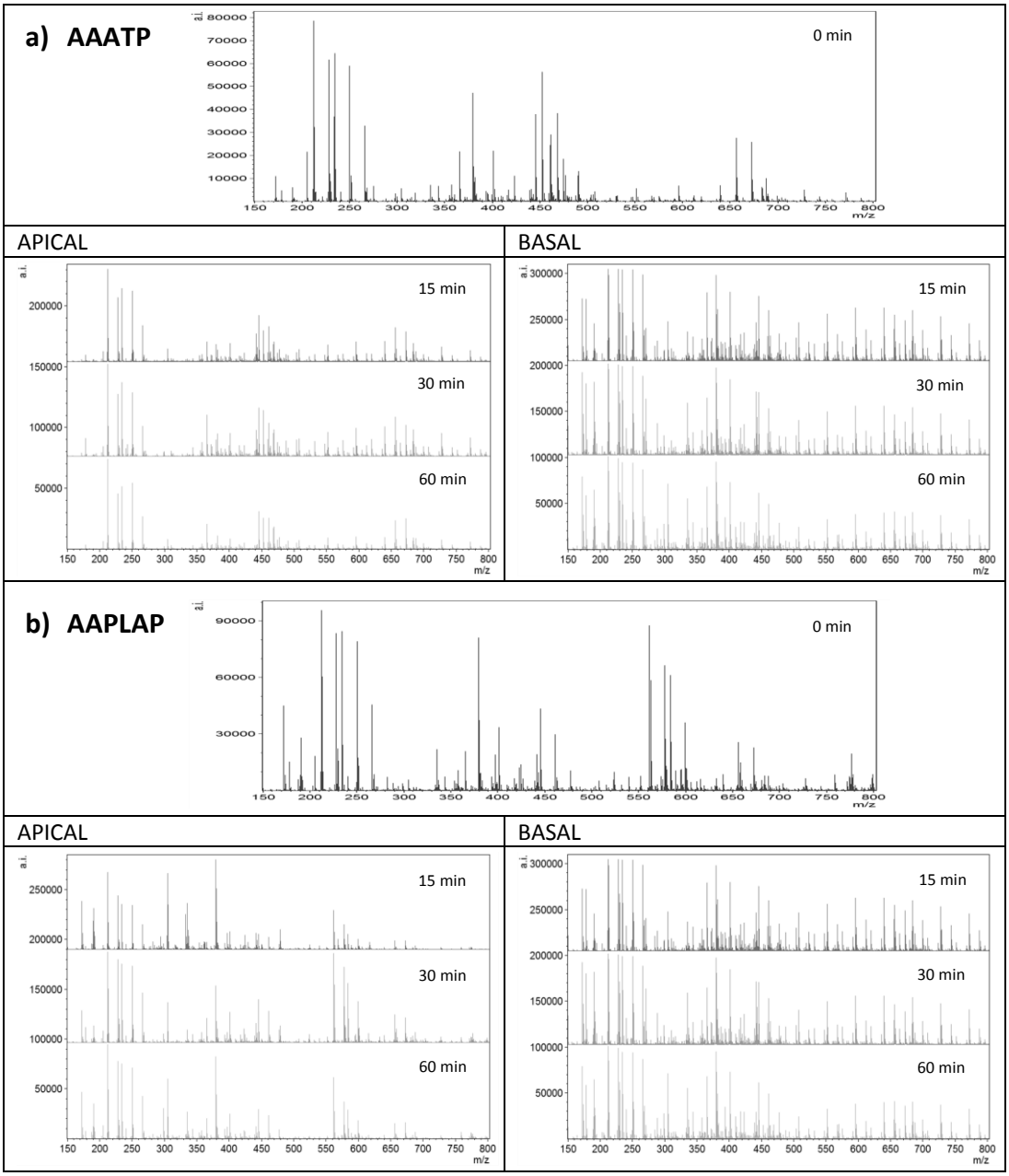

Figure 1A. MALDI-TOF mass spectra of samples taken from the transepithelial transport (apical and basal sides) through Caco-2 cell monolayer at different times (0, 15, 30, 60 min) after adding the purified peptides a) AAATP, and b) AAPLAP.

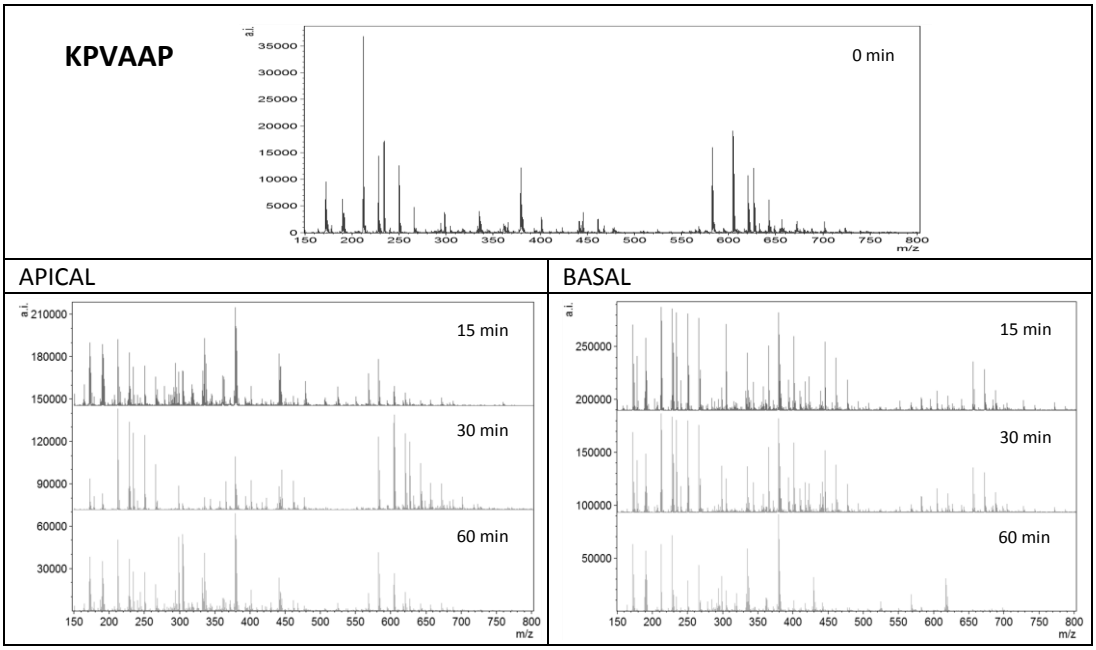

Figure 1B. MALDI-TOF mass spectra of samples taken from the transepithelial transport (apical and basal sides) through Caco-2 cell monolayer at different times (0, 15, 30, 60 min) after adding the purified peptide KPVAAP.

ACE inhibitory activity of samples taken from transport assays

Dry-cured ham extracts and peptide solutions taken from the apical and basal compartments at different transport times were analysed for ACE inhibitory activity, directly after sampling and in basal samples also after concentrating four times (**Figure 2**). Samples taken from the apical side showed high ACE inhibition, generally reaching the maximum values at 15 min of cellular transport in both types of samples, and with values very close to 100% inhibition for the purified peptides. These high values may be due to a strong action of cell peptidases on these peptides at this time, whereby the modification of the peptide structure may affect its biological activity and peptide fragments released can show even a higher degree of ACE inhibitory activity compared to the precursor peptides (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). In the case of dry-cured ham samples, M1 and S1 showed similar inhibition results even though

sample S1 contained only the fractions which the highest ACE inhibitory activity. Meanwhile the activity decreases more significantly after 30 min of transport for samples M2 and M3, which come from dry-cured hams with longer curing process. The ACE inhibition activity for sample M2 before the transport assay was very low (with a value of 4.5%), which is in agreement with a previous study (Escudero et al., 2014), and which is probably due to the fact that this sample has not been deproteinised compared to sample M3. Additionally, sample M2 did not show any ACE inhibitory activity in the apical side after 60 min of cellular transport, indicating that inhibitory peptides were either completely hydrolysed or transported to the basal side.

On the other hand, little to no ACE inhibition was detected in the basal samples except for peptides AAPLAP and KPVAAP after 60 min of transport, whereby ACE inhibitory activity reached values of 42% and 30%, respectively. For this reason, all basal samples were concentrated four times to check if samples show ACE inhibitory activity at higher concentration. Under these conditions, dry-cured ham extract samples showed activity although to a varying degree, obtaining values around 30-40% at 15 min of transport and then decreasing during time. However, for purified peptides, the ACE inhibitory activity of peptides AAATP and AAPLAP increased during incubation, reaching values up to 30% for AAATP and 70% for AAPLAP at 60 min of the assay. Also high percentages of inhibition (around 65%) were observed for KPVAAP after 15 and 60 min of cellular transport. These results indicate that some peptides as well as small derived fragments could be absorbed, maintaining their ACE inhibitory activity after cellular transport although at low concentration in non-concentrated samples (**Figure 2**). Also, synergic effects between antihypertensive peptides could explain higher values of ACE inhibition for peptide mixtures compared to the individual activity of individual peptides. In addition, it should be stressed that the concentration of peptides in the basal and concentrated basal samples does not show a linear but exponential response when ACE activity is plotted against the concentration of an ACE inhibitory peptide, as previously described by Sentandreu and Toldrá, 2006.

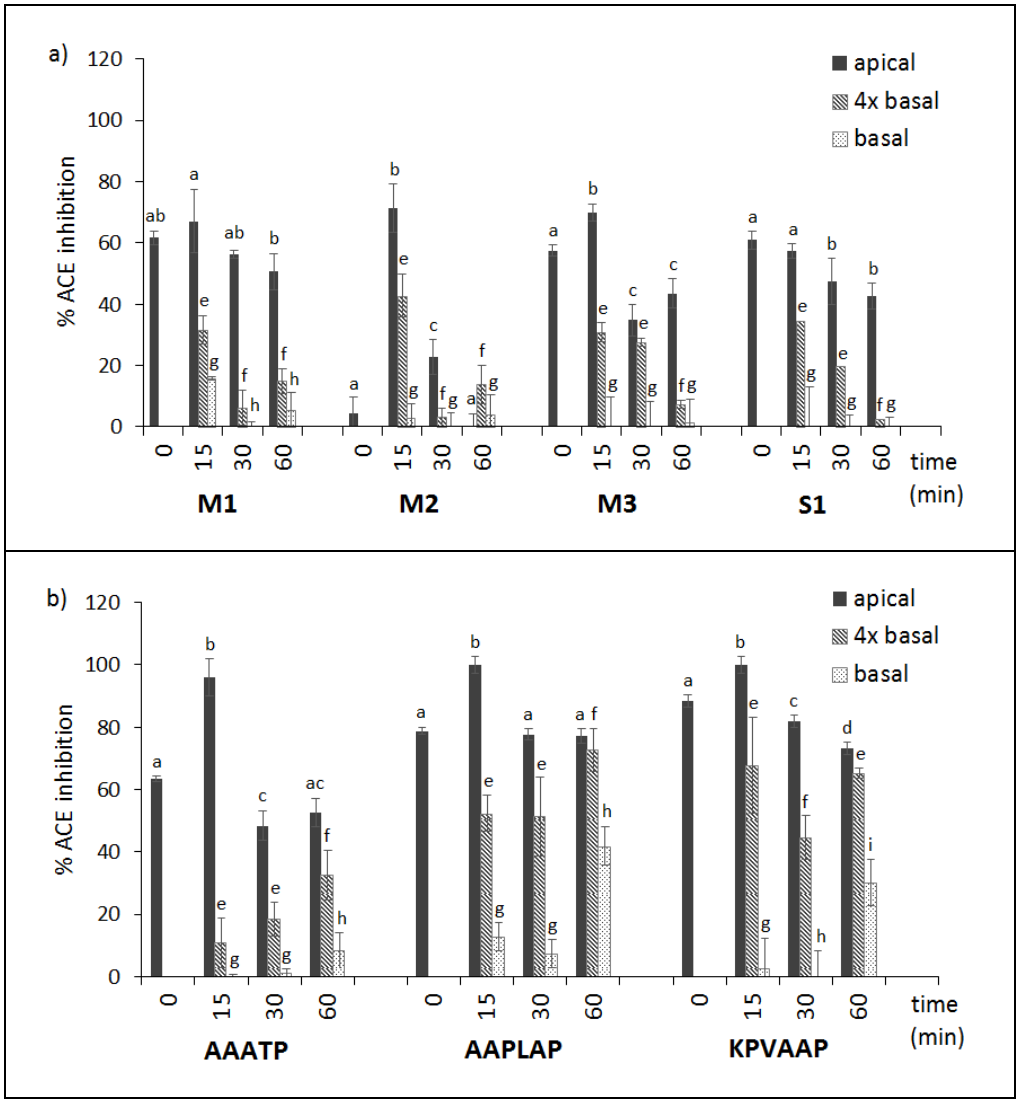

Figure 2. ACE inhibitory activity (%) observed in the apical, basal, and four times concentrated basal compartments obtained from the cellular transport after the application of a) dry-cured ham extracts and b) purified peptides. Different letters in each sample and compartment indicate significant differences at $p < 0.05$ (n=3).

Because angiotensin I converting enzyme is present in the brush border contributing to hydrolysis of peptides (Yoshioka, Erickson, Woodley, Gulli, Guan, & Kim, 1987), control samples containing only the Caco-2 cells and PBS were also analysed for ACE inhibitory activity, showing non-significant effects (data not shown). Nevertheless, these values were taken into account for determining the percentage of ACE inhibition of the samples.

Peptide degradation by epithelial peptidases

As dry-cured ham samples contain a very complex mixture of peptides, only the degradation of the purified peptides on the brush-border side was evaluated by MS-based techniques in order to detect those peptides that were transcellularly transported. **Table 1** shows the peptide sequences detected by MALDI-TOF/TOF MS in the apical and basal compartments at the different assayed times.

The analysis revealed the degradation of the intact peptide AAATP after 15 min of transport with generation of smaller sized peptides, some of which can cross the intestinal barrier up to the basal side. On the other hand, AAPLAP was detected in the apical chamber throughout the experiment, but analysis of the basal solutions showed that it was not transported intact. In fact, several shorter fragments were also detected in the apical and basal solutions, suggesting its partial degradation by brush border peptidases. Furthermore, peptide KPVAAP was detected up to 60 min of transport in both apical and basal samples, indicating that its chemical and structural properties allow it to be absorbed intact through the Caco-2 monolayer. However, it was also susceptible to peptidases and different derived fragments were also detected in apical and basal solutions. In this sense, among all the fragments detected in either apical or basal samples, several of them were previously reported as ACE inhibitory peptides (**Table 2**), being LAP and VAA those with the lowest IC_{50} values. The remaining peptides detected at different times of incubation as shown in **Table 1** (AATP, ATP, AAAT, APLA, PLAP, AAPL, VAAP, KPV, AAA, and VA) were synthetised and assayed for the *in vitro* ACE inhibitory activity in order to calculate their IC_{50} (**Table 3**).

Table 1. Peptide sequences detected by using MALDI-TOF/TOF MS in the cellular transport assay.

Precursor peptide	Peptide fragments[a]	Monoisotopic mass (Da)[b]	Apical – times (min)[c]				Basal – times (min)[d]		
			0	15	30	60	15	30	60
AAATP*		429.22	x	x					
	AATP	358.19		x	x	x			
	AAAT	332.17						x	x
	ATP	287.15		x			x	x	x
	AAT	261.13							
	AAA	231.12						x	
	TP	216.11							
	AT	190.10							
	AA*	160.08							
AAPLAP*		538.31	x	x	x	x			
	APLAP	467.27							
	AAPLA	441.26							
	PLAP	396.24		x	x	x			
	APLA	370.44					x	x	
	AAPL	370.22					x	x	
	APL	299.18							
	PLA	299.18							
	LAP*	299.18							
	AAP*	257.14							
	PL *	228.15					x	x	x
	LA *	202.13							x
	AP *	186.10							
	AA*	160.08							
KPVAAP*		581.35	x	x	x	x	x	x	x
	KPVAA	484.30							
	PVAAP	453.26							
	KPVA	413.26							
	PVAA	356.42							
	VAAP	356.21			x		x	x	
	KPV	342.23			x		x	x	
	PVA	285.17							
	VAA *	259.15							
	AAP*	257.14							
	KP*	243.16		x		x			
	PV	214.13							
	VA	188.12		x	x	x	x	x	x
	AP*	186.10		x	x	x			x
	AA *	160.08							

*Sequences previously identified showing ACE inhibitory activity according to BIOPEP database (see Table 2).

[a] Possible fragments derived from the degradation of the precursor peptide.

[b] Monoisotopic molecular mass in Daltons of the matched peptide.

[c] Peptides detected in the apical compartment at different transport times.

[d] Peptides detected in the basal compartment at different transport times.

Table 2. Peptide sequences previously identified as ACE inhibitors reported by other authors.

Peptides	IC_{50} (μM)	References
LAP	3.5	Fujita, Yokoyama, and Yoshikawa (2000)
KPVAAP	12.37	Escudero et al. (2014)
VAA	13	Miyoshi, Ishikawa, Kaneko, Fukui, Tanaka, and Maruyama (1991)
AAPLAP	14.38	Escudero et al. (2014)
KP	22	Ichimura, Hu, Aita, and Maruyama (2003)
AAATP	100	Escudero et al. (2013)
AP	230	Cheung, Wang, Ondetti, Sabo, and Cushman (1980)
LA	310	Cushman, Cheung, Sabo, and Ondetti (1981)
PL	337.32	Byun, and Kim (2002)
AA	620	Cushman, et al. (1981)
AAP	n.d.	Meisel (1993)

Table 3. ACE inhibitory activity (IC_{50}) of synthetic peptides.

Peptides	IC_{50} (μM)
VAAP	16.75
PLAP	76.50
AAA	111.47
AATP	300.74
ATP	406.56
AAAT	513.65
VA	607.96
KPV	> 1000
AAPL	> 1000
APLA	> 1000

These results highlight the potent inhibition of fragment VAAP resulting from KPVAAP degradation, and PLAP from AAPLAP, which showed IC_{50} values of 16.75 and 76.5 µM, respectively. Hence, the ACE inhibitory activity shown by the peptide fragments could also explain the inhibitory activity found previously in apical and basal samples (see **Figure 2-b**), suggesting that those fragments transported across the monolayer could also reach the blood stream to exert an antihypertensive activity. As such, brush border peptidases play a key role in the formation and degradation of bioactive peptides and therefore, in their bioavailability and physiological effect (Pihlanto-Leppälä, 2000; Vermeirssen, et al., 2005; Miguel & Aleixandre, 2006).

The study done by Escudero et al. (2013) showed *in vivo* ACE inhibitory activity for the peptide AAATP, although according to the present study it is not absorbed intact through the intestinal barrier (**Table 1**). These results suggest that either the fragments derived from its degradation are responsible for the decrease in blood pressure in the SHR model, or AAATP may exert antihypertensive activity through the interaction with receptors expressed in the gastrointestinal epithelia (Yamada, Matoba, Usiu, Onishi, & Yoshikawa, 2002), thereby inducing other mechanisms of blood pressure regulation such as through nitric oxide or endothelin production (Lifton et al., 2001).

The stability of peptides to enzymatic hydrolysis and absorption processes determine their bioavailability and bioactivity, being the main cause of differences found between *in vitro* and *in vivo* assays (Pihlanto-Leppälä, 2000; Vermeirssen, Van Camp, & Verstraete , 2004; Bejjani, & Wu, 2013). In fact, the amount of peptides absorbed *in vivo* could be higher than *in vitro* assays when Caco-2 cells are used as models, due to the lower expression of some intestinal transporters and tighter junctions in Caco-2 cell monolayers compared to *in vivo* intestinal tissues (Lennernäs, Palm, Fagerholm, & Artursson, 1996; Boisset, Botham, Haegele, Lenfant, & Pachot, 2000; Vermeirssen et al., 2005). Nevertheless, this study was useful to propose a mechanism that explains the beneficial impact of dry-cured ham peptides towards blood pressure.

CONCLUSIONS

Previous studies reported the ACE inhibitory activity of some dry-cured ham peptides and their stability to *in vitro* digestion. However, antihypertensive peptides need to resist the complete hydrolysis by brush-border peptidases and be absorbed actively across the intestinal epithelium to exert their activity. The transepithelial transport through a Caco-2 cell monolayer has been studied in dry-cured ham extracts and purified peptides, evaluating the degradation of peptides by mass spectrometry. Results showed that peptides AAATP, AAPLAP and KPVAAP are degraded throughout the transport assay, although KPVAAP can also be absorbed intact through the intestinal barrier. Moreover, this study evidences for the first time the absorption and generation of ACE inhibitory peptide fragments originating from dry-cured ham by the Caco-2 cell line. So, the antihypertensive action of the peptides or small fragments derived as well as the synergic effect between them could explain the ACE inhibitory results obtained in apical and basal samples, suggesting that dry-cured ham peptides could reach the circulatory system to exert an antihypertensive action.

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IV. Discusión general

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Durante el proceso de elaboración del jamón curado tienen lugar una serie de reacciones enzimáticas entre las que destaca la proteolisis, en la cual las enzimas musculares degradan progresivamente las proteínas sarcoplásmicas y miofibrilares (Toldrá y Flores, 1998). Estas enzimas endógenas son las endopeptidasas (catepsinas y calpaínas), que hidrolizan las proteínas dando lugar a grandes polipéptidos, y las exopeptidasas (principalmente peptidasas, aminopeptidasas y carboxipeptidasas), que degradan estos polipéptidos generando una gran cantidad de péptidos pequeños y aminoácidos libres responsables de las características organolépticas del producto final tales como color, textura, aroma y sabor (Aristoy y Toldrá, 1995; Toldrá et al., 1997; Toldrá y Flores, 1998; Lametsch et al., 2003).

La identificación de los péptidos generados por procesos proteolíticos ha sido posible gracias al reciente avance en técnicas proteómicas como la espectrometría de masas en tándem, las cuales presentan una gran precisión, especificidad, sensibilidad y versatilidad (Léonil et al., 2000). Consecuentemente, ha sido en la última década cuando se han podido identificar en jamón curado secuencias específicas de péptidos derivados de la degradación de proteínas miofibrilares como la actina (Sentandreu et al., 2007), titina y miosina (Mora et al., 2009a) o troponina T (Mora et al., 2010), así como de proteínas sarcoplásmicas como la creatina quinasa (Mora et al., 2009b), enzimas glicolíticas (Mora et al., 2011) o mioglobina (Mora y Toldrá, 2012), evidenciando la intensa acción de las enzimas musculares durante el proceso de elaboración del jamón curado. En este contexto, el método más comúnmente utilizado para la identificación de proteínas y péptidos se basa en la digestión con una enzima conocida (normalmente tripsina) que presenta sitios de corte específicos, el análisis por espectrometría de masas y la posterior identificación de la proteína de origen a partir de los espectros de masas de los fragmentos peptídicos en bases de datos de proteínas. Este análisis, denominado huella peptídica, necesita que las masas experimentales de un determinado número de fragmentos

coincidan con las masas teóricas de los péptidos contenidas en las bases de datos y que cubran parte de la secuencia de la proteína de origen para que la identificación sea correcta. En el caso de la identificación de los péptidos generados de forma natural durante el proceso de elaboración del jamón, éstos son demasiados pequeños para ser digeridos enzimáticamente, por lo que deben adoptarse otras estrategias proteómicas basadas en la separación por diferentes técnicas cromatográficas y el análisis por espectrometría de masas.

Teniendo en cuenta estos aspectos, la primera parte de esta tesis aborda la identificación de los péptidos generados a partir de la degradación de diferentes proteínas musculares a varios tiempos durante el proceso de elaboración del jamón (2, 3.5, 5, 6.5, y 9 meses). Además, se lleva a cabo el estudio de las posibles enzimas responsables de la intensa proteolisis que tiene lugar a lo largo de este proceso y la caracterización de péptidos potenciales marcadores del tiempo de curado, que podrían ser utilizados para prevenir posibles fraudes relacionados con el tiempo de proceso y asegurar la calidad del jamón. En primer lugar, se ha estudiado la degradación de la proteína 3 de unión al dominio LIM (LDB3) (capítulo 1), que aunque es un componente minoritario del sarcómero, es esencial para el mantenimiento de la estructura e integridad del músculo (Zhou et al., 2001; Luther, 2009). El análisis por ESI-LC-MS/MS mediante un analizador cuadrupolo-trampa iónica ha permitido identificar las secuencias de 107 péptidos y conocer los cambios proteolíticos que tienen lugar a lo largo del proceso de elaboración del jamón, evidenciando la compleja acción de las enzimas catepsinas y calpaínas y, mayoritariamente, de exopeptidasas tales como las aminopeptidasas, carboxipeptidasas, dipeptidilpeptidasas y tripeptidilpeptidasas en la liberación de pequeños péptidos y aminoácidos libres. Además, se han identificado 12 péptidos que contienen en sus secuencias residuos de metionina oxidadas, mostrando 6 de ellos esta modificación al final del proceso (9 meses). La oxidación de proteínas tiende a ocurrir a lo largo de todo el proceso de elaboración del jamón, pero ocurre más notablemente tras la etapa de postsalado (3.5 meses) y se mantiene hasta el final del curado (Koutina et al., 2012). De este modo, los péptidos identificados con oxidación de

metionina al final del curado en este estudio podrían proceder de la degradación de péptidos identificados en etapas anteriores del proceso de elaboración del jamón y que ya presentaban metioninas oxidadas. Además, el hecho de que algunos péptidos aparezcan sólo en determinadas etapas del proceso (por ejemplo, de los 2 a los 6.5 meses) o sólo al final del curado (9 meses), podría sugerir un uso potencial de estos péptidos como biomarcadores para controlar y estimar el tiempo de proceso del jamón. Por otro lado, y siguiendo la misma metodología experimental, el análisis por ESI-LC-MS/MS mediante un analizador cuadrupolo-tiempo de vuelo en tándem permitió la identificación de 68 péptidos resultantes de la degradación de la proteína ribosomal ubiquitina-60S (capítulo 2). En este estudio se ha demostrado de nuevo la complejidad, dinamismo y variabilidad de la proteolisis que tiene lugar a lo largo del proceso de elaboración del jamón debido a la acción de las enzimas endógenas. Además, 14 de los péptidos han sido identificados exclusivamente al final de la etapa de curado, de modo que podrían utilizarse como marcadores del tiempo de curado del jamón, indicando un tiempo mínimo de proceso del producto de 9 meses.

Habitualmente, los análisis de proteínas y péptidos implican la elección de una técnica proteómica u otra en función de varios aspectos como las necesidades del análisis a realizar, disponibilidad económica o accesibilidad a equipos. En los estudios previos, el análisis por LC-MS/MS con una fuente de ionización por electrospray (ESI) permitió la identificación de los péptidos generados de manera natural en el jamón y de los posibles péptidos marcadores del tiempo de curado con alta resolución, precisión y sensibilidad. Sin embargo, la ionización tipo MALDI acoplada a un detector tiempo de vuelo (TOF) también se ha descrito como una técnica muy efectiva para determinar las masas moleculares de los péptidos con una gran resolución y precisión (Careri y Mangia, 2003; Contreras et al., 2008), siendo un método más rápido, sencillo y económico que ESI-LC-MS/MS. Así pues, se decidió llevar a cabo el estudio de la degradación de la proteína titina a lo largo del proceso de elaboración del jamón (capítulo 3) mediante ESI-LC-MS/MS con un analizador híbrido cuadrupolo-tiempo de vuelo y mediante espectrometría de masas MALDI-TOF. El análisis por ESI-LC-MS/MS

permitió identificar las secuencias de 320 péptidos derivados de esta proteína, la cual es responsable de la elasticidad del sarcómero. Las enzimas calpaínas tiene gran importancia en la hidrólisis de la titina durante las primeras etapas del proceso de elaboración del jamón (Koohmaraie y Geesink, 2006), ya que posteriormente son inactivadas por el efecto de la sal (Rosell y Toldrá, 1996), mientras que las catepsinas y distintas exopeptidasas participan en su degradación durante todo el proceso de curado. Del total de péptidos identificados, 57 de ellos fueron únicamente detectados a los 9 meses, por lo que podrían ser utilizados como marcadores del tiempo de curado del jamón. No obstante, se utilizaron los resultados por MALDI-TOF para confirmar la presencia exclusiva de estos péptidos al final del curado y descartar aquellos que aparecen también a tiempos previos del proceso. Así, sólo 4 péptidos (KDEAAKPKGPIKGVAKK, KKLRPGSGGEK, KNTDKWSECAR y ISIDEGKVL) fueron identificados y confirmados por ambos métodos exclusivamente al final del proceso, los cuales podrían ser útiles como marcadores de un tiempo mínimo de curado del jamón de 9 meses. Las diferencias en la detección de péptidos por la distinta ionización entre ESI y MALDI son debidas a que la ionización por ESI es una técnica líquida en la que un mismo péptido puede aparecer con distintas cargas, extendiendo el rango de masas y permitiendo, por tanto, la identificación de péptidos relativamente grandes con un nivel de detección de masas pequeño, mientras que la ionización por MALDI genera mayoritariamente iones monocargados. Además, la composición en aminoácidos y características de los péptidos influye en el comportamiento durante la ionización (Stapels y Barofsky, 2004), lo que hace muy interesante el uso combinado de ESI y MALDI para obtener información complementaria en un estudio proteómico (Bodnar et al., 2003).

Además de los fenómenos proteolíticos, otros cambios químicos como la oxidación de proteínas y péptidos tienen lugar de forma importante durante el proceso de elaboración del jamón curado, afectando a la calidad del producto final. En este sentido, diversos estudios se han centrado en la oxidación de proteínas durante el proceso de elaboración, maduración, envasado, o

congelación de la carne y derivados cárnicos, así como en su efecto sobre los aspectos nutricionales y sensoriales de estos productos (Estévez, 2011; Wang et al., 2011; Koutina et al., 2012; Zhang et al., 2013). En cuanto a la oxidación de péptidos, los aminoácidos metionina y cisteína son los más susceptibles a cambios oxidativos debido a los átomos de sulfuro que contienen (Shacter, 2000). Durante el proceso de curado del jamón se ha observado la presencia de péptidos con oxidaciones de metionina procedentes de la degradación de la proteína creatina quinasa (Mora et al., 2009b) y mioglobina (Mora y Toldrá, 2012), así como de la proteína LDB3 estudiada en esta tesis (capítulo 1). No obstante, para poder entender mejor los mecanismos oxidativos que afectan a los péptidos durante el proceso de elaboración del jamón, fue necesario llevar a cabo un estudio más profundo que permitiese la caracterización de la oxidación de metionina en jamón de 14 meses de curado (capítulo 4). Así, el análisis por ESI-LC-MS/MS con un analizador cuadrupolo-tiempo de vuelo en tándem permitió la identificación de un total de 656 péptidos derivados de las principales proteínas miofibrilares del jamón, de los cuales 120 presentaron oxidación de metionina. Los mayores porcentajes de oxidación se encontraron en los péptidos procedentes de la degradación de las proteínas nebulina, titina, troponina I, y dos tipos de proteínas de la cadena pesada de miosina, mientras que la menor oxidación se produjo en los péptidos de alfa-actina y de dos tipos de proteínas de la cadena ligera de miosina. Estas diferencias son debidas a que la oxidación de las metioninas depende de su localización en la estructura de la proteína y accesibilidad a agentes oxidantes, así como de los residuos aminoacídicos próximos a ellas. De hecho, se ha descrito que aquellas metioninas próximas a residuos de ácido aspártico, ácido glutámico, serina, alanina o treonina son más susceptibles a ser oxidadas (Levine et al., 1996; Ghesquière y Gevaert, 2014). La importancia de la oxidación de aminoácidos esenciales como la metionina, se debe a su efecto sobre la calidad y valor nutritivo del producto, ya que supone un deterioro del aroma, sabor, color y textura, una disminución en la disponibilidad de aminoácidos y una menor digestibilidad de las proteínas oxidadas (Lund et al., 2011). El estudio llevado a cabo en esta tesis aporta información sobre las características de los péptidos oxidados y la influencia tanto del grado de

hidrólisis de las proteínas como de los aminoácidos próximos a la metionina en la oxidación de este aminoácido. Sin embargo, se necesitan estudios adicionales para conocer mejor los procesos oxidativos que tienen lugar durante el proceso de elaboración del jamón curado y su influencia sobre las características sensoriales y nutricionales de este producto.

Además de la identificación de proteínas y péptidos, la espectrometría de masas es una herramienta fundamental en la cuantificación de proteínas en muestras complejas como la carne y productos cárnicos (Aebersold y Mann, 2003; Bendixen, 2005). En este sentido, la cuantificación de péptidos marcadores de calidad del jamón supondría un paso adicional importante, debido al interés de conocer la cantidad de péptidos bioactivos presentes en el jamón, estudiar la evolución de los péptidos durante el proceso de elaboración o caracterizar posibles péptidos marcadores del tiempo de curado del jamón en base a su abundancia. Por ello, el siguiente objetivo de la presente tesis fue la optimización de un método sin marcaje ("label-free") para la cuantificación relativa de proteínas a partir de la cuantificación de los péptidos generados (capítulo 5). Esta metodología se ha basado en la medida de las intensidades de señal de los iones obtenidas tras el análisis por ESI-LC-MS/MS con un analizador cuadrupolo-tiempo de vuelo en tándem, que como ya se comentó en la introducción, presenta varias ventajas frente a otras metodologías. Brevemente, esta técnica presenta una mayor especificidad, precisión y rango dinámico que los métodos tradicionales utilizados hasta el momento para la cuantificación de proteínas en carne y productos cárnicos basados en la densidad de bandas electroforéticas. Además, presenta un menor coste y complejidad así como una mayor versatilidad con respecto a las metodologías que utilizan marcaje isotópico, así como una mayor linealidad de respuesta y precisión en la evaluación de cambios proteicos entre muestras que los métodos basados en el conteo espectral (Aebersold y Mann, 2003; Bendixen, 2005; Bantscheff et al., 2007). Para la optimización del método cuantitativo sin marcaje, parámetros tales como la linealidad, repetitividad y recuperación fueron evaluados mediante mezclas de patrones proteicos, obteniéndose muy buenos resultados. Con el fin de

conseguir un análisis robusto y preciso cuando se utilizan métodos sin marcaje es imprescindible una alta reproducibilidad en la preparación de la muestra, la inyección en el sistema LC-MS/MS, la separación cromatográfica y la ionización de los péptidos. Así pues, es muy importante tener bien establecidos los métodos de extracción de péptidos y digestión enzimática para asegurar que se lleven a cabo de manera completa y así evitar errores en la cuantificación. Sin embargo, frecuentemente se introducen variaciones incontrolables durante el proceso experimental que provocan, por ejemplo, pequeñas diferencias en el tiempo de retención, intensidad de señal o ruido de fondo durante el análisis. Para reducir estas fuentes de variabilidad y llevar a cabo la evaluación comparativa de péptidos de forma precisa y robusta, es necesario introducir un normalizador, es decir, una proteína conocida y que no está presente en la muestra que se introduce desde la primera etapa de la metodología. Consecuentemente, el análisis de datos se hace más complejo respecto a otros métodos, ya que implica la normalización de los datos, el alineamiento de los picos obtenidos en el espectro MS/MS y la evaluación estadística de los conjuntos de datos obtenidos en las distintas réplicas del análisis LC-MS/MS en un solo procedimiento (Wang et al., 2003; Zhu et al., 2010; Neilson et al., 2011). Tras la optimización del método y con el fin de demostrar su viabilidad y robustez, éste se utilizó para la cuantificación relativa de proteínas en carne de cerdo, demostrando ser una metodología rápida, simple y fiable para evaluar cambios en la abundancia de proteínas. Posteriormente, su aplicación en jamón curado permitió la caracterización de la degradación proteica que se produce a lo largo del proceso de elaboración, en concreto de las principales proteínas sarcoplásmicas (capítulo 6). De hecho, 20 proteínas han sido identificadas en una sola separación por cromatografía líquida del extracto de proteínas sarcoplásmicas, las cuales incluyen 12 enzimas relacionadas con procesos glucolíticos así como la creatina quinasa, involucrada en el metabolismo energético, y la mioglobina, principal responsable del color de la carne. Tradicionalmente, los procesos proteolíticos que tienen lugar en el jamón han sido evaluados mediante métodos electroforéticos (Toldrá et al., 1993; Córdoba et al., 1994) en los que la identificación de proteínas se ha llevado a cabo en

función del peso molecular de las bandas obtenidas en el gel (Larrea et al., 2006) y la cuantificación por medidas de densidad de las bandas electroforéticas (Larrea et al., 2006; Bermúdez et al., 2014). Esta metodología presenta varias limitaciones ya que no permite la identificación de las proteínas de bandas subyacentes, puede llevar a errores en la identificación cuando se evalúa la proteolisis ya que las proteínas sufren roturas generando fragmentos proteicos, y aporta resultados cuantitativos poco precisos, principalmente en el caso de proteínas solapadas (Bendixen, 2005; Bantscheff et al., 2007). Por otro lado, algunos autores han combinado la electroforesis en gel con espectrometría de masas MALDI-TOF para una identificación de proteínas precisa (Di Luccia et al., 1995), pero esta metodología implica etapas adicionales, laboriosas y más complejas en la preparación de las muestras, además que la identificación por huella peptídica es útil para proteínas individualizadas, complicándose conforme aumenta la complejidad de la muestra. Por ello, la metodología presentada en esta tesis supone un avance para evaluar la degradación de proteínas durante el proceso de elaboración del jamón, ya que permite la identificación y cuantificación de las proteínas de forma más simple, fiable y precisa que los métodos utilizados en estudios previos. Además, podría ser utilizada para la cuantificación de péptidos marcadores de calidad del jamón curado.

Por otro lado, una metodología experimental similar a la utilizada para identificar los péptidos generados de manera natural en el jamón curado también ha sido utilizada para la identificación de péptidos con propiedades bioactivas. En este sentido, se han identificado secuencias peptídicas en jamón curado que presentan actividad antioxidante (Escudero et al., 2013a) e inhibidora de la enzima convertidora de angiotensina I (ECA) (Escudero et al., 2013b; Escudero et al., 2014), lo que aporta un alto valor añadido a este producto. Sin embargo, para que estos péptidos bioactivos ejerzan su acción *in vivo* es necesario que alcancen el torrente circulatorio en forma activa, de modo que deben resistir previamente la completa degradación por enzimas gastrointestinales y además ser absorbidos a través de la pared intestinal preservando su actividad biológica (Vermeirssen et al., 2005). Debido a la importancia de la barrera gastrointestinal sobre la

disponibilidad y actividad de los péptidos bioactivos, se llevó a cabo un estudio del transporte intestinal *in vitro* de extractos peptídicos obtenidos a partir de diferentes tipos de jamón curado mediante un modelo de epitelio intestinal basado en monocapas de células Caco-2 (capítulo 7). Además, debido a que estos extractos contienen una mezcla compleja de péptidos, se realizó un estudio más detallado con los péptidos AAPLAP y KPVAAP, los cuales habían mostrado un gran poder de inhibición *in vitro* y una buena estabilidad tras una digestión gastrointestinal simulada (Escudero et al., 2014), y con el péptido AAATP, el cual había mostrado una significativa actividad antihipertensiva tanto *in vitro* como *in vivo* (Escudero et al., 2013b). Tras la simulación del transporte intestinal, el ensayo *in vitro* de la actividad inhibidora de la ECA de las muestras apicales evidenció la máxima inhibición tras los 15 minutos del transporte, mientras que las muestras basales no mostraron, en general, resultados significativos. Sin embargo, cuando las muestras basales fueron concentradas para asemejarse a la concentración de las muestras apicales, se obtuvieron valores de inhibición de la ECA muy notables, principalmente en el caso de los péptidos AAPLAP y KPVAAP. A la vista de estos resultados y debido a que los fragmentos peptídicos generados durante el transporte podrían también ejercer actividad inhibidora de la ECA, incluso mayor que los péptidos de los que proceden (Pihlanto-Leppälä, 2000; Miguel et al., 2004), se llevó a cabo el análisis de las muestras tomadas tras el transporte de los péptidos AAATP, AAPLAP y KPVAAP con el fin de identificar los posibles fragmentos generados. Así, un primer análisis por MALDI-TOF MS permitió determinar de manera rápida y sencilla el perfil de péptidos generados durante el proceso, mientras que la identificación de los posibles fragmentos peptídicos generados y transportados se realizó mediante MALDI-TOF/TOF, por ser una técnica robusta, con alta precisión, amplio intervalo de masas, y muy rápida en la adquisición de datos. Los resultados obtenidos han permitido estudiar la degradación de los péptidos por las peptidasas intestinales y conocer los fragmentos generados, así como evaluar qué péptidos y fragmentos han sido transportados a través de la monocapa de células Caco-2. Además, algunos de los fragmentos peptídicos detectados en el ensayo habían sido previamente identificados como antihipertensivos, mientras que el resto fueron ensayados *in*

vitro para evaluar su actividad inhibidora de la ECA y justificar los resultados de inhibición obtenidos en el ensayo previo. De esta forma, este estudio confirma el importante papel de las peptidasas intestinales en la formación y degradación de péptidos así como en la biodisponibilidad y efecto antihipertensivo de los mismos (Pihlanto-Leppälä, 2000; Vermeirssen et al., 2005; Miguel y Aleixandre, 2006), y evidencia la absorción que tiene lugar a través del epitelio intestinal de péptidos del jamón inhibidores de la ECA, los cuales podrían ejercer un efecto antihipertensivo *in vivo*.

Los resultados de la presente tesis se han centrado en la caracterización de los péptidos del jamón como marcadores de calidad, ya sea por su potencial como marcadores del tiempo de curado como por sus propiedades bioactivas. Además, estos péptidos tienen una gran influencia sobre las características sensoriales del producto final, y su estudio permite un mayor conocimiento de los complejos procesos bioquímicos que tienen lugar durante el proceso de elaboración del jamón. En este contexto, el uso de técnicas proteómicas como la espectrometría de masas es fundamental para la identificación y cuantificación de péptidos generados de manera natural en el jamón curado, siendo la base de investigaciones futuras para una mayor comprensión y control del proceso de elaboración de este producto tradicional.

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V. Conclusiones

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- Las técnicas proteómicas basadas en espectrometría de masas constituyen una herramienta fundamental para la identificación de péptidos generados de manera natural a lo largo del proceso de elaboración del jamón curado. Así, se ha evidenciado la intensa y compleja degradación de la proteína 3 de unión al dominio LIM, ubiquitina y titina por acción de las enzimas musculares endopeptidasas y exopeptidasas, así como el potencial de algunos péptidos como marcadores del tiempo de curado para garantizar la calidad del jamón. Esta metodología ha permitido además el estudio de péptidos generados a partir de la degradación de las principales proteínas miofibrilares del jamón curado y que han experimentado oxidación, cuya importancia radica en su efecto negativo sobre el valor nutritivo y características sensoriales del jamón.

- La optimización de una metodología sin marcaje ("label-free") para la cuantificación relativa de proteínas por espectrometría de masas ha permitido estudiar de manera rápida, sencilla y fiable los cambios en la abundancia de las principales proteínas sarcoplásmicas a lo largo del proceso de elaboración del jamón curado. Esta metodología supone un avance con respecto a otros métodos cuantitativos que presentan menor especificidad y precisión o requieren etapas más complejas de preparación de las muestras, y podría ser utilizada para la cuantificación de péptidos marcadores de calidad del jamón curado.

- El uso de células Caco-2 como modelo de epitelio intestinal ha evidenciado la absorción de péptidos del jamón curado que son inhibidores de la enzima convertidora de angiotensina I (ECA) así como la generación y absorción de fragmentos derivados de los mismos y que podrían ejercer un efecto antihipertensivo *in vivo*.

Anexo

Nomenclatura de aminoácidos

Aminoácido	Abreviatura de tres letras	Abreviatura de una letra
Alanina	Ala	A
Arginina	Arg	R
Asparagina	Asn	N
Ácido aspártico	Asp	D
Cisteína	Cys	C
Ácido glutámico	Glu	E
Glutamina	Gln	Q
Glicina	Gly	G
Histidina	His	H
Isoleucina	Ile	I
Leucina	Leu	L
Lisina	Lys	K
Metionina	Met	M
Fenilalanina	Phe	F
Prolina	Pro	P
Serina	Ser	S
Treonina	Thr	T
Triptófano	Trp	W
Tirosina	Tyr	Y
Valina	Val	V