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FORMACIÓN Y BIODISPONIBILIDAD DE PÉPTIDOS ALIMENTARIOS CON EFECTO SOBRE LA SALUD DURANTE LA DIGESTIÓN GASTROINTESTINAL DE PROTEÍNAS LÁCTEAS

RELEASE AND BIOAVAILABILITY OF FOOD-DERIVED PEPTIDES WITH HEALTH EFFECT DURING GASTROINTESTINAL DIGESTION OF MILK PROTEINS

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“Sow an act, and you reap a habit. Sow a habit and you reap a character. Sow a character and you reap a destiny. ”

Charles Reade

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ÍNDEX

ABSTRACT

In this thesis several aspects related to the production and subsequent modification of the peptide sequences within food matrices have been studied, taking into account the influence of technological treatments, and the modifications once they are ingested, including gastrointestinal digestion, absorption and metabolism. Liquid chromatography coupled to tandem mass spectrometry with different analyzers (ion trap, Q-TOF, MALDI TOF/TOF) has been used to perform sequencing and quantification of peptides within different dairy matrices (fermented milk, cheese, gastrointestinal digests) or biological samples (plasma). Special attention has been paid to the β-casein region 126-140, which is a well conserved domain among different species and hosts several active sequences, such as LHLPLP, f(133-138) , and HLPLP, f(134-138).

The production of active compounds and the impact of the packaging conditions on peptides have been also evaluated in this thesis. An alternative was provided to produce active hydrolysates, containing the antihypertensive peptides from β-casein, f(134-138), HLPLP, and f(133-138), LHLPLP, by casein fermentation using two food-grade yeast strains of *Debaryomyces hansenii*. In addition, the peptide profile of two cheeses packed using different technologies, vacuum and modified atmosphere, was studied during the shelf life of the product. No marked qualitative differences could be found in the peptide profile regarding the packaging technique and storage time, although the relative abundance of the peptides was different in both packaging systems. Once the production of active compounds and the impact of the packaging conditions on peptides have been evaluated, the peptidome generated through digestion under different conditions of several dairy matrices was studied. Skimmed milk powder (SMP) and a highly proteolysed blue cheese were subjected to static gastrointestinal digestion in order to assess the influence of the proteolytic state of the matrix in the release of peptides. Several regions of β-casein were shown to be

resistant to digestion i.e. 60-93, 128-140 and 193-209, in both matrices. The differences found among cheese and milk were attributed to the initial state of proteolysis of cheese, which hosted several precursor fragments for bioactive peptides, i.e. LHLPLP, before digestion, in contrast to SMP. On the other hand, the impact of heat treatment on the release of peptides during gastric digestion of heated and unheated milk has been studied using a dynamic digester, which represents next step after static models to better reproduce physiological conditions. The heat treatment elicited an increase in the resistance of casein fraction to digestion by pepsin, in contrast to β-Lg, which became more susceptible. Some resistant domains from β-casein (76-93, 126-140 and 190-209) were identified regardless the heat treatment, where the active sequences LHLPLP and HLPLP are located. Later on, an antihypertensive sequence included in the resistant domain of β-casein 126-140, i.e. HLPLP, f(134-138), was demonstrated for the first time to be absorbed into blood circulation in rats after its oral administration. The kinetic parameters of HLPLP after oral and intravenous administration were determined, being the effective absorption around 5.18 %, based on the quantification of the intact penta-peptide in plasma. However, it was found that several derived fragments, HLPL β-casein f(134-137) and LPLP β-casein f(135-138), were rapidly formed in plasma after both types of administration, and therefore, the absorption of the peptide is probably higher. The degradation of the penta-peptide by the action of plasma peptidases was confirmed by in vitro incubation of HLPLP in plasma, where several fragments i.e. HLPL, LPLP and HLP were released, and the antihypertensive activity of these fragments was demonstrated in a SHR model.

Subsequently, the implication of opioid receptors in the mechanism of action of two antihypertensive peptides from α_{s1}-casein (RYLGY, f(90-94) and AYFYPEL, f(143-149)) and of a casein hydrolysate, containing these, was shown by the co-administration of naloxone. The antihypertensive activity of YFYPEL, α_{s1}-casein f(144-

149) also present in the hydrolysate, was demonstrated for the first time, but its mechanism of action did not seem to involve opioid receptors.

The application and optimisation of peptidomic techniques has permitted to characterise the peptide fraction within dairy matrices and to evaluate the influence of the technological treatments, the changes during digestion, absorption, metabolism, and ultimately estimate the *in vivo* bioavailability of an active sequence.

RESUMEN

En esta tesis se abordan varios aspectos relacionados con la producción y las modificaciones de la fracción peptídica en matrices alimentarias, teniendo en cuenta la influencia de los tratamientos tecnológicos, y los cambios que sufren tras ser ingeridas, incluyendo la digestión gastrointestinal, absorción y metabolismo. Se ha utilizado cromatografía de líquidos acoplada a espectrometría de masas en tándem con distintos analizadores (trampa de iones, Q-TOF, MALDI TOF/TOF) para llevar a cabo tanto la secuenciación como la cuantificación de péptidos en distintos productos lácteos (leche fermentada, quesos, digeridos gastrointestinales) o muestras biológicas (plasma). Se ha prestado especial atención a la región (126-140) de la β-caseína, donde se encuentran incluidas algunas secuencias activas de interés, por ejemplo LHLPLP, f(133-138) y HLPLP, f(134-138).

Se propone una alternativa para la producción de hidrolizados activos que contienen los péptidos antihipertensivos de la β-caseína f(134-138), HLPLP, and f(133-138), LHLPLP, mediante la fermentación de caseína comercial con dos cepas de levaduras de *Debaryomyces hansenii* de grado alimentario. Se evaluaron en quesos semicurados el efecto del envasado, a vacío y en atmósfera modificada, en el perfil peptídico durante su vida útil. No se encontraron diferencias cualitativas destacables en el perfil peptídico de los quesos respecto al tipo de envasado pero sí en la intensidad relativa de los péptidos identificados. Una vez se hubo examinado la producción de secuencias activas y el impacto del envasado en el perfil peptídico, se estudió el peptidoma generado durante la digestión de varios productos lácteos, utilizando distintas condiciones. Se comparó el peptidoma obtenido al someter leche en polvo y queso azul a un proceso de digestión gastrointestinal estática, con el fin de evaluar la influencia del estado proteolítico de la matriz en la formación de péptidos. Se observó la resistencia a la digestión de ciertas regiones de la β-caseína, concretamente los residuos 60-93, 128-140 y 193-209, tanto en los digeridos de leche

en polvo como de queso. Sin embargo, también se observaron diferencias que pueden ser atribuidas al estado proteolítico inicial del queso, que antes de su digestión ya contenía fragmentos precursores de algunos péptidos activos estudiados como el péptido LHLPLP. Por otra parte, se estudió la formación de péptidos, en leche con distinto tratamiento térmico, a lo largo de la digestión gástrica empleando un digestor dinámico, cuyo uso representa un nivel más sofisticado con respecto a los modelos estáticos para reproducir las condiciones fisiológicas. Se pudo concluir que el tratamiento térmico provoca un aumento de la resistencia de las caseínas a la digestión por pepsina, en contraposición a la β-Lactoglobulina, que resultó ser más susceptible. Se identificaron algunos dominios de la β-caseína resistentes a la digestión gástrica independientemente del tratamiento térmico, por ejemplo las regiones 76-93, 126-140 y 190-209, una de las cuales alberga las secuencias activas LHLPLP y HLPLP, mencionadas anteriormente. Más adelante, se demuestra por primera vez que una secuencia antihipertensiva perteneciente al dominio de la β-caseína 126-140, resistente a la digestión, HLPLP f(134-138), se absorbe al torrente sanguíneo de ratas tras su administración oral. Además, se calcularon los parámetros farmacocinéticos de absorción y eliminación de dicho péptido. Su absorción total efectiva, determinada en base a la cantidad intacta del pentapéptido fue estimada en 5.18%. Sin embargo, se constató que tras su administración intravenosa y oral se formaban rápidamente varios fragmentos *in vivo* derivados de él como el HLPL, β-caseína f(134-137) y LPLP β-caseína f(135-138), por lo que la absorción efectiva probablemente es mayor a la calculada. La incubación del péptido HLPLP *in vitro* permitió confirmar la degradación del péptido HLPLP por acción de las peptidasas plasmáticas, encontrándose los fragmentos HLPL, LPLP y HLP, que presentaron actividad antihipertensiva en un modelo de ratas espontáneamente hipertensas.

Asimismo, se muestra que los receptores opioides están implicados en la actividad antihipertensiva de los péptidos RYLGY, α_{s1}-caseína f(90-94) y AYFYPEL,

α_{s1}-caseína f(143-149) y el hidrolizado activo que los contiene, a través de su coadministración con naloxona. Se ha demostrado por primera vez la actividad antihipertensiva del péptido YFYPEL α_{s1}-caseína f(144-149), presente también en el hidrolizado, pero cuyo mecanismo de acción no parece implicar receptores opioides.

La aplicación y optimización de las técnicas peptidómicas ha permitido realizar una caracterización de la fracción peptídica de distintos productos lácteos y evaluar la influencia de los tratamientos tecnológicos, los cambios durante la digestión, absorción y metabolismo, llegando a estimar la biodisponibilidad *in vivo* de una secuencia activa.

ABBREVIATIONS

ACE: Angiotensin Converting-Enzyme

α-La: α-Lactalbumin

β-Lg: β-Lactoglobulin

CPP: Caseinophosphopeptides

CM: Casomorphins

C_{max}: Maximum Concentration

CMP: Caseinomacropeptide

ESI: Electrospray ionization

GRAS: Generally recognized as safe

HPLC: High performance liquid chromatography

IT: Ion trap

LF: Lactoferrin

MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight

MS: Mass scpectrometry

MS/MS: Tandem mass spectrometry

N: Nitrogen

NO: Nitric oxide

Q-TOF: Quadrupole-time of flight

SDF: Simulated duodenal fluid

SGF: Simulated gastric fluid

SHR: Spontaneously hypertensive rat

SMP: Skimmed milk powder

T_{max}: Time required to reach the maximum concentration

UHT: Ultra high temperature

OBJECTIVES AND WORK PLAN

In the last decades, there has been an increasing interest on protein hydrolysates produced by enzymatic hydrolysis or by fermentation, containing biologically active peptides, such as, opioid (Teschemacher et al., 2003), antihypertensive (Martínez-Maqueda et al., 2012), antimicrobial (López-Expósito et al., 2008); or to improve mineral bioavailability (Phelan et al., 2009). When assessing the bioactivity of a given compound, i.e food-derived peptide, using animal models, the peptide is usually orally administered, and an observed effect is associated to its administration. However, the different phenomena occurring from the ingestion of the bioactive compound to the observation of the physiological effect remains unknown in most cases. Thus, it is crucial to explore the events occurring either before food ingestion (i.e. stability during shelf-life of the product) or once ingested, i.e., during gastrointestinal digestion, absorption, metabolism, mechanism of action, in order to elucidate the relationship between a bioactive compound and a given biological effect and also to identify the active form of this compound.

With this in mind, the primary objectives of the research were:

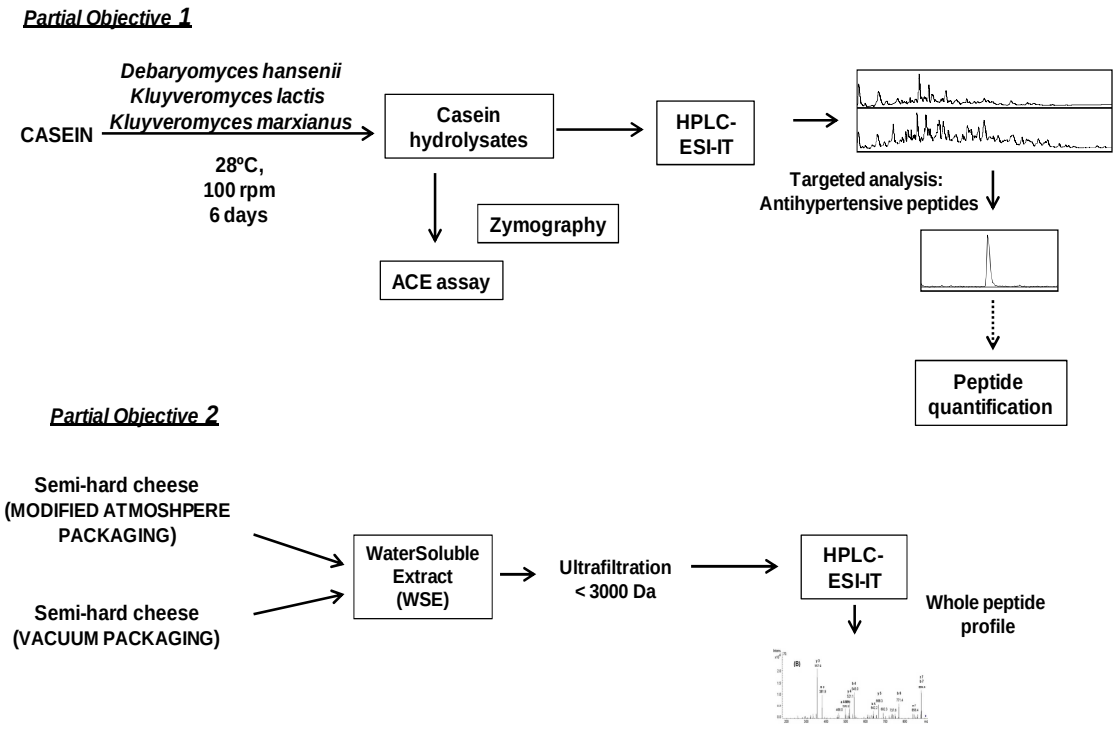
Objective 1

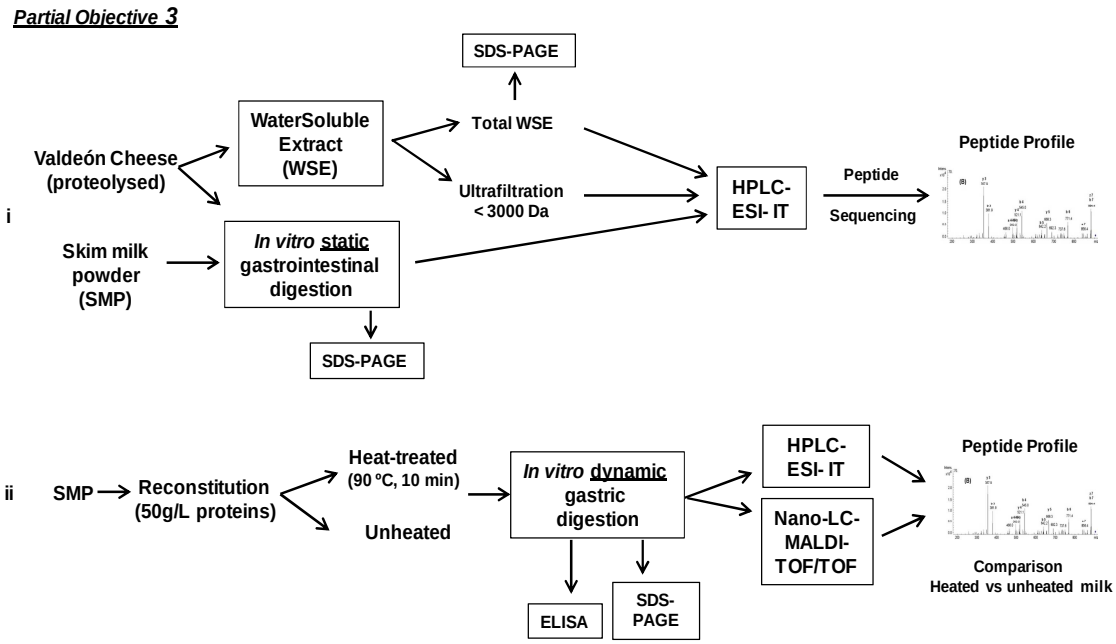
Application of a peptidomic strategy to the identification of peptides during production, shelf-life of the product and changes during digestion. Concretely, this first objective comprises three partial objectives:

1. Targeted search of antihypertensive peptides in casein fermented by yeasts
2. Evolution of the peptide profile of cheese during shelf-life when different packaging technologies are used (vacuum and modified atmosphere).

3. Evaluation of the changes undergone by dairy proteins during in vitro simulated gastrointestinal digestion to assess (i) the impact of proteolysis degree of the dairy matrix on the final digestome and (ii) the impact of heat treatment of milk protein on the subsequent release of peptides during dynamic gastric digestion.

To carry out these partial objectives (1, 2 and 3), this working plan was followed:

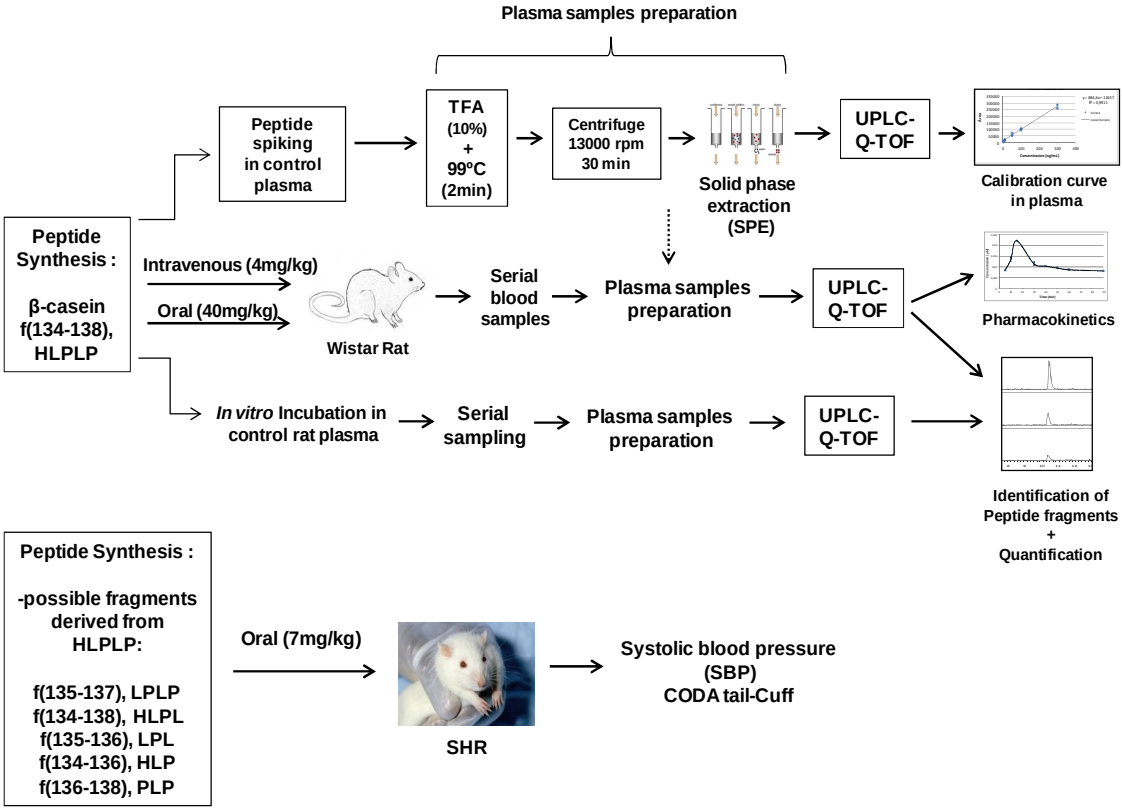




Scheme 1. Working plan objective 1

Objective 2

The aim of this study was to characterize whether the β-casein-derived peptapeptide HLPLP is bioavailable in rats, and to calculate the main kinetic parameters of absorption and elimination. Furthermore, the stability of the peptide in plasma was studied, as well as, the antihypertensive activity of the derived fragments.

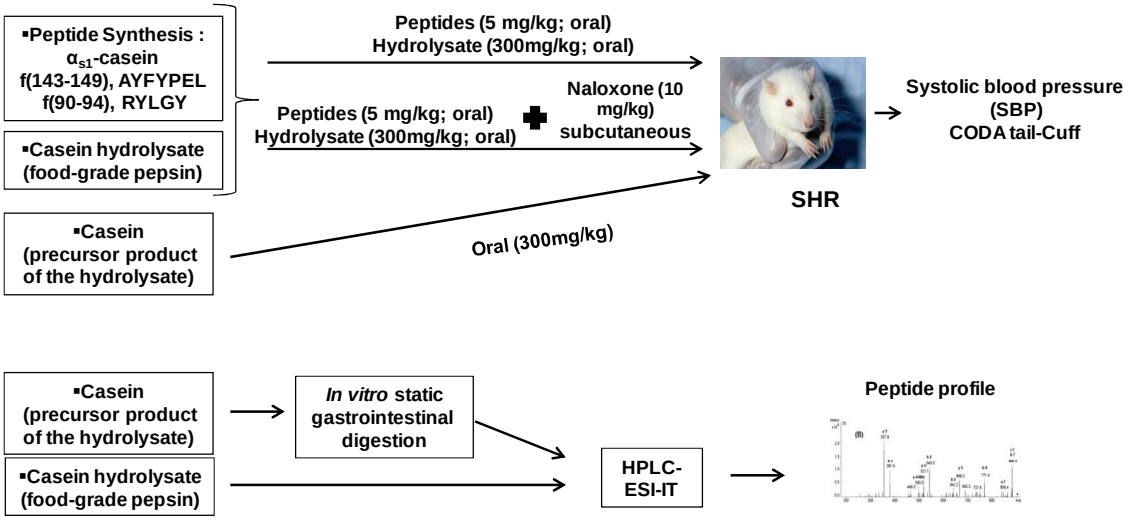


Scheme 2. Working plan objective 2

Objective 3

To evaluate the involvement of the opioid system in the mechanism of action of the active peptides [RYLGY f(90-94), AYFYPEL f(143-149) and YFYPEL f(144-149)] and an antihypertensive casein hydrolysate containing them.

To carry out these objectives, this working plan was followed:



Scheme 3. Working plan objective 3

1. INTRODUCTION

In the last years, much research has been done regarding the health effects of food proteins and on the biologically active peptides included in their sequences. There is evidence on different physiological processes occurring in the body mediated by endogenous peptides that can act as hormones or signalling peptides. Food proteins can lead to the release of peptides structurally similar to those found endogenously. Therefore, they could act as agonist or antagonist at the same target as their endogenous homologous peptides (Meisel et al., 1998). Therefore, recent research has been focused on the biological effect produced by peptides, such as, opioid (Teschemacher et al., 2003), antimicrobial (Agyei et al., 2012; López-Expósito et al., 2008), or antihypertensive (Martínez-Maqueda et al., 2012).

Likewise, much effort has been dedicated to the investigation of foodstuff itself, and to the processing thereof to produce certain bioactive sequences within the matrix, by fermentation or hydrolysis (Korhonen, 2009.) However, little information is available about the changes that active sequences undergo from ingestion to arrival at target organ. Therefore, it is important to understand these changes, since it would permit to establish a relationship with bioactivity, and attribute it to a certain compound. For instance, one of the most important parameters to consider when studying a bioactive compound is its bioavailability. From the nutritional perspective it is defined as the fraction thereof available to be employed in physiological functions or stored by the organism. This term includes another one, less extended, called bioaccesibility, which is the portion of the compound of interest released from the food matrix in its soluble form, becoming absorbable (Fernández-García et al., 2009). Both concepts are mostly determined by the process that food should undergo upon ingestion, gastrointestinal digestion. Digestion process provokes important modifications on foodstuff, and especially on food proteins, which determines the release, among others, of peptides encrypted in the proteins sequences. In order to exert their physiological effect, these peptides need to be available for absorption and subsequent use by the organism, or

for interaction with intestinal receptors (Shimizu et al., 2007; Shimizu, 2010). However, few studies have been carried out on the absorption of food-derived peptides, and their detection in plasma. Once it has been shown that a certain peptide is absorbed into blood circulation and could reach target organ, it is important to evaluate its metabolism, distribution, elimination and mechanism of action, to elucidate how and at which dose the peptide induces a positive effect on the organism, in order to establish a relationship between food and health. Up to date, no food-derived peptide has been approved by the EFSA, because the evidence presented was not sufficient to establish a cause and effect relationship between their consumption and a given health claim, such as, the maintenance of normal blood pressure. Therefore, there is a need to shed some light into the factors affecting the food from its ingestion to the final physiological effect of a peptide, which also involves the identification of the active form *in vivo* and the elucidation of its mechanism of action (Figure 1).

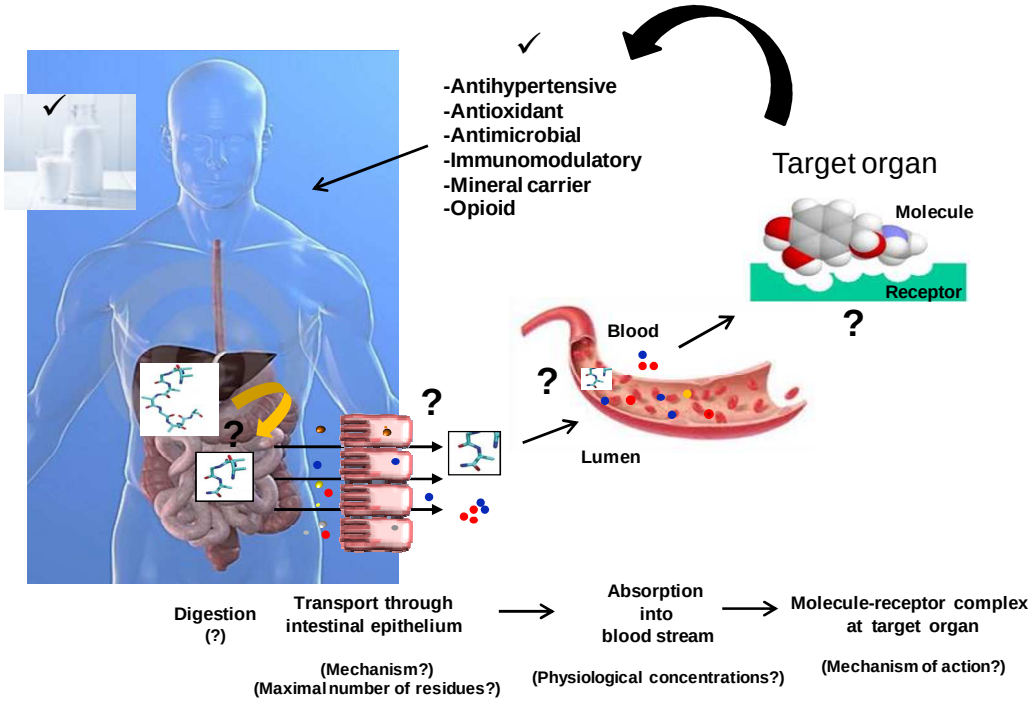


Figure 1. Path from ingestion to physiological effect.

1.1 Milk composition

Milk is an oil/water emulsion containing numerous substances, either in dissolution or in colloidal state. In addition to carbohydrates, lipids, water and minerals, milk contains proteins. The amount of the latter present in ovine milk is 4.75-7.20%, in caprine milk 2.6-4.1% and in bovine milk 2.5-3.5%. In all cases the total content of proteins depends on the breed, on individual animal variations, on lactating stage, and on nutritional and health state of the animal (for review see Raynal-Ljutovac y col., 2008; Dalgleish, 1993).

The protein composition of milk can be divided in two groups: whey proteins and caseins. The whey proteins are mainly comprised by 4 units: β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), seralbumin, and immunoglobulins (Ig). In addition, other minor proteins are present in milk, such as, Lactoferrin (LF), proteose peptone (fragments from N-terminal end of β-casein), enzymes and globular proteins from fat globule membrane. Whey proteins are globular proteins that undergo heat-induced denaturation. Moreover, they are not susceptible of phosphorylation, they are not labile to calcium and they display intramolecular disulfide bonds that stabilise their structure. The two major whey proteins are β-Lg and α-La. The first one is the most abundant one in ovine, caprine and bovine whey, although is not present in human milk.

The casein fraction comprises 4 proteins: α_{s1}-, α_{s2}-, *κ*-, and β-casein. Another minor protein exists in the casein fraction, γ-casein, generated by the action of proteolytic enzymes on β-casein. The total content of casein, as well as the relative ratio of each protein is characteristic for each species. In the case of ovine milk, the casein fraction is present at 45 g/L, being the percentages of caseins 33-35%, 8-14%, 30-38% and 14-17% for α_{s1}-, α_{s2}-, β- and *κ*-casein, respectively. In bovine milk, the amount of caseins is 29 g/L and the different caseins represent 37.6-39.5%, 7.8-12.1%, 33.4-44.6% and 9.5-19.7%, respectively. On the other hand, the content of caseins in caprine milk can vary from 27 g/L to 32 g/L, depending on the genetic polymorphism of

α_{s1}-casein, and the amount of them is 18-21%, 11-13%, 48-63% and 8-15%, respectively. Overall, caprine milk contains less α_{s1}-casein compared to other ruminant's milk (Bramanti et al., 2003; Raynal-Ljutovac y col., 2008). The caseins are proteins that may be phosphorylated in Serine residues and precipitate at pH 4.6 (could slightly change depending on the species), whereas the whey proteins are soluble at this pH value. Moreover, caseins are found in micellar structures. These structures are supramolecular protein aggregates containing caseins combined with calcium phosphate, magnesium, potassium, sodium and little amount of citrate (Fox, 1992).

1.2 Gastrointestinal digestion of milk proteins. Application of peptidomic-based techniques

Upon ingestion, food undergoes complex chemical and physical changes mediated by peristaltic movements and the different enzymes involved in gastrointestinal digestion that will finally disintegrate food. As a result of the specific or unspecific action of enzymes on food, macro- and micronutrients are released, and when they finally reach the small intestine, the absorption process occurs, mainly at the jejunum (Langerholc y col., 2011). In the case of proteins, they are differently susceptible to proteases depending on their conformation, the presence of cross-linkages between protein chains, fixed metals, antinutritional factors, like trypsin or chymotrypsin inhibitors, particle size of proteins ingested or biological differences between individuals. Milk proteins belong to the easily utilized class of proteins with a digestibility of 95% (Damodaran, 1997).

Given the undeniably important role of digestion on food components that finally will influence health, it is essential to know how food proteins are degraded during this process. The *in vivo* experiments provide the most valuable information in this sense. Intervention studies have been carried out in humans in order to evaluate digestion of milk proteins (bovine unless otherwise indicated). Nevertheless, animal models have

been widely used as a preliminary approach on the study of *in vivo* digestion of milk proteins. Porcine models are one of the most commonly used models, to whom cannulas at stomach and jejunum are usually placed to collect the effluents (Figure 2); although reports exist on the use of rats or calves at this aim.

Figure 2. Cannulated pig.

1.2.1. Human studies on digestion of milk proteins

Several studies have been conducted in humans to study the in vivo digestion of milk proteins. Milk proteins have high oro-ileal digestibility, and protein quality, assessed in terms of the net postprandial protein utilization in healthy humans (Bos et al., 1999). The influence of diet composition on the postprandial utilization of milk proteins has been pointed out (Gaudichon et al., 1999). Indeed, in this work the ingestion of milk supplemented with sucrose delayed the absorption of protein longer than supplementation with milk fat, but digestibility of proteins remained unchanged. In addition, the different supplementation modified the metabolic utilization of nitrogen (N) while the N absorption did not vary. Moreover, a study in patients with a high jejunostomy revealed that the digestion of milk proteins seemed to be incomplete in the upper jejunum; thus, the lower part of jejunum and the ileum play a key role to accomplish complete digestion and absorption processes (Mahé et al., 1991a). In addition, in this study they detected 64% and 44% of β-Lg and α-La, respectively, in an intact antigenic form at jejunum. Troost et al. (2001) reported over 62% of intact LF after gastric digestion in humans, during which the iron saturation of LF did not

influence significantly the degradation of the protein. The hydrolysis of ^{15}N-labeled protein in milk and yogurt was evaluated in humans (Gaudichon et al. 1995). They observed that the fermentation process only modified the gastric emptying rate but not the level of hydrolysis of the protein, the endogenous N stimulation or the digestibility rate. Likewise, Mahé et al. (1991b; 1995) observed different behavior for β-Lg, caseins, skim milk and yogurt in the intestine of healthy humans, due to differences in their gastric emptying rates. On the other hand, Calbet et al. (2004) observed similar values of gastric emptying after the administration of casein, whey proteins and their corresponding hydrolysates to humans; as well as the amino acids absorption values, except in the case of casein, which showed slower rates. In addition, these authors observed that the hydrolysates compared to the whole protein solutions induced an increase of 50% of gastric secretion and of glucose-dependent insulinotropic polipeptide (GIP), a gut peptide that counterbalance the secretagogue effect of amino acids. The basal gastro-jejunal secretions have been demonstrated to be stable in composition, which are partly comprised of proteins with rapid rate of synthesis (Gaudichon et al., 1994a). Moreover, the influence of the protein amount on the digestion kinetics of ^{15}N labeled β-Lg and casein has been also evaluated in humans (Mahé et al., 1996). In this study, they reported differences in the amino acid delivery and in the nature of products present in lumen between both proteins. This fact could be attributted to the different gastric emptying, which occurs as peptides for casein, and for β-Lg mainly in intact form. These two proteins have been described as “slow” and “fast” proteins, respectively, in regard of the kinetics of hydrolysis during digestion and the amino acid absorption rate (Boirie et al., 1997). This may have been elicited by the coagulation of casein in the stomach leading to a longer retention, in contrast to β-Lg, which is soluble and can be rapidly emptied from stomach. Dangin et al. (2001) observed that “fast”-protein meal produced a strong, rapid and transient increase in aminoacidemia, L-1-^{13}leucine flux and oxidation *vs.* the “slow”-protein meal, which increased these parameters, but they were maintained longer over time. Furthermore,

kinetics of dietary amino acid delivery from different food proteins could ultimately influence the anabolic responses in the body since “slow” digested proteins can induce better postprandial use of dietary N (Lacroix et al., 2006a). Likewise, Deglaire et al. (2009a) evaluated the postprandial utilization of proteins after ingestion of two diets based on intact and hydrolyzed casein in humans. The hydrolyzed casein diet elicited faster absorption rate, which led to stronger and earlier hyperaminoacidemia and hyperinsulinemia, and resulted in less N peripheral availability for anabolic use after its ingestion compared to casein ingestion. Thus, the fast supply of amino acids favors the splanchnic use of N at the expense of their anabolic use in peripheral tissues such as muscle, as it was previously reported for the fast soluble milk or soy proteins compared to caseins in humans (Fouillet et al., 2006; 2002).

Some studies on the characterization of the resulting products from milk protein digestion in humans have been reported, as shown in table 1, where the peptides identified within in vivo digests can be observed. The management and analysis of samples from *in vivo* digestion is difficult. Nevertheless, several examples can be found on the identification of peptides released from in vivo digestion of milk proteins, although few studies have reported information about the whole profile of in vivo digests in humans. Among the human trials conducted on this topic, in 1985, Svedverg et al. identified immunoreactive material from β-casomorphins (β-CM)-4, 6 y 7, originated from β-casein fragment (f)(60-63), f(60-65) y f(60-66), respectively in small intestine of humans. All of these sequences had been previously reported as opioid peptides (Brantl et al., 1981). Later on, Chabance et al. (1998) conducted a human trial, at stomach and duodenal level, and in which several peptides derived from the casein fraction, could be recorded, after ingestion of milk or yogurt. After milk ingestion, a total of 26 peptides were identified from α_{s1}-, α_{s2}-, β and *κ*-casein in gastric contents, in contrast to eight peptides found after yogurt ingestion (from α_{s1}-, β and *κ*-casein). In both effluents the caseinomacropeptide (CMP) [f(106-169) from *κ*-casein) and its N-terminal part i.e. f(106-117), MAIPPKKNQDKT, were found, which results from the

cleavage of the peptide bond Phe^{105}-Met^{106}, commonly known to be cleaved by pepsin during gastric phase, and their resistance during intestinal phase. In duodenal content the peptides were shorter than in stomach, and the sequences belonged to α_{s1}-, β and *κ*-casein, and as well as to LF. Among these sequences, some active peptides from α_{s1}-casein could be identified, such as the antihypertensive peptide AYFYPEL, f(143-149), found in the stomach content. The peptide YFYPEL, f(144-149), known as antioxidant (Suetsuna et al., 2000) or stimulant of mucin secretion and expression (Martínez-Maqueda et al., 2013); and VAPFPEVF f(25-32) as angiotensin converting-enzyme (ACE)-inhibitor (Contreras et al., 2009), were found in duodenal fluids, and the latter one also in stomach content. Moreover, several fragments from the β-casein domain comprised between residues 29-48 were found in stomach content, which suggested the resistance of this region to gastric digestion. Later on, reports appeared on the identification of the CMP in digestive fluids isolated from jejunum of humans after consumption of casein, yogurt and whey proteins (Ledoux et al., 1999).

Table 1. Peptides sequences from β- and α_{s1}-casein identified in gastrointestinal effluents from adult humans

Sequence	Protein fragment	Location	Substrate	Reference
RELEELNVPGEI	β-CN 1-12	Stomach; duodenum; jejunum	Milk; CN[1]	Chabance et al., 1998; Boutrou et al., 2013
RELEELNVPGEIVE* (19 fragments included in this domain)	β-CN 1-14	Jejunum	CN	Boutrou et al., 2013
LNVPGEIVE	β-CN 6-17	Stomach	Milk	Chabance et al., 1998
NVPGEIVE	β-CN 6-14	Jejunum	CN	Boutrou et al., 2013
LNVPGEIVELS	β-CN 7-14	Jejunum	CN	Boutrou et al., 2013
NVPGEIVEL	β-CN 7-16	Duodenum	Milk	Chabance et al., 1998
NVPGEIVELSS	β-CN 7-18	Duodenum	Milk	Chabance et al., 1998
KIEKFQSEEQQQ	β-CN 29-40	Stomach	Milk	Chabance et al., 1998
KIEKFQSEEQQQT	β-CN 29-41	Stomach	Milk	Chabance et al., 1998
IEKFQSEEQQQT	β-CN 30-41	Stomach	Milk	Chabance et al., 1998
FQSEEQQQTED	β-CN 33-44	Stomach	Milk	Chabance et al., 1998
FQSEEQQQTEDELQDK	β-CN 33-48	Jejunum	CN	Boutrou et al., 2013
TEDELQDKIHPF* (6 fragments included in this domain)	β-CN 41-52	Jejunum	CN	Boutrou et al., 2013
DKIHPF	β-CN 47-52	Jejunum	CN	Boutrou et al., 2013
SLVYPFPGPIPNSL* (22 fragments included in this domain)	β-CN 57-70	Jejunum	CN	Boutrou et al., 2013
YPFP (β-CM 4)	β-CN 60-63	Duodenum	Milk	Svedverg et al., 1985;
YPFPGP (β-CM 6)	β-CN 60-65	Duodenum; jejunum	Milk; CN	Svedverg et al., 1985; Boutrou et al., 2013
YPFPGPI (β-CM 7)	β-CN 60-66	Duodenum; jejunum	Milk; CN	Svedverg et al., 1985; Boutrou et al., 2013
PGPIPN	β-CN 63-68	Jejunum	CN	Boutrou et al., 2013
YPFPGPI (β-CM 9)	β-CN 60-68	Jejunum	CN	Boutrou et al., 2013
PNSLPQNIPPLTQTPVVVPPFLQPEV* (12 fragments included in this domain)	β-CN 67-92	Jejunum	CN	Boutrou et al., 2013
SLPQNIPPLTQT	β-CN 69-80	Duodenum; jejunum	Milk; CN	Chabance et al., 1998; Boutrou et al., 2013
VPPFLQPEV	β-CN 84-92	Duodenum	Milk	Chabance et al., 1998
VPPFLQPEVM	β-CN 84-93	Duodenum	Milk	Chabance et al., 1998
GVSKVKEAMAPK	β-CN 94-105	Stomach	yogurt	Chabance et al., 1998
HKEMPFPKY* (5 fragments included in this domain)	β-CN 106-114	Jejunum	CN	Boutrou et al., 2013
EMPFPKY	β-CN 108-114	Jejunum	CN	Boutrou et al., 2013
HKEMPFPKYPVQPFT	β-CN 106-120	Stomach	Milk	Chabance et al., 1998
KEMPFPKY	β-CN 107-114	Stomach	Milk	Chabance et al., 1998
YPVQPF	β-CN 114-118	Duodenum	Milk	Chabance et al., 1998
YPVQPFTESQS*	β-CN 114-124	Jejunum	CN	Boutrou et al., 2013

(8 fragments included in this domain)				
TLTDVENLHLPLPLL*	β-CN 128-140	Jejunum	CN	Boutrou et al., 2013
(24 fragments included in this domain)				
LHLPLP	β-CN 133-138	Jejunum	CN	Boutrou et al., 2013
WMHQPHQPLPPTVMFPPQSVL*	β-CN 144-163	Jejunum	CN	Boutrou et al., 2013
(26 fragments included in this domain)				
HQPHQPLPPTVM	β-CN 145-156	Duodenum	Milk	Chabance et al., 1998
VMFPPQSVLSL	β-CN 155-165	Duodenum	Milk	Chabance et al., 1998
SLSQSKVLPVPVPE	β-CN 164-175	Stomach	Milk; yogurt	Chabance et al., 1998
SKVLPVPVQK*	β-CN 168-176	Jejunum	CN	Boutrou et al., 2013
(6 fragments included in this domain)				
KAVPYPQRMPIQ*	β-CN 176-187	Jejunum	CN	Boutrou et al., 2013
(4 fragments included in this domain)				
FLLYQEPVLGPVRGPFPIIV*	β-CN 190-209	Jejunum	CN	Boutrou et al., 2013
(29 fragments included in this domain)				
HPIKHQGLPQEV	α_{s1}-CN 4-15	Stomach; jejunum	Milk; CN	Chabance et al., 1998; Boutrou et al., 2013
HPIKHQGLPQEVLNENLL*	α_{s1}-CN 4-21	Jejunum	CN	Boutrou et al., 2013
(9 fragments included in this domain)				
EVLNE	α_{s1}-CN 14-18	Duodenum	Milk	Chabance et al., 1998
FFVAPFPEVFG*	α_{s1}-CN 23-33	Jejunum	CN	Boutrou et al., 2013
(15 fragments included in this domain)				
FVAPFPEVF	α_{s1}-CN 24-32	Jejunum	CN	Boutrou et al., 2013
VAPFPEVF	α_{s1}-CN 25-32	Stomach; duodenum; jejunum	Milk; CN	Chabance et al., 1998; Boutrou et al., 2013
FVAPFPEVFGKE	α_{s1}-CN 25-34	Stomach	yogurt	Chabance et al., 1998
FPEVFGKE	α_{s1}-CN 28-35	Duodenum	Milk	Chabance et al., 1998
EKVNELSKDIGSEST	α_{s1}-CN 35-49	Stomach	Milk	Chabance et al., 1998
DQAMEDI	α_{s1}-CN 51-57	Jejunum	CN	Boutrou et al., 2013
AMEDIKQMEAE	α_{s1}-CN 53-63	Jejunum	CN	Boutrou et al., 2013
EIVPNSVEQKHIQ	α_{s1}-CN 70-82	Stomach	yogurt	Chabance et al., 1998
HIQKEDVPSERY*	α_{s1}-CN 80-91	Jejunum	CN	Boutrou et al., 2013
(5 fragments included in this domain)				
YLGYLEQ	α_{s1}-CN 91-97	Jejunum	CN	Boutrou et al., 2013
YLGYLEQL	α_{s1}-CN 91-98	Jejunum	CN	Boutrou et al., 2013
YKVPQLEIVPNSAEERLH*	α_{s1}-CN 104-121	Jejunum	CN	Boutrou et al., 2013
(25 fragments included in this domain)				
VPQLEIVPN	α_{s1}-CN 106-114	Duodenum; jejunum	Milk; CN	Chabance et al., 1998; Boutrou et al., 2013
VPQLEIVPNSAEER	α_{s1}-CN 106-119	Jejunum	CN	Boutrou et al., 2013
LHSMKEGIHAQQ	α_{s1}-CN 120-131	Stomach	Milk	Chabance et al., 1998
EGIHAQQKEPMIGVNN*	α_{s1}-CN 125-140	jejunum	CN	Boutrou et al., 2013

(8 fragments included in this domain)				
EPMIGV	α_{s1}-CN 133-138	Duodenum	Milk	Chabance et al., 1998
AYFYPEL	α_{s1}-CN 143-149	Stomach	Milk	Chabance et al., 1998
AYFYPELFRQF	α_{s1}-CN 143-149	Stomach	yogurt	Chabance et al., 1998
YFYPEL	α_{s1}-CN 144-149	Duodenum	Milk	Chabance et al., 1998
QLDAYPS	α_{s1}-CN 155-161	Jejunum	CN	Boutrou et al., 2013
YYVPLGTQYTDAPSFSDIPNPI*	α_{s1}-CN 165-186	Jejunum	CN	Boutrou et al., 2013
(14 fragments included in this domain)				
SDIPNPIGSENS	α_{s1}-CN 180-191	Stomach	yogurt	Chabance et al., 1998
GSENSEKTTMPLW	α_{s1}-CN 187-199	Jejunum	CN	Boutrou et al., 2013
TDAPSFSDIPN	α_{s1}-CN 174-184	Duodenum	Milk	Chabance et al., 1998

[1]CN: casein; *Protein domain where several peptides have been identified in samples, not specifically indicated in order to simplify the tables, unless they had been previously described as active sequences, in which case they have been highlighted separately; **In bold**: active sequences (commented in the text).

The improvement of the analytical techniques, such as mass spectrometry (MS), combined with a suitable preparation of these samples has permitted to obtain greater amount of information with respect to the identification of peptides. In this regard, in 2013, the detection of 347 and 146 peptides from casein and whey protein, respectively, in jejunal fluids gathered from humans after ingestion of these proteins was possible by the use of a nanoLC-Q-TOF (Boutrou et al., 2013). Several identified sequences were known as bioactive peptides, for instance, the immunomodulator β-casein peptide f(63-68), PGPIPN (Migliore-Samour et al., 1989), the antihypertensive peptide LHLPLP, β-casein f(133-138) (Miguel et al., 2006); or the κ-casein sequence KNQDK, f(112-116), known as antithrombotic peptide (Bal Dit Sollier et al., 1996). In this study, various peptides were quantified in the effluents, and some β-CMs [f(57-, 58-, 59, and 60-66)] were present in sufficient amount to elicit their physiological effect. Overall, several cores from β- and α_{s1}-casein seem to be resistant to digestion in adult humans as it can be observed in Table 1, since numerous fragments were identified during gastrointestinal digestion of milk proteins i.e. β-casein 1-14, 57-70, 67-92, 128-140, 144-163, and the C-terminal part of the protein [region 190-209]. The resistant domains from α_{s1}-casein seemed to be 23-33, 104-121 and 165-186. Indeed, several active sequences above mentioned belong to some of these regions of the protein (57-70 and 128-140) i.e LHLPLP, PGPIPN or some β-CMs (Boutrou et al., 2013). Interestingly, β-CMs 6 and 7 are coincident peptides detected after milk (Svedverg et al., 1985) and casein (Boutrou et al., 2013) ingestion, regardless of the food matrix. Moreover, as it can be observed in Table 1, various peptides are present throughout the whole gastrointestinal track since they were generated in stomach and also identified in duodenum after milk ingestion (Chabance et al., 1998), being also found in jejunum after casein ingestion (Boutrou et al., 2013), for instance β-casein f(1-12) and α_{s1}-casein f(25-32).

Recently, a peptidomic analysis of human milk from 3 mothers and the stomach contents from their 4 to 12-day newborns after feeding was carried out using a nano-

LC-Q-TOF (Dallas et al., 2014.) This study revealed approximately 135 peptides exclusively detected in the “intact” milk, due to the over-activity of plasmin system proteolysis in mammary gland. This fact is believed to arise from an evolutionary selected natural mechanism capable to compensate the early immaturity of the human newborn gastro-intestinal system. On the other hand, 586 distinct peptides were identified in stomach contents from the infants. Overall, 64 peptides were common for both “intact” milk and gastric digest. Most of these peptides found either in intact milk or in gastric samples belonged to β-casein, which indicates a protein-selective degradation within milk or in stomach, since this protein does not represent even half of milk proteins. Several of these sequences were coincident or displayed structural similarities to some previously described bioactive peptides. For instance KVLPIPQ, from human β-casein, with Ile/Val replacement compared the known antihypertensive sequence KVLPVPQ (Maeno et al., 1996), and YPYY from human β-casein, which matches with the opioid antagonist (casoxin-B) from bovine *κ*-casein (Chiba et al., 1989). These results suggest that gastric digestion occurs within the first weeks of life in the infant, releasing peptides that can exert biological activities.

1.2.2. Milk protein digestion in animal models

Among the studies on animal models, Miranda & Pelissier (1983) studied the *in vivo* digestion of milk proteins in rats after ingestion of raw milk. The proteins α-La and β-Lg were not much susceptible of hydrolysis during gastric digestion, even after 240 min. Likely, whey proteins were also shown to be resistant to gastric digestion in calves (Yvon et al., 1985). Later on, the amount of N in the digests remaining in the stomach of neonatal pigs suggested that whey proteins were emptied from stomach faster than caseins (Newport et al., 1985). Later on, Moughan et al. (1991) studied the rate of gastric emptying and the postprandial change on pH values in calves fed infant formula containing either intact bovine proteins or a hydrolysate thereof. The type of diet did not

affect the pH after ingestion, however, the gastric emptying occurred faster in the case of the hydrolysate vs intact protein (12% and 22% of hydrolysate and intact protein, respectively, remaining in the stomach, after 3 hours). The gastric emptying was claimed to be a major factor controlling the absorption kinetics of milk ^{15}N-labeled protein during a study conducted in mini-pigs fed milk and yogurt (Gaudichon et al. 1994b). In this study, milk proteins were observed to be rapidly absorbed once they reached the intestine. Overall, the pig is a suitable model to predict differences among dietary protein digestibility in men, showing good inter-species correlation in terms of true N and amino acid digestibility (Deglaire et al., 2009b). Besides, it is monogastric (incontrast to ruminants), and omnivore like humans.

In regard to the characterisation of in vivo digests of milk proteins, some reports have been published on the peptides present in the gastrointestinal tract in animals (Table 2). In 1986, the β-casein f(60-70), known as pro-CM YPFPGPIPNSL, was found in intestinal content of mini-pigs after ingestion of casein, finally adopting the name of β-CM-11, due to its opioid activity (Meisel, 1986). This sequence was later on identified in distal jejunal effluents from mini-pig after ingestion of casein, in addition to the caseinphosphopeptide (CPP), f(66-74), from α_{S1}-casein with three phosphoserines (Meisel & Frister, 1988; 1989). Several peptides originated from casein were detected in the stomach contents of calves after ingestion of a diet based on milk or casein (Yvon & Pelissier, 1987). Among them, two active sequences from α_{S1}-casein can be pointed out: the antihypertensive peptide AYFYPEL, f(143-149) (Contreras et al., 2009); Isracidine, RPKHPIKHQGLPQEVLNENLLRF, f(1-23), known as antibacterial peptide (Lahov & Regelson, 1996); and the immunomodulatory peptide from β-casein, YQEPVLQPVRGPFPIIV, f(193-209) (Coste et al., 1992). In 1991, the presence of various phosphopeptides derived from casein [β-casein f(1-28) (4 maximum phosphorylation sites, 4P), β-casein f(33-48)1P, α_{s1}-casein f(43-58)2P, α_{s1}-casein f(59-79)5P and α_{s2}-casein f(46-70)4P] was reported in duodenum, jejunum and ileum of rats

fed whey proteins, casein and CPPs (Brommage et al., 1991). The CPP f(59-79) showed good calcium binding properties, favored by the presence of the phosphoseryl cluster (SpSpSpEE) in its sequence (Cross et al., 2005). However, it is known that the intestinal epithelium has a strong phosphatase activity, and CPPs are easily de-phosphorylated in neutral or slightly alkaline conditions (Bader et al., 1984; Moss et al., 1992). A year later, Scanff et al. (1992), could identify the CMP f(106-169) from κ-casein, in fluids gathered from stomach of calves. In addition, they also identified a precursor peptide of β-CMs [f(58-93)], and a CPP derived from α_{s1}-casein [f(110-142)1P].

Table 2. Peptide sequences from β- and α_{s1}-casein identified within in vivo digests from animals

Sequence	Protein fragment	Location; animal	Substrate	Reference
RELEELNVPGEIVESLSSSEES… …ITRINK	β-CN 1-28	Duodenum, jejunum and ileum (rats)	CN[1], CPPs	Brommage et al., 1991
NVPGEI	β-CN 7-12	Jejunum (piglets)	IF[2]	Bouzerzour et al., 2012
NVPGEIVE	β-CN 7-13	Jejunum (piglets)	IF	Bouzerzour et al., 2012
NVPGEIVESLSSSEESIT	β-CN 7-24	Ileum (rats)	CPPs	Hirayama et al., 1992
FQSEEQQQTEDELQDK	β-CN 33-48	Duodenum, jejunum and ileum (rats)	CN, CPPs	Brommage et al., 1991
LVYPFPGPIPNSLPQNIPPLTQTP… …VVVPPFLQPEVM	β-CN 58-93	Stomach (Calves)	Milk	Scanff et al., 1992
YPFPGPI (β-CM 7)	β-CN 60-66	Jejunum (piglets)	IF	Bouzerzour et al., 2012
YPFPGPIPNSL (β-CM 11)	β-CN 60-70	Duodenum;Jejunum (minipigs)	CN	Meisel et al., 1986;1989
PGPIPN	β-CN 63-68	Jejunum (piglets)	IF	Bouzerzour et al., 2012
NIPPLT	β-CN 73-78	Jejunum (piglets)	IF	Bouzerzour et al., 2012
IPPLTQTP	β-CN 74-81	Jejunum (piglets)	IF	Bouzerzour et al., 2012
IPPLTQTPVVVP	β-CN 74-85	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PLTQTPV	β-CN 76-82	Ileum (piglets)	IF	Bouzerzour et al., 2012
PLTQTPVV	β-CN 76-83	Ileum (piglets)	IF	Bouzerzour et al., 2012
TPVVVP	β-CN 80-85	Jejunum (piglets)	IF	Bouzerzour et al., 2012
TPVVVPP	β-CN 80-86	Jejunum (piglets)	IF	Bouzerzour et al., 2012
TPVVVPPFL	β-CN 80-88	Jejunum (piglets)	IF	Bouzerzour et al., 2012
TPVVVPPF	β-CN 80-87	Jejunum (piglets)	IF	Bouzerzour et al., 2012
TPVVVPPFLQ	β-CN 80-89	Jejunum (piglets)	IF	Bouzerzour et al., 2012
TPVVVPPFLQP	β-CN 80-90	Jejunum (piglets)	IF	Bouzerzour et al., 2012
TPVVVPPFLQPEV	β-CN 80-92	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PVVVP	β-CN 81-85	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PVVVPP	β-CN 81-86	Ileum (piglets)	IF	Bouzerzour et al., 2012
PVVVPPFLQ	β-CN 81-89	Jejunum (piglets)	IF	Bouzerzour et al., 2012
VVVP	β-CN 82-85	Jejunum (piglets)	IF	Bouzerzour et al., 2012
VVVPP	β-CN 82-86	Ileum (piglets)	IF	Bouzerzour et al., 2012
VVVPPFLQPEV	β-CN 82-92	Jejunum (piglets)	IF	Bouzerzour et al., 2012
VVPPF	β-CN 83-87	Jejunum (piglets)	IF	Bouzerzour et al., 2012
VVPPFL	β-CN 83-88	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PPFLQP	β-CN 85-90	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PFLQP	β-CN 86-90	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PFLQPE	β-CN 86-91	Jejunum (piglets)	IF	Bouzerzour et al., 2012
PFLQPEV	β-CN 86-92	Jejunum (piglets)	IF	Bouzerzour et al., 2012
FLQP	β-CN 87-90	Jejunum (piglets)	IF	Bouzerzour et al., 2012
QPEV	β-CN 89-92	Jejunum (piglets)	IF	Bouzerzour et al., 2012

QPEVMGVSSK	β-CN 89-98	Ileum (piglets)	IF	Bouzerzour et al., 2012
VMGVSSKV	β-CN 92-99	Ileum (piglets)	IF	Bouzerzour et al., 2012
KVKE	β-CN 97-100	Ileum (piglets)	IF	Bouzerzour et al., 2012
TLTDVENLHLPLPL	β-CN 128-139	Stomach (Calves)	CN	Yvon & Pelissier, 1987
QSWMHQPHQPLPP	β-CN 141-153	Jejunum (piglets)	IF	Bouzerzour et al., 2012
WMHQPHQPLPP	β-CN 143-153	Jejunum (piglets)	IF	Bouzerzour et al., 2012
QPHQPLP	β-CN 146-152	Jejunum (piglets)	IF	Bouzerzour et al., 2012
QPHQPLPP	β-CN 146-153	Jejunum (piglets)	IF	Bouzerzour et al., 2012
VLSLSQSK	β-CN 162-169	Ileum (piglets)	IF	Bouzerzour et al., 2012
SKVL	β-CN 168-171	Jejunum (piglets)	IF	Bouzerzour et al., 2012
QRMPIQA	β-CN 182-188	Jejunum (piglets)	IF	Bouzerzour et al., 2012
LLYQEPVLG	β-CN 191-199	Jejunum (piglets)	IF	Bouzerzour et al., 2012
LYQEPVLG	β-CN 192-199	Jejunum (piglets)	IF	Bouzerzour et al., 2012
YQEPVLGPVRGPFPIIV	β-CN 193-209	Stomach (Calves)	CN;milk	Yvon & Pelissier, 1987; Scanff et al., 1992
RPKHPIKHQGLPQEVLNENLLRF	α_{s1}-CN 1-23	Stomach (Calves)	CN	Yvon & Pelissier, 1987
KHQGLPQEVLNENLLRFF	α_{s1}-CN 7-24	Ileum (rat)	CPPs	Hirayama et al., 1992
LRF	α_{s1}-CN 21-23	Stomach (Calves)	CN	Yvon & Pelissier, 1987
DIGSESTEDQAMEDIK	α_{s1}-CN 43-58	Duodenum, jejunum and ileum (rats)	CN, CPPs	Brommage et al., 1991
TEDQAMEDIK	α_{s1}-CN 49-58	Jejunum (piglets)	IF	Bouzerzour et al., 2012
QAMEDIK	α_{s1}-CN 52-58	Jejunum (piglets)	IF	Bouzerzour et al., 2012
QMEAESISSSEEIVPNSVEQK	α_{s1}-CN 59-79	Duodenum, jejunum and ileum (rats)	CN, CPPs	Brommage et al., 1991
EAESISSSEEIVPN	α_{s1}-CN 61-74	Ileum (rat)	CPPs	Hirayama et al., 1992
ISSSEEI	α_{s1}-CN 65-71	Jejunum (piglets)	IF	Bouzerzour et al., 2012
SSSEEIVPN	α_{s1}-CN 66-74	Jejunum (minipigs)	CN	Meisel et al., 1989
SSEEIVPN	α_{s1}-CN 67-74	Jejunum (piglets)	IF	Bouzerzour et al., 2012
SEEIVPN	α_{s1}-CN 68-74	Jejunum (piglets)	IF	Bouzerzour et al., 2012
LGY	α_{s1}-CN 92-95	Stomach (Calves)	CN	Yvon & Pelissier, 1987
EIVPNSAEERLHSMKEGIHAQQK… …EPMIGVNQEL	α_{s1}-CN 110-142	Stomach (Calves)	Milk	Scanff et al., 1992
AYFYPEL	α_{s1}-CN 143-149	Stomach (Calves)	CN	Yvon & Pelissier, 1987
YQL	α_{s1}-CN 154-156	Stomach (Calves)	CN: milk	Yvon & Pelissier, 1987; Scanff et al., 1992
DAYPSGAW	α_{s1}-CN 157-164	Stomach (Calves)	CN; milk	Yvon & Pelissier, 1987; Scanff et al., 1992
YYVPL	α_{s1}-CN 165-169	Stomach (Calves)	CN	Yvon & Pelissier, 1987
YYVPLGTQYTDAPSF	α_{s1}-CN 165-179	Stomach (Calves)	CN	Yvon & Pelissier, 1987
YYVPLGTQYTDAPSFSDIPNPI… …GSENSEKTTMPLW	α_{s1}-CN 165-199	Stomach (Calves)	CN	Yvon & Pelissier, 1987
FSDIPNPIG	α_{s1}-CN 179-187	Jejunum (piglets)	IF	Bouzerzour et al., 2012
SDIPNPIGSENSEKTTMPLW	α_{s1}-CN 180-199	Stomach (Calves)	Milk	Scanff et al., 1992

[1] CN: casein; [2] IF: Infant Formula; **In bold**: active sequences (commented in the text)

The same year, Hirayama et al. (1992) found only two CPPs, f(61-74) from α_{S1}-casein and f(7-24) from β-casein, in the intestine of rats after ingestion of CPPs. Furthermore, CMP was found in the stomach and duodenum of rats fed a diet containing 48% of Phe-CMP-1P (Fosset et al., 2002), and in addition, the presence of some derived peptide fragments was reported. For instance, CMP-1P and Phe-CMP, both from variants A and B. Recently, in 2012, Bouzerzour et al. could keep track of numerous milk peptides that reached ileum and jejunum of piglets fed infant formula at different times postprandial using a nano- LC-Q-TOF. They reported the region comprised between residues 74 and 91 from β-casein, as one of the most resistant domains to digestion, as shown in table 2. Indeed, the peptide TPVVVPPFLQP, f(80-90), which had previously shown ACE inhibitor activity (Abubakar et al., 1998), was identified. As well, the opioid sequence β-CM 7 was detected in jejunum of piglets. Furthermore, the same technique was employed to identify approximately 3000-4000 sequences in duodenal effluents of mini-pigs fed raw (Figure 3 and 4) and heated milk, and their corresponding rennet gels (Barbé et al., 2014). In this study, the structure of dairy matrices had little influence on the cleavage sites of peptides, but mainly affected the number of sequences. Approximately 29 of them were previously reported bioactive peptides. However, a peptide mapping of the whole peptide profile was provided, giving information about the pattern and kinetic of peptide release *in vivo*. Some resistant cores of the β- and α_{s1}-casein can be observed in the Figures 3 and 4, respectively. For instance, β-casein domains 30-60 and the C-terminal part of the protein comprised between residues 170-209. In the case of α_{s1}-casein, the N-terminal region (residues 1-20), especially at very short times of digestion, the core composed of residues 110-140 and the C-terminal part of the protein, gave rise to numerous peptides.

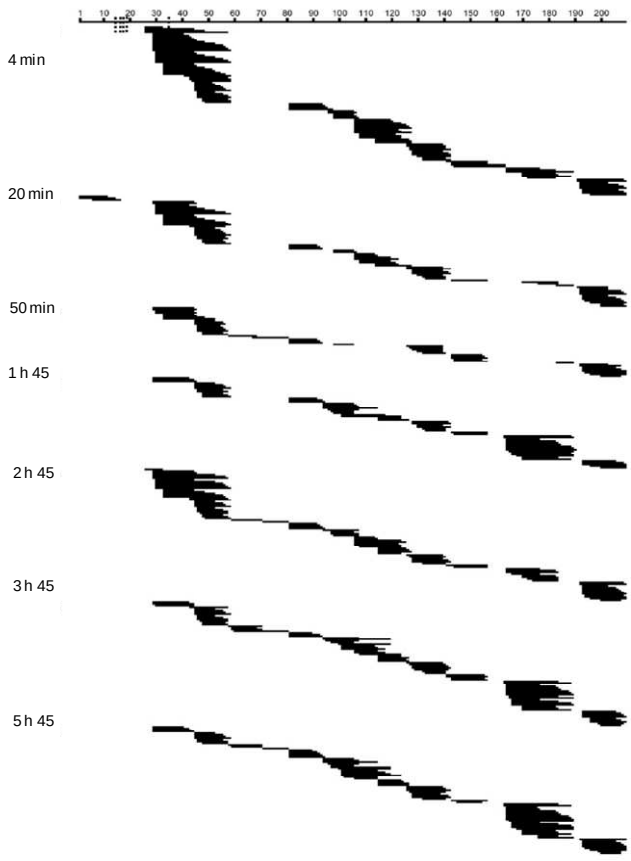


Figure 3. β-casein peptides identified in duodenal effluents of mini-pigs fed raw milk (Barbé et al., 2014)

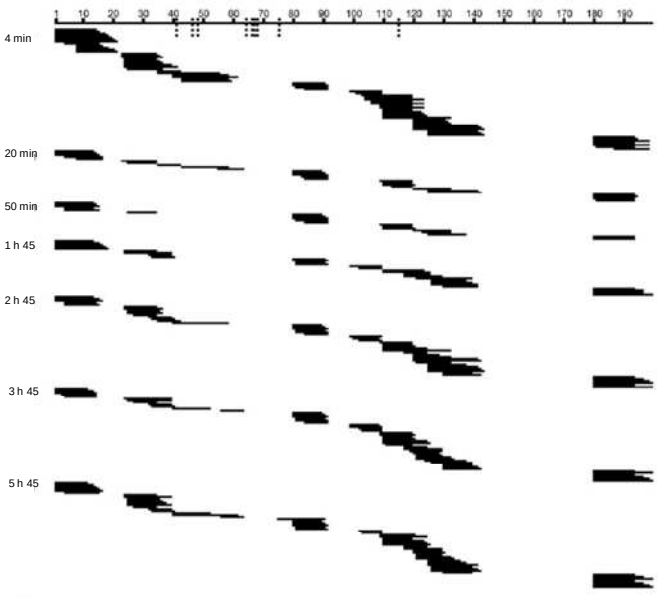


Figure 4. α_{s1}-casein peptides identified in duodenal effluents of mini-pigs fed raw milk (Barbé et al., 2014)

In summary, although during the last years the information available on the identification of peptides released through *in vivo* digestion of milk proteins in humans or in animal models has increased, little is known about the concentration available in small intestine. The information provided by *in vivo* digestion studies is valuable, on one hand, because the presence of certain peptides in small intestine indicates that they are actually generated through *in vivo* digestion, and they can reach small intestine, where they may be transported through the epithelium to plasma or interact with intestinal receptors in situ. However, the mere presence of the peptide would not always elicit their biological activity, but they need to be in sufficient amount. Therefore, in addition to the identification of the sequences it is important to evaluate if the peptides formed during digestion are released in physiological concentrations. This has only been reported in few cases. On the other hand, this information obtained from *in vivo* digestion trials could be used to validate and develop the *in vitro* digestion models. It has to be underlined that these studies concern bioaccesibility of peptides, although not their bioavailability and systemic distribution. In this regard, they could undergo further hydrolysis by the peptidases under the mucus of intestinal layer, or intracellular or plasma degradation.

1.2.3. In vitro digestion of milk proteins

The in vivo digestion trials performed in animals or in humans provide the most physiologically relevant results. Nevertheless, these studies are associated to significant ethical implications, and they are also expensive and time consuming. Thus, this had led to the development of different in vitro approaches, which has given as a result the development of different models of simulated gastrointestinal digestion. A great variety of these in vitro models have been employed in the last years in order to evaluate the structural and chemical changes that take place during food digestion (Hur et al., 2011; Wickham et al., 2009; Guerra et al., 2012); and whose differences mainly

lie on the number of steps of digestion, type of fluids (enzymes, salts, buffers) in each step, or the type of mixing. Likewise, other aspects have been proposed as important parameters to be considered when studying digestion: i) sequential secretion of enzymes at physiological concentrations, ii) suitable pH for the enzymes, and the use of cofactors in the process, such as bile salts, iii) removal of digestion products, iv) adequate mixing at each step and vi) physiological transit time for each step of digestion (Minekus et al., 1995). Two types of *in vitro* digestion models exist, the static and the dynamic models. The dynamic models can perform continuous changes in pH and sequential secretion of gastric or pancreatic juices containing the enzymes; they also consider the gastric emptying, or the removal of digestion products. Overall, the dynamic digestion models reproduce better the physiological conditions occurring *in vivo* (Guerra et al., 2012).

In vitro digestibility of milk proteins (bovine origin unless otherwise specified) has been widely assessed by static digestion models. The β-Lg was shown to be resistant to gastric digestion simulating physiological adult conditions (pH around 2), and susceptible to duodenal phase, although being less degraded in presence of phosphatidylcholine (Mandalari et al., 2009). This protein has been proved to be resistant to pepsin when subjected to adult and infant digestion models, in contrast to β-casein (Dupont et al., 2010a), as also observed in the previous work. In addition, in this study numerous peptides could be identified, giving information about the peptide profile generated throughout digestion using nano-LC-Electrospray ionization (ESI)-Q-TOF. Other whey proteins, such as α-La, have been shown to be partly resistant to gastric digestion (Picariello et al., 2010), although more susceptible than β-Lg. The reason why is the conformational transition of α-La. This protein at pH lower that 3 partially loses its terciary compact structure leading to a lax conformation, where some residues (hydrophobic) become exposed to the medium and more accessible to the proteases, although it keeps some traits of the molten globule (Yang et al., 2006). Nevertheless, milk proteins from different species such as equine, bovine, caprine and

human show differences in digestibility (Inglingstad et al., 2010). Overall, the proteins from equine milk were found to be rapidly digested compared to the others. Nonetheless, the pH of gastric digestion is an important factor which significantly influences the hydrolysis of milk proteins. LF and caseins from donkey's milk was markedly more susceptible to digestion with human gastrointestinal fluids at pH 2 compared to pH 4 since pepsin is more active; as it also occurred in the case of β-Lg, although in the latter case this effect was not as pronounced (Tidona et al., 2011). Nonetheless, β-Lg from donkey's milk was found quite resistant to digestion; it suffered more degradation compared to that from bovine or goat's milk (Almaas et al., 2006; Almaas et al., 2008). Interestingly, in the latter study, caprine α-La was shown to be very resistant to digestion with human juices, however, it was fully degraded by commercial enzymes.

Some works using MS-based techniques have focused on targeted analysis of certain sequences generated through digestion, and other studies have been untargeted to obtain information about the whole peptide profile. In this sense, the formation of active peptides was reported during digestion of human milk and infant formulas using two-step model by adding pepsin for the gastric phase and pancreatin for the duodenal step (Hernández-Ledesma et al., 2007). In this work, the peptides HLPLP and WSVPQPK that showed ACE-inhibitory activity and antioxidant activity, were identified in human milk digests by HPLC-ESI-IT. Likely, Beaufort cheese was digested using pepsin for gastric phase, also followed by incubation with pancreatin, although they employed nano-LC-MS/MS for the identification of the peptides, in this case targeted at CPPs (Adt et al., 2011). Most of the CPPs found in this cheese digest were monophosphorylated. Furthermore, other authors have studied the formation and resistance of CPPs through gastrointestinal digestion after the addition of pepsin and subsequent incubation with pancreatin-bile extract solution, analysed by HPLC-ESI-IT (García-Nebot et al., 2010; Miquel et al., 2005). Cheese ripening and proteolytic systems involved in the elaboration of dairy products are the processes that more likely

could lead to the formation of β-CM 7, from β-casein. However, the potential of gastrointestinal digestion (pepsin and Corolase PP®) to release this sequence in different dairy products, regardless the type of matrix has been pointed out i.e 10 cheeses, 4 liquid milks, 4 fermented milks, 7 infant formulas, 4 dried-milk products (De Noni & Cattaneo, 2010). Some studies have applied the MS-based techniques to reveal the whole peptide profile generated during in vitro digestion. For instance, Picariello et al. (2010) have reported the use of nano-LC-Q-TOF and MALDI-TOF to map the peptides generated through a multi-step in vitro digestion. In this study, the resistance of β-Lg f125-135 to digestion was underlined due to the importance for cow's milk allergy. On the other hand, Eriksen et al. (2010) detected more extensive hydrolysis of the peptide profile from β-Lg digested with porcine enzymes compared to human fluids by nano-LC-ESI-Q-TOF, despite no noticeable differences were found in SDS-PAGE analysis. Likely, Almaas et al. (2011) performed an in vitro digestion on caprine whey proteins with human gastrointestinal juices. The analysis by nano-LC-Q-TOF revealed that only two peptides matched with the profile previously described with non-human enzymes. These results suggest that the human enzymes are more complex and have different cleavage sites compared to non-human enzymes. Likewise, the same technique was used to analyze two types of Norwegian cheeses digested with human gastrointestinal juices following a three-step model, including oral, gastric and duodenal phase (Qureshi et al., 2013). This work reported the depletion of Pro after gastric digestion, while no changes were detected for some others such Phe, Lys, Tyr, Arg, Leu or Trp. Conversely, the duodenal phase provoked an increase of these residues. Furlund et al. (2013) identified several peptides released during LF digestion with gastrointestinal human juices and also after gastric digestion in human volunteers, where some of the sequences matched in vivo and in vitro, using nano-LC-Q-TOF. The study revealed that gastric pH and the activity of human gastrointestinal juices significantly influenced the formation of peptides. There is evidence about the importance of another step during digestion, which few authors actually take into

account; this is the use of brush border enzymes (Petrilli et al., 1984; Oshawa et al., 2008; Picariello et al., 2010). This step would finally simulate the fraction of peptides that could really be transported intact through intestinal epithelium. Given the variety of *in vitro* models and the fact that none has been commonly accepted, there is a need of correlating *in vivo-in vitro* data to validate and develop a model that simulate as closely as possible the physiological conditions. In this regard, an *in vitro* model was developed using a correlation with data obtained from *in vivo* digestion of proteins, lipids and carbohydrates present in literature (Kopf-Bolanz et al., 2012). In this study it is noteworthy to highlight the preparation of the digestive fluids at physiological final concentration of ions, the application of three steps, starting with oral phase, where lysozyme was included; moreover, mucins I and II were added to the digestive fluids. Recently, a standardized harmonized *in vitro* static model (Figure 5) has been developed in the framework of Infogest COST Action (Minekus et al., 2014).

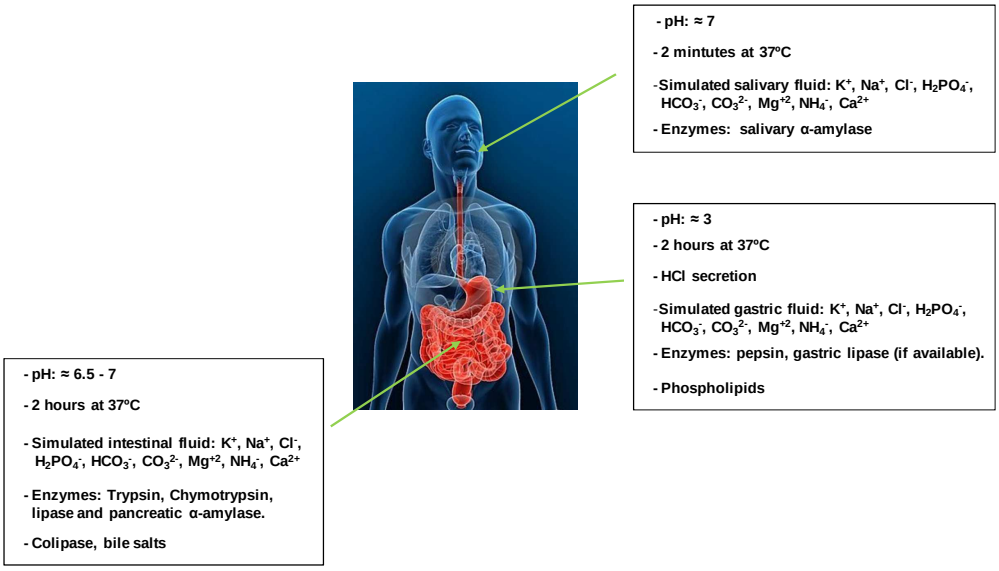


Figure 5. Digestion scheme. Oral, gastric and intestinal steps (Minekus et al., 2014).

Some of the dynamic systems used for milk protein digestion are bi- or multi-compartmental models. Among the dynamic digestion models, the mono-compartmental are normally focused on the gastric step (Kong et al., 2010; Mercuri et

al., 2011; Hoebler et al., 2002), although they consider the gastric emptying and the gradual decrease of pH. There is evidence of the existence of some devices developed to simulate digestion and the movement in small intestine, to face the transport phenomena occurring in the lumen and the influence of the concentration of species available for absorption (Tharakan et al., 2010). A semi-continuous digestion model was described by Vermeirssen et al. (2003a), where the pH was controlled by pH stat and a pump carried the content from stomach to intestinal vessel, and out of the latter at the end of digestion. The performance of this device was compared with that of batch digestion in order to achieve the best conditions for the generation of ACE-inhibitory peptides from whey and pea proteins. They count with systems to simulate the gastric and intestinal steps, usually small intestine. They can control the pH in stomach and small intestine, the gastric emptying, and usually also the intestinal transit. Figure 6 shows an example of a model with gastric and intestinal stirred compartments. Moreover, Minekus et al. (1995) developed a model (TIM-1) with 4 compartments representing stomach, duodenum, jejunum and ileum, and, in addition, possessed a system of passive transport provided with a dialysis membrane. The conditions for the performance of this device were based on those obtained in healthy humans in terms of meal transit time from stomach (milk and yogurt; Marteau et al., 1990; 1991), pH (yogurt; Marteau et al., 1991), bile-salt concentrations and glucose absorption. The results showed good mimicking of the in vivo data in regard of the gastric and ileal delivery of blue dextran, used as substrate in this study. Almost all the glucose (96%) that reached duodenum was absorbed through the dialysis membrane. In addition, the bile-salts were found to be within the correct range, and the pH values simulated correctly the pre-set curve based on in vivo data. This system reproduced very closely the in vivo conditions occurring in man based on the above mentioned parameters.

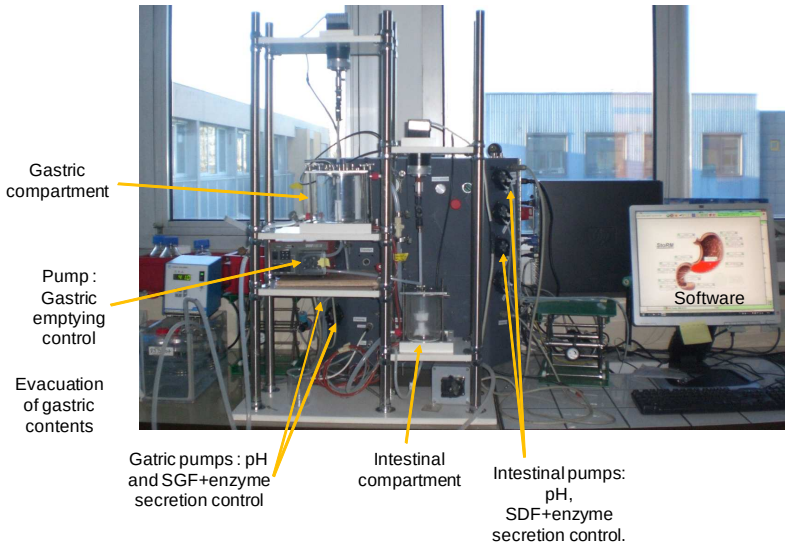


Figure 6. Bi-compartmental dynamic digester available at STLO-INRA (Rennes).

The correlation between in vitro and vivo data for milk proteins in dynamic digestion systems has been done in few studies. For instance, Yvon et al. (1992) performed a correlation of in vitro with in vivo data obtained from animal experiments to create an in vitro dynamic tool for gastric digestion of milk by controlling the emptying rate, the pH, the nature of enzymes, and the ratio of substrate/enzyme. In this study, the rate of pH decrease was 2-fold faster in vitro compared to in vivo experiments. In addition, the percentage of the intake (in terms of fresh matter) leaving the stomach recovered after 6 h during in vivo experiments was achieved in half of the time in the case of in vitro digestion. However, a wide variability of data was found in vivo for these parameters. In this comparison, the nitrogen emptying rate was similar between in vivo and in vitro, suggesting a close in vitro simulation of the clotting process in vivo. Similarly, Ménard et al. (2014) developed a gastrointestinal dynamic digestion model for infant formula through the comparison of in vivo and in vitro data. The hydrolysis kinetics of caseins and β-Lg resulted similar in both in vivo and in vitro experiments, although the percentage (%) of immunoreactive β-Lg present in the small intestine was higher in the in vitro model. Good correlation was found for food transit and for casein and β-Lg concentrations in stomach and intestine between in vivo and in vitro

experiments. Differences in the kinetics of proteolysis of both proteins were found. Casein was hydrolysed faster than β-Lg, which remained intact in stomach even after 120 min, as previously found in static models in vitro.

In summary, the dynamic models are important tools not only applicable from nutritional point of view (Martin et al., 2012; Yvon et al., 1992), but also from the toxicological and pharmacological perspective. In addition, they represent a model to assess the potential risk of allergens present in food stuff, since they allow a more realistic evaluation of the interactions thereof with the food matrix and the manner in which they are degraded (Wickham et al., 2009). Although some static models have been employed in pharmacological studies, as an initial approach on the development and testing of drugs, the advantages of the dynamic models have made of them the model of choice. The dynamic device TIM has been used in pharmacological trials in order to evaluate the effect of the dosage form, the transit time on the behavior of drugs (Blanquet-Diot et al., 2012; Blanquet et al., 2004), of the feeding state and the interaction with food (Brouwers y col., 2011; Souliman et al., 2006). Likewise, the bioaccesibility of some contaminants has been studied by the application of TIM-1, and compared to that obtained by using a static model (Torres-Escribano y col., 2011). Nevertheless, other dynamic models have been used to assess the effect of the dosage form of drugs (Mercuri y col., 2011). However, although these models have been used to evaluate the behavior certain food components through digestion process, few information is available focused on the resulting products, such peptides, released during dynamic digestion of milk proteins.

1.2.4 *Influence of food processing on gastrointestinal digestion of milk proteins*

Food processing could influence the behavior of milk proteins during digestion and subsequent formation of peptides. Heat treatment, homogenization, vacuum

concentration and drying are some of the most common treatments applied to dairy products in industry (Schuck et al., 2013). Other kinds of processes have been less extended, such as high pressure application (Pedras et al., 2014). Heat treatment affects the structural and nutritional characteristics of food proteins. Indeed, these modifications may result in a loss of amino acid bioavailability, such as Lys or Met, and of protein efficiency ratios; and even proteolysis can occur during heat treatment (Guo et al., 1999). Likewise, Maillard reaction induced by heat treatment in presence of carbohydrates leads to the blockage of Lys and the subsequent loss of its bioavailability, and could depend on the type and extent of treatment (Finot et al., 1981). In this regard, blocked Lys would not be recognized by digestive enzymes, for instance trypsin. Heat treatment or drying could also produce the formation of non-natural amino acids, i.e. isopeptide bonds in lysinoalanine or lantionine, which are no longer substrate of digestive proteases. In addition, casein and whey proteins aggregates induced by heat have been reported in some studies (Patel et al., 2006; Jean et al., 2006). The proteins usually involved in these aggregates are β-Lg (higher proportion) and α-La with κ- and α_{S2}-casein, depending on the size of aggregates, the amount of κ-casein available and the casein/whey protein ration in the product (Guyomarc'h et al., 2003). These modifications have been reported to change the behavior of milk proteins during digestion, *in vivo* and *in vitro*. Moreover, they affect the profile of bioactive peptides generated through digestion, since different protein bonds will be available for the action of the gastrointestinal enzymes (Meisel et al., 1998), or they could have toxicological implications from the point of view of food allergies (Mills et al., 2009). In regard to physiological parameters, heat treatment applied to milk resulted in an increase of the gastric emptying rate of caseins in rats (Miranda & Pelissier, 1987). Likewise, the effect of technological treatments on digestion of milk proteins was evaluated in the stomach of calves after ingestion of raw and pasteurized milks and yogurt (Scanff et al., 1990). In this study, the heat treatment on the liquid milks produced a slower coagulation; in yogurt, the kinetic of casein digestion was

slower compared to that observed for the liquid milks; and the β-Lg was not susceptible of gastric hydrolysis in any case. Recently, the heat treatment was reported to be important factor influencing the digestion kinetics of milk proteins and the subsequent amino acids absorption in mini-pigs (Barbé et al., 2013). Proteomic analyses of raw, pasteurized, sterilized and ultra-high temperature (UHT) milks subjected to in vivo (rat pup model) and in vitro digestion revealed higher lactulosyl-lysine content in milks with higher heat treatment (sterilized and UHT milks), which implied a decrease of α–La, β-Lg and casein digestibility (Wada et al., 2014). These authors suggest that the improvement of the digestibility of certain proteins produced by heat-induced denaturation would be offset by other modifications, such as non-enzymatic posttranslational modifications i.e Maillard reaction, that finally led to a decrease in digestibility. The nutritional consequences of the heat treatment have also been pointed out by other authors. For instance, the bioavailability of different raw products such as caseins, whey protein and microfiltered milks, was not significantly different in rats compared to different heat-treated milks, despite their differences in the distribution of N between them; and the different postprandial N metabolism found only for spray-dried milk (Lacroix et al., 2003; 2006b). The UHT treatment on milk influenced the digestive kinetics and the postprandial metabolism of milk proteins in humans, leading to a higher transfer of dietary N into serum amino acids and proteins, and to body urea after ingestion compared to other processes, such pasteurization or microfiltration, due to the heat-induced modifications of proteins (Lacroix et al., 2008; 2006c). The effect of heating on milk proteins digestion has also been described *in vitro*, for instance, an increased resistance of caseins with the heat treatment was observed in infant formulas subjected to different processes (Dupont et al., 2010b); or in different dairy matrices, such as raw, pasteurised and sterilised milks, and yogurt subjected to digestion using an infant model (Dupont et al., 2010c). Furthermore, digestion of caprine and bovine proteins was affected by the heat treatment, although the application of different heat-treatment processes did not cause any difference in the

degradation of caprine proteins, in contrast to bovine ones (Almaas et al., 2006). The authors explained this discrepancy due to the higher resistance of bovine vs caprine β-Lg to digestion, differences in composition and tertiary structure between the proteins from both species. The protein degradation of different technologically processed matrices i.e. raw, pasteurized, UHT, yogurt, cheeses, kefir etc was monitored before and after *in vit*ro gastrointestinal digestion (Kopf-Bolanz et al., 2014). In this study, β-Lg was differently degraded during gastric phase in fermented or unfermented matrices, an also depending on type of heat-treatment. This protein was fully degraded by pepsin in fermented products while in heated milks it could be still detected; and after the intestinal step was completely hydrolysed. The peptidomic analysis revealed the impact of the heat-treatment on β-Lg behavior, but it was not that evident on α_{s1}-casein; and showed that the higher the heat-treatment, the higher the number of peptides released from β-Lg. Although it is known, that β-Lg is resistant to digestion by pepsin at low pH there is also evidence that under certain conditions it can be susceptible to hydrolysis by this enzyme. Heating at temperatures higher than 90ºC, and especially at 100ºC induces conformational changes in this protein, which provokes the exposure of the hydrophobic regions, increasing its susceptibility to hydrolysis by pepsin (Guo et al., 1995; Peram et al., 2013). Likewise, other conditions have been described to increase the susceptibility of this protein to gastric digestion, for instance heating at low pH or under pressure, presence of alcohols and esterification (Bateman et al., 2010; Dalgalarrondo et al., 1995; Chobert et al., 1995; Zeece et al., 2008).

1.2.5 Influence of digestion on biological activity of peptides and proteins

Gastrointestinal digestion is a key step to evaluate the arrival of a peptide in its active form to duodenum or jejunum, where they could be transported through the intestinal epithelium, reach blood and be distributed to target organs; or interact in situ with gastrointestinal receptors. If some of these peptides are generated in sufficient

concentration, they can be physiologically and metabolically relevant as signals implicated in the control of the gastric and intestinal motility, pancreatic secretion, food intake, and in the ethiology of autoimmune diseases or food allergies. Likewise, the evaluation of the formation of epitopes involved in food allergies by means of digestion assessment has gained interest in the last years (Whickham et al., 2009; Benedé et al., 2014). Indeed, an in vitro digestion method was developed to evaluate the potential of a protein to become a food allergen by pepsin hydrolysis (Astwood et al., 1996). This method was incorporated to the protocol of assessment of possible allergen risk from novel food (FAO-WHO, 2001).

Digestion does not only affect the formation or release of peptides, when they were present in the food matrix, as it is the case of cheese fermented products, they can be further hydrolyzed during digestion (Korhonen, 2009; Sforza et al., 2012; Griffiths et al., 2013). In this regard, peptides can be either stable to digestion or be hydrolyzed, and the latter fact could imply the decrease or increase of bioactivity. For example, in vitro digestion produced the hydrolysis of the ACE-inhibitor sequence [(Lactokinin from β-Lg f(142-148)] to negligible concentrations, leading to the subsequent loss of its potential hypotensive effect in vivo (Walsh et al., 2004). Likely, the partial digestion and fragmentation of α-La led to a reduction in its activity, which had been demonstrated to inhibit proliferation of adenocarcinoma cell lines (Caco-2 and HT29) in its monomeric form (Brück et al., 2014). On the contrary, the release of the active form of certain sequences may occur during gastrointestinal digestion. Maeno et al. (1996), observed that the sequence KLPVPQ, f(169-175), from β-casein, possessed a low in vitro ACE-inhibitory activity, but produced a significant blood depressor effect after its oral administration to spontaneously hypertensive rats (SHR). This sequence was shown to lose the Gln at C-terminal end during simulated gastrointestinal digestion, giving rise to KLPVP, which had *in vivo* blood pressure lowering effect, as well as, *in vitro* ACE-inhibitory activity. On the other hand, in vitro digestion, in batch (non-optimal, physiological and prolonged-physiological conditions)

and semi-continuous, was studied regarding the ACE-inhibitory activity of peptides (Vermeirssen et al., 2003b). In this study, batch digestion, using physiological conditions, led to the highest ACE-inhibitory activity, with no correlation between the degree of proteolysis and such activity, since the highest proteolysis degree was achieved when longer incubation in physiological conditions was applied (prolonged-physiological conditions). This suggests that the generation of ACE-inhibitory peptides reached saturation point, at which the equilibrium between formation and degradation of active peptides occurred. Thus, physiological conditions were enough to obtain maximal ACE-inhibitory activity. In addition, when physiological conditions were reproduced in the semi-continuous system, no significant differences were observed in the degree of proteolysis and the values of ACE inhibition compared to batch physiological conditions. Therefore, the ACE-inhibitory activity of whey hydrolysates may be controlled by the conditions of in vitro gastrointestinal digestion, influencing the bioavailability of the peptides. Likewise, two Norwegian cheeses (Gamalost and Norvegia) were subjected to gastrointestinal digestion with human's fluids and Gamalost cheese increased its ACE-inhibitory activity after gastric digestion but somewhat it decreased after duodenal phase (Qureshi et al., 2013). Conversely, the ACE-inhibitory activity of Norvegia cheese followed an increasing trend through both steps, but lower values were observed compared to the other cheese. On the other hand, Oshawa et al. (2008) demonstrated the resistance of the antihypertensive β-casein peptides IPP and VPP to simulated gastrointestinal digestion with pepsin and pancreatin, or trypsin and chymotrypsin, followed by incubation with brush border enzymes expressed by Caco-2 cells. Likely, Quirós et al. (2009) demonstrated the resistance of the antihypertensive β-casein f(133-138), to *in vitro* gastrointestinal digestion. However, this sequence was further hydrolyzed by the brush border enzymes during incubation with Caco-2 cell culture, releasing the penta-peptide f(134-138), HLPLP (Quirós et al., 2008), the latter retaining antihypertensive activity (Miguel et al., 2010). The antihypertensive sequences RYLGY, AYFYPEL [α_{s1}-casein f(90-95)

and f(143-149), respectively)] and YQKFPQY [α_{s2}-casein f(89-95)] were subjected to in vitro gastrointestinal digestion and the first two sequences were partly hydrolyzed, although the latter one was totally degraded (Contreras et al., 2013). After digestion, the two α_{s1}-casein peptides maintained similar ACE-activity. In addition, some of the derived fragments maintained moderate ACE-inhibitory activity, which could explain the ACE-inhibitory activity of digests. Moreover, the peptide fragments derived from RYLGY showed antihypertensive activity suggesting that digestion could contribute to the effect of the precursor sequence through these fragments. Likely, the bovine LF digested with human gastric juice showed higher inhibition of *Echovirus* 5 replication than the native bovine protein, regardless the gastric digestion conditions, suggesting the release of peptides with antiviral activity (Furlund et al., 2012). However, they observed the strongest antiviral activity for the fraction of LF digested pH at 2.5 (doing fast decrease of pH instead of gradual), simulating adult gastric digestion and non-buffering conditions, which led to complete degradation of the protein. Moreover, Tidona et al. (2011) observed higher antimicrobial activity against *Escherichia coli* 10208355 and *Listeria monocytogenes* 2230/92 in digested donkey's milk compared to the undigested one. These results were due to the synergic effect of the generated peptides and intact proteins resistant to digestion process, as lysozyme.

The information obtained from these studies would not only be valuable to the application on the field of food, but also could be extended to the prevention and treatment of chronic diseases by nutraceuticals (Erdmann et al., 2008) or to the assessment of bioavailability of drugs (Brouwers y col., 2011).

1.3 Absorption, distribution, metabolism, and elimination of bioactive peptides

Once the resistance of certain peptides to digestion and their transport through the intestinal epithelium have been assessed, it is essential to evaluate the bioavailability properties of the peptides of interest. This term has been defined in a

previous section from nutritional point of view, as the fraction of the nutrient or bioactive available to be used in physiological functions or to be stored. Nevertheless, this term may adopt other meanings, for instance from pharmacological point of view. The Food and Drug Administration defines this term as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action" (Food and Drug Administration, 2013). This term includes the availability of the nutrient for absorption, distribution, metabolism and elimination. However, because of the difficulty, for practical and ethical reasons, to carry out these kind of trials, it is usually defined as the portion of the compound that reaches bloodstream (Fernández-García et al., 2009). Nonetheless, it has to be underlined that some active compounds can interact in situ with intestinal receptors present in the epithelium, being the absorption process a secondary factor for the active compound to exert the physiological effect.

Few studies have been performed on the absorption of bioactive peptides derived from milk proteins. Given the complexity of the biological samples, the technique of analysis needs to be sensitive in order to detect peptides in plasma. This is the case of the method developed by van Platerink et al. (2006), based on MS-based technique, and whose sensitivity and selectivity permitted the detection and quantification of small amounts of 17 peptides in plasma, achieving limits of detection of 0.01 ng mL^{-1} and of quantification from 0.05 to 0.2 ng mL^{-1} . By using this method, the tri-peptide IPP was detected in plasma from humans after ingestion of a milk beverage enriched in tri-peptides, containing IPP among others (Foltz et al., 2007). The ingestion of the beverage produced an increase in the plasma concentration of IPP, whose maximum concentration (C_{max}) was 1.6-fold that obtained after placebo ingestion. They observed that the time required to reach C_{max} (T_{max}) tended to be shorter after the ingestion of the enriched milk beverage compared to the placebo drink. The pharmacokinetic parameters of this peptide, IPP, were modified when the milk beverage was administered 30 min after a meal. In this case, greater values of

area under the curve (1.3 fold) and of half-time (1.5 fold) were observed compared to those obtained in fasted state; however, T_{max} and C_{max} were not affected by the feeding state of subjects. Later on, a pharmacokinetic study was carried out after intragastric and intravenous administration of the antihypertensive peptides IPP, LPP and VPP in pigs (van der Pijl et al., 2008). In this study they demonstrated the absorption of the three peptides after its intragastric administration to pigs, with different half-times of absorption between 2 and 4.6 min. No significant differences were found between the peptides, neither in the times of absorption, nor in T_{max} and half-time of elimination. However, significant differences were reported in C_{max} between IPP and LPP vs VPP; as well as in the half-time of elimination of the peptides after intravenous administration. In any case, the effective absorption of the peptides exceeded 0.1%, and considering that half-time of absorption and elimination did not exceed 5 and 15 min, respectively, an acute effect of the peptides under these conditions was concluded. Matsui et al. (2002a) studied the absorption and elimination kinetics of the peptide VY in normotensive humans after ingestion of a beverage containing the di-peptide. They found the peptide in plasma samples collected from subjects, reaching the maximum peak of absorption 2 h postprandial, and it was dose-dependent. The half-time of elimination was estimated at 3.1 h. This sequence is known to have blood depressor effect in humans with mild hypertension (Kawasaki et al., 2000). However, despite the detection of the intact peptide in plasma (Matsui et al., 2002a), the values of blood pressure of these normotensive male subjects did not change during the pharmacokinetic study. The same authors (Matsui et al., 2002b) found the di-peptide VY in plasma of subjects with mild hypertension after ingestion of the milk beverage containing the peptide. In this case, they obtained similar concentration values of the peptide in plasma as in the previous study. Therefore, it was demonstrated that the absorption process of this peptide was no affected by the physiological state of subjects; however, no changes in blood pressure were reported. These results point out the difficulty of establishing the physiological ranges for the administration of

peptides correlated to their biological effect *in vivo* and to their pharmacokinetic parameters. Nevertheless, although little is known about these aspects, these trials are essential to understand the behavior of peptides in the organism to establish a clear relationship between activity and bioavailable fraction.

Moreover, once the absorption of a peptide is demonstrated, it is important to evaluate its resistance to plasma peptidases with the goal of assessing its metabolism, since it can play a key role in the manifestation of the effect *in vivo* (Vermeirssen et al., 2004). In this regard, some authors have evaluated the impact of plasma peptidases on the stability of peptides during in vitro incubation of the peptides. For instance, the peptide IVY was incubated in rat and human plasma in vitro, releasing the active peptide fragment VY (Matsui et al., 2000). Likewise, Quirós et al. (2008) also performed an incubation in vitro of the β-casein f(134-138) in human plasma in order to assess its resistance to plasma peptidases, once they had demonstrated its transport through a Caco-2 cell monolayer. However, little information in this regard has been reported in vivo. For instance, studies focused on the metabolism and the distribution of active peptides have been carried out in different rat organs (lung, kidney, aorta, heart and brain). Indeed, the tri-peptides VPP and IPP were found in abdominal aorta of rats, after the ingestion of fermented milk that contained those sequences (Masuda et al., 1996).

Overall, few studies have been focused on the absorption, metabolism, distribution and elimination of food-derived peptides and little is known about pharmacokinetic properties of peptides derived from milk proteins. However, there is a need to deepen into this field, to clear up some of the questions that remain unknown and that are important factors when dealing with bioactive peptides. This information is valuable in order to achieve a broader knowledge on the behavior of peptides in the organism and with the final aim of establishing a direct relationship with health.

1.4 Mechanism of action of antihypertensive peptides

The antihypertensive effect of food-derived peptides has been widely studied (Martínez-Maqueda et al., 2012; Ricci-Cabello et al., 2012; Jäkala et al., 2010). Several milk-derived peptides have demonstrated antihypertensive activity in mild-hypertensive subjects, as reported in a meta-analysis carried out with 17 clinical trials (for review see Pripp et al., 2008). One of the systems involved in the control of blood pressure is the rennin-angiotensin system (Figure 7), where the ACE is included. This enzyme is responsible for the conversion of the deca-peptide angiotensin I in the octa-peptide angiotensin II, a vasoconstrictor. By a separate parallel pathway, ACE is also implicated in the inactivation of bradikinin, which possess vasodilator activity. Most of the peptides derived from milk proteins with a reported activity related to the reduction of blood pressure have been described as ACE inhibitors. However, other systems are involved in blood pressure regulation. The systems endothelin-converting enzyme and the kinin-nitric oxide are also involved in this function, by the release of vaso-regulatory peptides, independent from ACE, and of nitric oxide (NO), a vasodilator antiplatelet agent. Nevertheless, ACE is also related to the endothelial NO synthase. The latter enzyme is activated by angiotensin II, and the production of NO inhibits the action of ACE by negative feedback (Phelan & Kerins, 2011).

The relationship that in the last years has been outlined for the food-derived peptides and the cardiovascular health lies on the blood pressure lowering effect mediated by several pathways. Among the wide range of antihypertensive peptides, IPP and VPP are two of the most studied ones, in regard to the Renin Angiotensin-system. These peptides have been described as ACE-inhibitors *in vitro* (Nakamura et al., 1995a), and their antihypertensive activity has been proven in rats after single oral administration (Nakamura et al., 1995b) and long-term intake (Sipola et al., 2001; 2002a), as well as in humans (for review see meta-analysis Xu et al., 2008; Qin et al., 2013; Cicero et al., 2013).

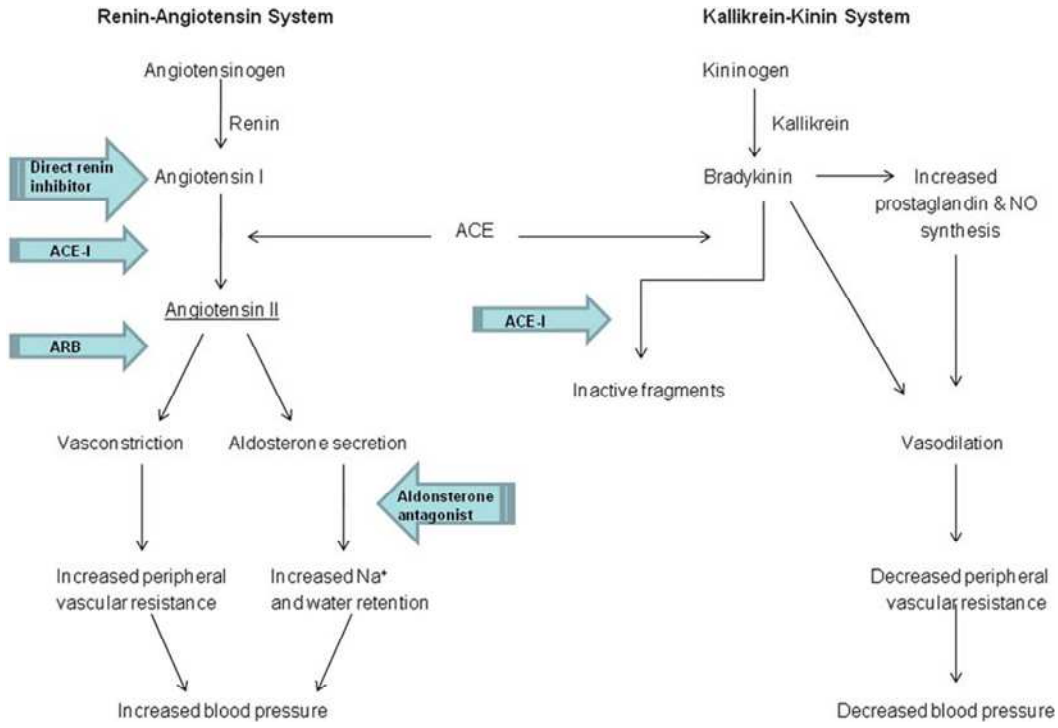


Figure 7. Renin-angiotensin and Kalikrein-kinin pathway (Phelan and Kerins, 2011).

For instance, a reduction in vascular reactivity to angiotensin I and II in mesenteric arteries from rats was observed after their incubation with IPP and VPP (Jakäla et al., 2009a). Moreover, a decrease in plasmatic ACE activity was observed after their administration to SHR, although no significant differences were reported (Jakäla et al., 2009b). Likewise, these tri-peptides were found intact in abdominal aorta of rats after the ingestion of fermented milk containing those sequences, and the values of ACE activity in this tissue decreased compared to the control (Masuda et al., 1996). Furthermore, there is evidence about the improvement of endothelial function induced by these food-derived tri-peptides (IPP and VPP), and also by whey peptide NOP-47, where NO was involved (Ballard et al., 2009; Hirota et al., 2011). Indeed, IPP and VPP produced a significant increase in the endothelial NO synthase gene after their administration to SHR during five days (Yamaguchi et al, 2009). Likely, a peptic casein hydrolysate, containing two antihypertensive peptides, RYLGY and AYFYPEL, that also showed ACE-inhibitory activity (Contreras et al., 2009), later on improved the

endothelial function in aortic and mesenteric rings, and increased the expression of endothelial NO synthase in aorta (Sánchez et al., 2011).

The interaction of peptides with opioid receptors has been suggested as an alternative mechanism of action on the regulation of blood pressure. The presence of opioid receptors, as well as endogenous opioid peptides has been demonstrated in different organs of peripheral cardiovascular system (Schultz et al., 2001). Some studies have reported the involvement of endogenous opioid peptides in the regulation of blood pressure (Siren et al., 1992; Czapla et al., 1998). The endogenous opioid peptides interact with opioid receptors (μ, δ y κ), whose stimulation may induce direct or indirect functional changes on heart or cardiomyocytes (Barron et al., 1999). The administration of the opioid agonist morphine, with high affinity for μ receptors, induced a depressor response mediated by interactions with opioid receptor (Randich et al., 1993). Nevertheless, it seems that morphine may also interact with δ and κ receptors (Ela et al., 1997). Evidence on this subject has led to consider that the interaction of these opioid receptors with the cardiovascular system is also related to the release of NO. In this sense, it has been demonstrated that morphine produced endothelial-dependent relaxation in aortic rings from rats, associated to the release of NO, and it was reverted by naloxone (Stefano et al., 1995). In this regard, little information is available on peptides derived from milk proteins whose antihypertensive activity has been related to the opioid system. For instance, the blood lowering effect of the milk-derived peptide α-Lactorphin [α-La f(50-53)], has been related to the opioid system, as agonist of μ opioid receptors (Nurminen et al., 2000). The evaluation of the participation of opioid receptors in the regulation of cardiovascular system is complex, due to the high number of them present either centrally or peripherally. In this respect, it has been demonstrated that α-Lactorphin, in contrast to morphine, does not exert its antihypertensive effect mediated by central opioid receptors (Ijäs et al., 2004). Moreover, this peptide was shown to improve endothelial function associated to the release of NO (Sipola et al., 2002b).

1.5 *Peptidomic-based techniques for quantification and monitoring of peptides in food matrices*

The application of MS to identify peptides, usually preceded of high resolution separation techniques, has become one of the indisputable tools when dealing with bioactive peptides, in terms of discovery, bioavailability and monitoring through different processes. Given its development during the last years, and its versatility, it is one of the election techniques, over other analytical choices.

In the field of the biologically active peptides, these techniques have had an enormous application to monitor the production of peptides by fermentation or by hydrolysis, to check their stability or to establish their bioavailability. Some peptidomic analyses are focused on targeted or untargeted products present in the food matrix due when manufactured i.e cheese ripening. For instance, a study aimed at seeking particular bioactive sequences in cheeses using an IT as mass analyzer (Bütikofer et al., 2007). Sometimes, the objective was to perform a mapping of peptides conferring bitter flavor to cheese (Toelstede et al., 2008). In addition, some products, such as human milk may undergo proteolysis in the mammary gland, and as a consequence, numerous peptides with antimicrobial properties can be formed, which could have further health implications for the neonate (Dallas et al., 2013). Two antihypertensive peptides from α_{s1}-casein, RYLGY, f(90-94) and AYFYPEL, f(143-149) were monitored for a scaled-up production of an antihypertensive casein hydrolysate by HPLC-ESI-IT (Contreras et al., 2010). In addition, Quirós et al. (2006) developed and validated a mass spectrometry method based on the same instrument to quantify the antihypertensive sequence LHLPLP, f(133-138) present in a complex matrix, such as fermented milk produced by *Enterococcus faecalis* that caused hypotensive effect after single oral (Muguerza et al., 2006) and long-term administration (Miguel et al., 2005). Likewise, fermentation by yeasts such as *Kluyveromyces marxianus* has been proposed as an alternative to enzymatic hydrolysis to produce antihypertensive

peptides from α-lactalbumin and β-lactoglobulin (Hamme et al., 2009). In this sense, various antihypertensive peptides generated by *Kluyveromyces marxianus* from LF substrate were identified using HPLC-ESI-IT (García-Tejedor et al., 2013). However, little information is available about the peptide profile of these hydrolysates sequenced by MS-based techniques.

MS techniques are useful tools to monitor the stability of the peptides through technological processes (heat treatment, packaging, storage, drying etc). In addition, food processing may induce changes in the profile of peptides present in food matrices. For instance, the release of peptides during cheese proteolysis (Piraino et al., 2007); or a detailed study on the evolution of peptide fraction along 24 months of aging (Sforza et al., 2012). On the other hand, food processing such heat-treatment can induce modifications of proteins, such as glycosylation that may have an influence on the bioavailability of certain amino acids involved in these changes. The pattern of protein glycosylation is susceptible of detection by MS-based techniques (Siciliano et al., 2013; Montgomery et al., 2013). Some technological processes could also modify the concentration of bioactive peptides in food. In this regard, the application of MS-based techniques to quantify and monitor food-derived bioactive peptides has been pointed out as a suitable tool (Contreras et al., 2008). The stability of two antihypertensive peptides (RYLGY and AYFYPEL), present in a peptic casein hydrolysate, to drying and storage was assessed by HPLC-ESI-IT (Contreras et al., 2011).

1.6 Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. Review article.

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Review

Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides

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ABSTRACT

In the last years, the identification and characterization of bioactive peptides have become emerging research subjects. Food peptidomics can be considered a subfield of the food proteomics focused on composition, interaction and properties of peptides present in a food matrix. On the basis of the description of recent works, the objective of this review is to highlight the increasing role of peptidomics as indispensable tool in the fields of discovery, bioavailability and monitoring of dairy bioactive peptides. The enhanced peptide identification, resulting from the valuable mass spectrometry development and the regular use of high-resolution techniques, supports the application of peptidomic approaches in the case of empirical bioactive peptide identification workflow. Bioinformatic-driven approaches have gradually gained importance through the wider application of in silico analysis, structure activity relationship models, chemometrics and peptide database management. Investigations of bioactive peptide modifications during digestion, whether it be selective or untargeted search using peptidomic tools have been discussed, as well as peptide changes along absorption, distribution, metabolism and elimination, including studies in cellular and animal models. Examples of application of peptidomics in the analysis of bioactive peptide occurrence in dairy products together with peptide monitoring during scaling up, industrial treatments and storage have been also described.

Contents

1. Introduction

Nutrition exerts an important life-long environmental impact on human health, and this interplay between nutrition and health has been known for centuries (Kussmann, Panchaud, & Affolter, 2010).

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The quality of a dietary protein source depends not only on the amino acid composition, their digestion, absorption, and availability for subsequent anabolism, but also on the peptides that are released (Awati et al., 2009). Many physiological functions in the organism are mediated by peptides, acting as neurotransmitters, hormones or antibiotics (Hruby & Balse, 2000). Because peptides from food sources can be structurally similar to these endogenous peptides, it is reasonable that they can interact with the same receptors and play a role as modifiers of food intake, growth factors, immune regulators, or antimicrobials in the host organism (Kamau et al., 2010; Meisel, 1998). Bioactive peptides can be released in vivo, during gastrointestinal digestion, by the action of host or microbial enzymes but they may also be originated in vitro, whether it be from ripening, fermentation (naturally occurring enzymatic reactions) or targeted food hydrolysis with selected enzymes. Besides, preparation of bioactive peptides can be performed using recombinant DNA technology or chemical synthesis (Hernández-Ledesma, Contreras, & Recio, 2011). Once they are released in the body, bioactive peptides may act as regulatory compounds with hormone-like activity, exhibiting a wide range of biological functions, including antihypertensive, antioxidant, opioid, antimicrobial, and immunostimulating activities (Hartmann & Meisel, 2007). Although other animal as well as plant proteins contain potential bioactive sequences, milk proteins are currently the main source of biologically active peptides. Thus, they account for most of the researches on bioactive peptides that apply peptidomics. This review will focus on studies dealing with peptides from milk and related dairy products.

The food peptidome can be defined as the whole peptide pool present in food products or raw materials, or obtained during processing and storage. Food peptidomics can be considered a subfield of the food proteomics focused on composition, interactions, and properties of peptides present in a food matrix (Gagnaire, Jardin, Jan, & Lortal, 2009). Some issues are common between the food proteomics and food peptidomics fields, like the occurrence of non-sequenced proteins, which makes mandatory to use de novo sequencing. This approach can be also helpful for identifying new single amino acid polymorphisms. Working with complex matrices is inherent to these fields as well. However, while in food proteomics a certain coverage is enough to find the selected protein, in the case of food peptidomics, peptides may be unique (variants or modifications) whereas many similar species can be found in the studied matrices. Furthermore, food peptides are released by the action of various unspecific and specific proteases, as opposed to the tryptic peptides generated in the proteomic experiments (Panchaud, Affolter, & Kussmann, 2012). An additional difficulty arises from the need to follow up these specific molecules not only in the food matrix but also upon ingestion and absorption.

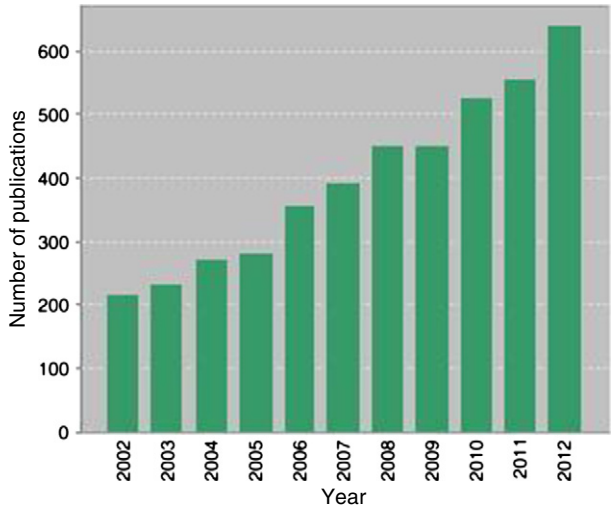

Fig. 1. Number of publications including the topic "bioactive peptide" in the 2002–2012 period. Web of Science®.

In biomarker proteomics, identification and quantification rely on several peptides that can unambiguously be inferred back to one parent protein sequence accounting for the key biological activity. By contrast, bioactive peptides are the active molecules per se and their identification relies on the detection of this particular sequence in its full length (Panchaud et al., 2012). Furthermore, their quantification is needed when the effective dose has to be calculated. Peptidomics can also have a role in biomarker search. For instance, a peptidomic approach has permitted to identify peptide panels which discriminate between two bacterial causes of infection in bovine mastitis, as it has been recently reported (Mansor et al., 2013). In human milk, the identification of the complete set of peptides naturally occurring suggests that protein cleavages in the mammary gland are not random events and represents the first step in understanding where and when milk peptides exert specific functions (Dallas et al., 2013).

In the past ten years, the identification and characterization of bioactive peptides have become emerging research subjects as shown by the increasing number of publications with the term "bioactive peptides" as topic (Fig. 1). As a result of its analytical versatility and power for structure elucidation and, to a lesser extent, quantification, mass spectrometry (MS) has developed into the major contributor to proteome and peptidome-wide assessment in food (Kussmann et al., 2010). This is a review in the fields of discovery, bioavailability, and monitoring of dairy bioactive peptides emphasizing those contributions where peptidomics has constituted an indispensable tool to achieve the objective.

2. Peptide discovery

Nowadays, the research focused toward the discovery of new dairy bioactive peptides continues mostly based on empirical strategies, including advanced analytical techniques as MS in different configurations. Despite the current prevalence of empirical approaches, in the last years emerging bioinformatic tools have achieved an increasing importance in the discovery of dairy bioactive peptides, through the prediction of their biological activity and the optimization of the empirical procedure.

2.1. Empirical approaches

The use of high-resolution separation techniques along with the enhanced peptide identification, resulting from the combination of MS methods and databases, support the present peptidomics status regarding the empirical discovery of dairy bioactive peptides. Usually, the empirical approach involves a series of steps: 1) release of the bioactive sequences; 2) initial screening for a targeted bioactivity; 3) purification and separation; 4) further determination of biological activity; 5) peptide identification by MS; 6) in vitro and in vivo validation of biological activity. A representative example of this workflow is found in the discovery of casein (CN)-derived peptides with antihypertensive properties (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Initially, peptic hydrolysis of CN was carried out, its angiotensin-converting enzyme (ACE)-inhibitory activity was monitored at different intervals of time and the antihypertensive activity was also verified in an animal model. Once the activity was determined, the hydrolysate was firstly subjected to ultrafiltration and later fractionated by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC). Those chromatographic fractions with relevant ACE-inhibitory activity were analyzed by RP-HPLC coupled to tandem MS (MS/MS) and the peptides comprised in the active fractions were identified. Finally, the ACE-inhibitory activity was verified for selected peptides, besides the verification of their in vivo antihypertensive activity.

In addition to in vitro enzymatic hydrolysis, the release of bioactive peptides from the parent dairy proteins may occur during gastrointestinal

digestion, through proteolysis processes involved during microbial fermentation (mainly by lactic acid bacteria because their advanced proteolytic system), and food manufacturing (ripening and cooking) (Rutherfurd-Markwick, 2012). The variety of enzymes becomes wider in recent years due to the increasing use of non-digestive enzymes like microbial- and vegetable-derived enzymes (Choi, Sabikhi, Hassan, & Anand, 2012).

The complexity of the dairy hydrolysates, fermented products, or digests and the analytical limitations of the equipment make necessary the implementation of pretreatments to remove interfering components and concentrate peptides with the aim of obtaining a successful separation and identification (Poliwoda & Wieczorek, 2009). The peptide extraction using homogenization in water or organic solvents is mandatory for the study of bioactive peptides from cheeses, followed by a deproteinization step through selective precipitation, filtration, and/or centrifugation, as occurs in the search of ACE-inhibitory peptides in different Cheddar cheeses (Ong & Shah, 2008) or fermented milk (Pihlanto, Virtanen, & Korhonen, 2010). Acid precipitation can be also used like a preliminary sub-fractionation, as performed by Ferranti et al. (2004) during the study of the bioactive peptide occurrence in human milk. Regarding the sample fractionation step, ultrafiltration with different molecular cut-off membranes, the use of solid phase extraction cartridges, low pressure liquid chromatography, and RP-HPLC at semi-preparative scale represent the main techniques employed (Martínez-Maqueda, Hernández-Ledesma, Amigo, Miralles, & Gómez-Ruiz, 2013). The combination of techniques based on different selectivities is recommended in support of optimizing the bioactive peptide identification. For instance, in the determination of ACE-inhibitory peptides derived from yak milk CN, the serial ultrafiltrations (10 & 6 kDa) were further fractionated by low pressure size exclusion chromatography (SEC) and finally by RP-HPLC (Jiang, Chen, Ren, Luo, & Zeng, 2007). Similarly, in the fractionation of antibacterial peptides from ovine α_{s2}-CN, the use of low pressure ion exchange chromatography (IEX) was followed by semi-preparative RP-HPLC (López-Expósito, Gómez-Ruiz, Amigo, & Recio, 2006).

The large number of peptides contained in studied samples usually leads their prior separation before identification by MS. HPLC in their different modes have become the preferred technique to the separation of peptides because its versatility, efficiency, and automation capabilities (Hernández-Ledesma, Martínez-Maqueda, Miralles, Amigo, & Gómez-Ruiz, 2013). RP-HPLC constitutes the most employed method to analyze milk-derived bioactive peptides, highlighted by its ability to effectively separate small peptides (Recio & López-Fandiño, 2010), that is a common characteristic of most bioactive peptides (Roberts, Burney, Black, & Zaloga, 1999). Nevertheless, other HPLC modes have been also used in bioactive peptide separation such as IEX, SEC, hydrophilic interaction chromatography (HILIC), hydrophobic interaction chromatography, and affinity chromatography (Hernández-Ledesma et al., 2013). A representative example is found in the promising application of use metal oxide affinity chromatography in the field of "phosphopeptidomics" (Leitner, 2010). Sometimes, it is mandatory the implementation of more than one HPLC step, that is orthogonal combination of separation techniques, occurring that the last dimension is usually RP-HPLC (D'siva & Mine, 2010; Sandra et al., 2009). On the other hand, capillary electrophoresis (CE) represents another separation option supported by its versatility and low consumption of sample, reagent, and time (Herrero, Ibañez, & Cifuentes, 2008; Kašička, 2012). Català-Clariana, Benavente, Giménez, Barbosa, and Sanz-Nebot (2010) identified several bioactive peptides in infant milk formulas using CE-MS. Miniaturized techniques like capillary electromigration chromatography and nano liquid chromatography (nanoLC) represent a promising separation strategy that may be increasingly applied to empirical discovery of bioactive peptides given their optimized design (Issaq, Chan, Blonder, Ye, & Veenstra, 2009). Direct nanoLC-MS was carried out by Zhu and FitzGerald (2010) to identify caseinophosphopeptides (CPPs) generated via tryptic hydrolysis of CN.

MS has been notably developed over the last decade, becoming the undisputed tool in bioactive peptide identification, reducing the use of other identification techniques like amino acid analyzers or protein sequencers (Picariello, Mamone, Addeo, & Ferranti, 2012). Occasionally MS is complemented with some traditional identification methods as shown by Pihlanto et al. (2010) who carried out the identification of ACE-inhibitory peptides in fermented milk by using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and Edman degradation sequencing. Benefits associated with MS equipment involve sensitive and accurate mass information, likely structure elucidation and short time analysis (D'siva & Mine, 2010). Nowadays, the quantity of available MS configurations is higher, featuring enhanced different capabilities (Hernández-Ledesma et al., 2013) and depending mainly on the combination of the ionization source and mass analyzer (Recio & López-Fandiño, 2010). Fast atom bombardment practically represents an extinct ionization methodology over the last decade, despite its application was crucial for the discovery of several milk-derived bioactive peptides such as CPPs, ACE-inhibitory, opioid, and antioxidant peptides (Contreras, López-Expósito, Hernández-Ledesma, Ramos, & Recio, 2008). The emergence of the electrospray (ESI) displaced previous ionization methods and presently continues being the predominant ionization choice, as shown in Table 1. The ease of on-line coupling with HPLC entails a crucial benefit that has been extensively exploited like in the discovery of novel antihypertensive peptides from a peptic hydrolysis of lactoferrin (Ruiz-Giménez et al., 2012) and the identification of ACE-inhibitory peptides in Mexican Fresco cheese (Torres-Llanez, González-Córdova, Hernandez-Mendoza, Garcia, & Vallejo-Cordoba, 2011). Both studies used ESI as ionization source and ion trap mass analyzer (IT). The progression of ESI technology is today a fact with the increasing implementation of miniaturized instrumentation such as nano-ESI which allows working with small samples and buffer volumes, e.g., the discovery of dipeptidyl peptidase IV (DPP IV)-inhibitory peptides from Gouda cheese using nanoESI-IT (Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). ESI also permits the appropriate on-line coupling with CE as proved in the characterization of bioactive peptides in infant milk formulas by ESI-IT and ESI-TOF, where the effectiveness of both mass analyzers was additionally evaluated (Català-Clariana et al., 2010). The combination of more than one mass analyzer seeks the enhancement of the identification through their particular advantages and several existing MS configurations are a sign of this reasoning like the combination of quadrupole (Q) and TOF mass analyzers used in the discovery of a novel peptide with mucin stimulatory activity (Plaisancié et al., 2013) or during the development of an at-line method for the identification of ACE-inhibitory peptides (van Platerink, Janssen, & Haverkamp, 2007). MALDI constitutes another broadly employed ionization source that exhibits significant differences compared to ESI, i.e., higher sensitivity, single charged ions, and lower susceptibility to salt and impurities although does not allow on-line chromatographic coupling (Mamone, Picariello, Caira, Addeo, & Ferranti, 2009). MALDI is normally presented with TOF (MALDI-TOF) and is especially focused to the identification of longer peptides (Saavedra, Hebert, Minahk, & Ferranti, 2013). The use of MALDI-TOF in the identification of milk-derived bioactive peptides is clearly demonstrated through the examples included in Table 1, like the identification of ACE-inhibitory peptides in Idiazabal cheese (Sagardia, Iloro, Elortza & Bald, 2013) or antimicrobial peptides in a peptic hydrolysate of lactoferrin (Chan & Li-Chan, 2007). Regarding small peptide identification, MALDI matrix might interfere the analysis due to the appearance of matrix signals at low masses. Nevertheless a suitable method named NALDI (nanostructure laser desorption/ionization) without matrix has been recently introduced and could represent a potential option for low mass peptide analysis (Saavedra et al., 2013). Analysis of peptides in milk has been already carried out by NALDI (Kütt, Malbe, & Stagsted, 2011) although future development in bioactive peptide field is expected.

Once performed the identification of the peptides potentially responsible for the observed activity, the verification of the in vitro or

Table 1
Examples of mass spectrometry application for dairy bioactive peptide discovery.

Origin[a]	Activity	Ion source	Mass analyzer	Reference
Fermented milk	ACE-inhibitory Antihypertensive	ESI	IT	Quirós et al. (2007)
Fermented caprine whey	ACE-inhibitory	ESI	IT	Didelot et al. (2006)
Commercial caprine kefir	ACE-inhibitory Antimicrobial Antioxidant	ESI	IT	Quirós et al. (2005)
Different Spanish cheeses	ACE-inhibitory	ESI	IT	Gómez-Ruiz, Taborda, Amigo, Recio, and Ramos (2006)
Mexican Fresco cheese	ACE-inhibitory	ESI	IT	Torres-Llanez et al. (2011)
Different Italian cheeses	Antimicrobial	ESI	IT	Losito et al. (2006), Rizzello et al. (2005)
Fermentation of sodium caseinate	ACE-inhibitory	ESI	IT	Hayes et al. (2007), Robert, Razaname, Mutter, and Juillerat (2004)
Hydrolysis of milk proteins with thermolysin	ACE-inhibitory	ESI	IT	Otte, Shalaby, Zakora, Pripp, & El-Shabrawy (2007)
Ovine κ-CN and whole CN hydrolysed with digestive enzymes	ACE-inhibitory	ESI	IT	Gómez-Ruiz, Ramos, & Recio (2007)
In vitro gastrointestinal digestion of human milk	ACE-inhibitory Antioxidant	ESI	IT	Hernández-Ledesma et al. (2007)
WPC hydrolysate from *Cynara cardunculus*	ACE-inhibitory	ESI	IT	Tavares et al. (2011)
Chymosin and peptic hydrolysis of CN	Antibacterial	ESI	IT	McCann et al. (2005, 2006)
Peptic hydrolysis of ovine α_{S2}-CN and bovine κ-CN	Antibacterial	ESI	IT	López-Expósito, Gómez-Ruiz, et al. (2006), López-Expósito, Minervini, Amigo, and Recio (2006)
Peptic hydrolysis of lactoferrin	Antihypertensive	ESI	IT	Ruiz-Giménez et al. (2012)
Peptic hydrolysis of CN	Antihypertensive	ESI	IT	Contreras et al. (2009)
Hydrolysis of α-LA and β-LG with Corolase PP™	Antioxidant	ESI	IT	Hernández-Ledesma, Dávalos, Bartolomé, and Amigo (2005)
Tryptic hydrolysis of WPC	DPP-IV inhibitory	ESI	IT	Silveira et al. (2013)
Tryptic hydrolysis of WPC	Mucin secretory	ESI	IT	Martínez-Maqueda, Miralles, Ramos, and Recio (2013)
Infant milk formula	Several activities	ESI	IT & TOF	Català-Clariana et al. (2010)
Tryptic hydrolysis of ovine β-LG and milk yogurts	ACE-inhibitory	ESI	QTOF	Chobert et al. (2005)
Commercial hydrolysed caseinate	ACE-inhibitory	ESI	QTOF	van Platerink et al. (2007)
Yogurt	Mucin secretory	ESI	QTOF	Plaisancié et al. (2013)
Commercial antihypertensive yogurt	Several activities	ESI	TOF	Kunda et al. (2012)
Gouda cheese	DPP-IV inhibitory	nanoESI	IT	Uenishi et al. (2012)
Mozzarella cheese whey	Antioxidant Cytomodulatory	ESI & MALDI	(ESI)-IT & (MALDI)-TOF	De Simone et al. (2009)
CN hydrolysated with a proteinase from *Lactobacillus helveticus* PR4	ACE-inhibitory Antibacterial	MALDI	TOF	Minervini et al. (2003)
Fermented milk	ACE-inhibitory Antihypertensive	MALDI	TOF	Pihlanto et al. (2010)
Sodium caseinate fermented with *Lactobacillus acidophilus* DPC 6026	Antimicrobial	MALDI	TOF	Hayes, Ross, Fitzgerald, Hill, and Stanton (2006)
Peptic hydrolysis of lactoferrin	Antimicrobial	MALDI	TOF	Chan and Li-Chan (2007)
Idiazabal cheese	ACE-inhibitory	MALDI	TOF	Sagardia, Iloro, et al. (2013)
Tryptic hydrolysis of milk proteins	Phosphopeptides	MALDI	TOF	Lo, Chen, Chen, and Chen (2007), Wang, Chen, Wu, Guo, and Xia (2007)

α-LA, α -lactalbumin; CN, casein; β-LG: β -lactoglobulin; WPC: whey protein concentrate; ACE: angiotensin-converting enzyme; DPP-IV: dipeptidyl peptidase IV; ESI: electrospray; MALDI: Matrix-assisted laser desorption/ionization; IT: ion trap; TOF: time-of-flight; QTOF, quadrupole-time-of-flight.
[a] Unless indicated, milk proteins from bovine origin.

in vivo biological activity for identical synthetic peptides is required to establish their bioactivity. This step is generally performed in peptide discovery studies. For instance, in a recent report, the DPP-IV inhibitory activity of two novel β-lactoglobulin (β-LG)-derived peptides was verified by determining the activity of the synthetic sequences (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). Same strategy was followed to identify two novel antioxidant peptides after screening the activity of eight synthetic peptides found in the simulated gastrointestinal digestion of human milk (Tsopmo et al., 2011). Furthermore, peptidomics can be helpful in the study of related issues as the bioavailability or the bioactive peptide monitoring, according to the following sections.

2.2. Bioinformatic-driven approaches

The recent development and combination of computational tools and bioactive peptide databases have led to a growing importance of bioinformatic-driven approaches or in silico analysis in the bioactive peptide discovery. Unlike empirical approaches that typically involve a significant expense, bioinformatics provide a cost-effective strategy through the reduction of different steps of the traditional workflow (Saavedra et al., 2013). Major contribution of in silico analysis in bioactive peptide field is related to the prediction of biological activity and the evaluation of food proteins as potential precursors, as observed in the in silico analysis of 34 proteins as potential sources of DPP-IV inhibitory peptides (Lacroix & Li-Chan, 2012). Furthermore, bioinformatic-driven studies are often supported by the possibility of predicting the proteolysis of dietary proteins due to the knowledge of the specific cleavage site of certain enzymes (Panchaud et al., 2012). The prediction of peptide activity is closely related to the study of interactions between target receptor and the peptide structure. Fig. 2 exhibits how two conformers of a β-CN peptide interact in different ways with the ACE active site, which results in different inhibitory behavior (Gómez-Ruiz, Recio, & Belloque, 2004). In recent years, the quantitative structure activity relationship model (QSAR) has reached a notable occurrence in the bioactive peptide discovery as reviewed by Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez, Gutiérrez-López, and Dávila-Ortiz (2012). QSAR analysis is based on elucidating structure–activity relationships by physicochemical descriptors to predict biological activities of other peptide sequences. QSAR approaches have been successfully used in

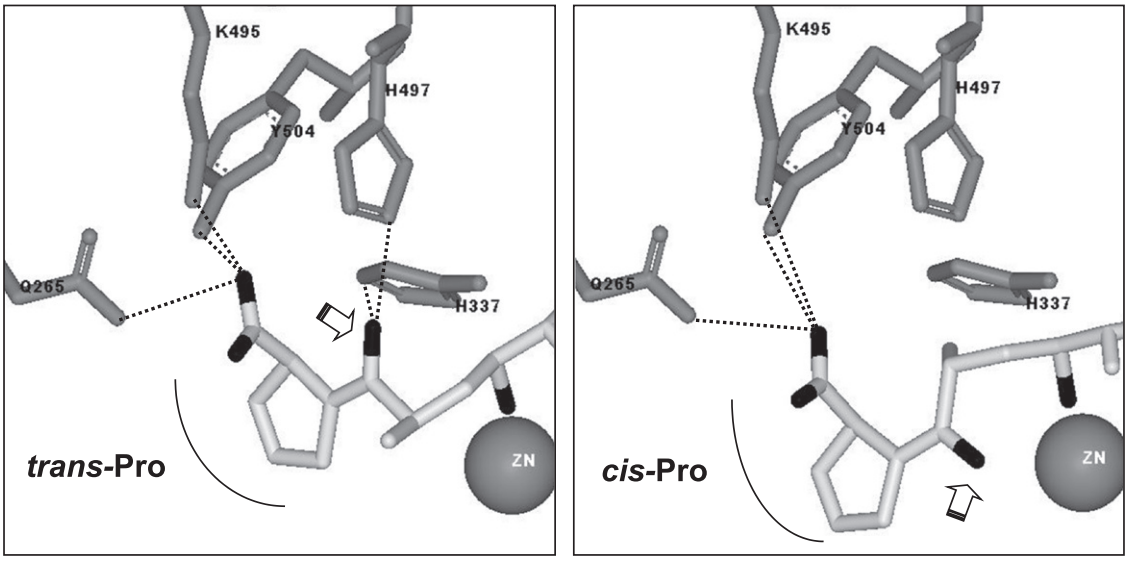

Fig. 2. Models showing the interactions between a peptide inhibitor, with trans- and cis-Pro at the C-terminal end (light gray), and the active site of the angiotensin-I-converting enzyme (dark gray). Model modified from the crystal coordinates of Drosophila angiotensin-I-converting enzyme bound to lisinopril (Protein Data Bank, PDB code 1J36) (11), modeled with DS Viewer Pro 5.0 (Accelrys). H-bonds are labeled with dashed lines. Carboxyl groups (CO and COO –) are labeled in black. The change of trans-Pro to cis-Pro results in the displacement of the CO group of the previous residue of the inhibitor (labeled with an arrow) to the opposite side of the peptide chain, losing its original H-bonding with the enzyme.
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studies of ACE-inhibitory activity, e.g., applied to oligopeptides and tripeptides (Sagardia, Roa-Ureta, & Bald, 2013; Toropova et al., 2012), or for the evaluation of different food proteins as ACE-inhibitory peptide precursors (Gu, Majumder, & Wu, 2011). The antioxidant activity has also been evaluated via QSAR analysis, as Li and Li (2013) developed via 17 physicochemical descriptors or Li, Li, He, and Qian (2011) performed focusing on the second C-amino terminal position, as well as in the prediction of antimicrobial peptides (Shu et al., 2013). QSAR approaches broadly exploit the peptide and protein databases, which may include extensive information like bioactivity, physicochemical parameters, references, and more. Among bioactive peptide databases, BIOPEP emerges as one of the most valued with more than 2500 entries classified according to specific biological activities and the opportunity of in silico proteolysis of more than 700 proteins (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). Other databases are Pepbank, BioPD, and SwePep as reviewed by Carrasco-Castilla et al. (2012).

The remarkable improvement of computational tools and analytical equipments, including MS, has supported the growing applicability of chemometrical analysis to different topics related to food bioactive discovery (Minkiewicz, Dziuba, Darewicz, et al., 2008). The response surface methodology (RSM) has demonstrated to be very useful in the optimization of proteolysis variables such as time, temperature, pH, enzyme or bacterial strain as well as substrate ratio when searching for a specific activity (Carrasco-Castilla et al., 2012). For instance, the production of antioxidant hydrolysates from a whey protein concentrate with thermolysin (Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011) and the fermentation of sour milk by *Lactobacillus helveticus* to get ACE-inhibitory activity (Pan & Guo, 2010) were assisted by RSM prior to the bioactive peptide identification. Chemometrics, as the art of extracting chemically relevant information from processes and transforming it into valuable data, can also be helpful in the bioactive peptide identification by predicting retention (HPLC) or migration (CE) times on the basis of the knowledge of peptide physicochemical properties and the use of complex algorithms. Recent examples of the use of semi-empirical models of elution time prediction to improve the peptide identification reliability are found in the study of hypoallergenic infant milk formulas by CE–ESI-TOF (Català-Clariana, Benavente, Giménez, Barbosa, & Sanz-Nebot, 2013), as well as, a functional yogurt by microLC–ESI-TOF (Kunda et al., 2012).

3. Bioavailability

When studying the effect that an active compound could have in our organism, it is important to assure that the active form reaches the target organ. For this purpose, stability to digestion has to be assessed and, if it is absorbed, it is also important to evaluate its distribution, metabolism, and excretion, in order to establish the bioavailability properties of a selected peptide (Foltz, van der Pijl, & Duchateau, 2010).

3.1. Modifications during gastrointestinal digestion

The ingested food composed of complex molecules is digested through physical and chemical processes followed by absorption of the refined macro- and micronutrients, which mainly takes place in the duodenum and upper jejunum (Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011). Digestion process determines the formation of peptides derived from food proteins, which can be physiologically and metabolically relevant for the digestion process itself, but also for novel food formulation, concerning not only nutritional but also technological and toxicological aspects, such as, identification of potential epitopes involved in allergies (Wickham, Faulks, & Mills, 2009).

The MS application, normally preceded of high-resolution separation techniques, is becoming a useful tool for the study of digestion processes, since it allows to obtain not only descriptive-type results but also the identification of resulting products. Several authors have recently employed this technique to characterize the products formed during digestion of different milk proteins, as bovine (Dupont, Mandalari, Molle, Jardin, Léonil, et al., 2010; Picariello et al., 2010), ovine (Gómez-Ruiz, Ramos, & Recio, 2004a), caprine (Almaas et al., 2011), donkey (Bermeosolo-Bidasolo, Ramos, & Gomez-Ruiz, 2011), and human (Hernández-Ledesma, Quiros, Amigo, & Recio, 2007). The identification by MS can be targeted at some products of interest through digestion (potential epitopes, bioactive monitoring, stability of certain regions); or exhaustive untargeted analysis, intended to elucidate the kinetics of

protein digestion to finally develop and validate digestion protocols and models. Some authors have used this targeted identification to assess the stability of several peptides during digestion, for instance to monitor their ACE-inhibitory activity in cheese (Gómez-Ruiz et al., 2004a), or to follow the generation of antioxidant and ACE-inhibitory peptides in human milk and infant formulas digested in vitro with porcine pepsin and pancreatin (Hernández-Ledesma et al., 2007). De Noni and Cattaneo (2010) studied the occurrence and stability of β-casomorphins (β-CMs) 5 and 7 in dairy products during in vitro gastrointestinal digestion using porcine pepsin followed by the addition of Corolase PP™. Likewise, the survival of certain antihypertensive regions of β-CN (e.g. f(133–138) mature protein sequence always considered), which turned out to be resistant to in vitro digestion, was evaluated by Quirós, Contreras, Ramos, Amigo, and Recio (2009) by using a two-stage hydrolysis with porcine pepsin and Corolase PP™. However, peptides can be hydrolyzed during gastrointestinal digestion and either maintain their activity, decrease it, or increase it. As an example of the latter case, the antihypertensive sequence KLPVPQ, which had a low in vitro activity, showed higher activity by losing its C-terminal Gln residue after in vitro digestion with pancreatin (Maeno, Yamamoto, & Takano, 1996).

Other authors have used MS-based techniques to identify phosphopeptide formation during gastrointestinal digestion. For instance, the resistance of CPPs to in vitro gastrointestinal digestion was monitored when they were incorporated into milk-based fruit beverage or into a fruit beverage using two enzymatic solutions for digestion: porcine pepsin and pancreatin-bile solution (García-Nebot, Alegría, Barberá, Contreras, & Recio, 2010). Likewise, Miquel et al. (2005) carried out the identification of CPPs generated in different infant formulas after in vitro gastrointestinal digestion using porcine pepsin from gastric mucosa, followed by the addition of pancreatin from porcine pancreas and bile extract. Also, Adt et al. (2011) studied the content of CPPs in Beaufort cheese using selective precipitation before and after subjecting cheese to digestion with pepsin acid solution followed by incubation with pancreatin. They highlighted the increase of CPPs after digestion with gastrointestinal enzymes, being most of them, monophosphorylated.

The untargeted analysis to characterize gastrointestinal digests by using MS-based techniques has also been reported by several authors. The peptide profile of two Norwegian cheeses before and after in vitro gastrointestinal digestion with human enzymes was compared by Qureshi, Vegarud, Abrahamsen, and Skeie (2013) using nanoLC–ESI-QTOF. In this study, digestion was performed using a three-stage model that included the incubation of the samples in a Stomacher to mimic the chewing step, followed by the addition of human gastric and duodenal juices. It was reported the resistance of some peptides, although others seemed to be totally degraded and disappeared after digestion. Also, it was pointed out that generation of free amino acids was affected during this process. Gastric digestion caused a significant decrease on Pro content. Whereas the aromatic amino acids such as Tyr, Phe, and Trp; the positively charged ones (Arg and Lys); and Leu did not undergo any changes. On the contrary, duodenal digestion produced a significant increase on these amino acids. Recently, Sánchez-Rivera et al. (in press) studied the peptidome of a proteolyzed cheese (Valdeón) and skim milk powder after a two stage in vitro static digestion by adding porcine pepsin, followed by trypsin, chymotrypsin, lipases and bile salts, using RP-HPLC–ESI-IT. The great homology of the digests found in this study suggests that the gastrointestinal digestion could bring closer the profile of resulting products whose matrices differed in their proteolytic state. However, regarding the occurrence of bioactive sequences, there are exceptions attributed to peptide precursor differences that are in accordance with the different biological activity observed. Eriksen et al. (2010) used a nanoLC–ESI-QTOF to compare the peptide formation from caprine whey proteins after two-step static in vitro digestion with human gastric and duodenal juices and porcine enzymes (pepsin and Corolase PP™). Although there was not a great difference at protein digestion level revealed through SDS-PAGE assays, it was reported in this study that, the peptide profiles of β-LG revealed differences and showed a more extensive digestion by porcine enzymes than by human ones under the same conditions. Likely, the comparison of protein digestion patterns for two in vitro static digestions models, infant and adult, have been assessed using nanoLC–ESI-QTOF by Dupont, Mandalari, Molle, Jardin, Léonil, et al. (2010). Likewise, the peptide survival during an in vitro multi-step static digestion model of milk proteins was studied by Picariello et al. (2010), by using MALDI-TOF, nanoESI-QTOF, and nanoLC–ESI-QTOF. CPP-enriched fractions obtained with a TiO_2 column were analyzed in this study in order to increase protein coverage. Furthermore, the authors highlighted the relevance of certain regions of β-LG, resistant to proteolysis and their implications for cow's milk allergy. There are some valuable in vivo studies conducted to understand the breakdown of proteins, and its kinetics during digestion. Bouzerzour et al. (2012) conducted a study on digestion of infant formula in piglets, in order to evaluate protein digestion kinetics and peptide release. The β-CN region between residues 74 and 91 was reported to be resistant to proteolysis during these experiments, which was in agreement with in vitro studies of infant digestion carried out with different dairy matrices such as raw, pasteurized, sterilized milks and yogurt (Dupont, Mandalari, Molle, Jardin, Role-Répécaud, et al., 2010). Also, some authors have characterized human digests after ingestion of milk or yogurt (Chabance et al., 1998). Sequences f(25–32) and f(143–149) from α_{s1}-CN were identified by Edman degradation using an automated gas-phase sequencer. Likewise, more recently, Boutrou et al. (2013) conducted an in vivo study in humans to assess the release of peptides after ingestion of CN or whey protein. A total of 356 peptides from β-CN and 165 from whey proteins were identified in jejunum. The amount of several peptides found postprandial, (i.e. different β-CN including β-CN f(60–66) and f(108–113)) was claimed to be enough to allow these peptides to exert their biological activity, known to have opioid and antihypertensive activity, respectively. In order to evaluate the effect that a dietary peptide could exert in the organism, the digestion process is to be as close as possible to in vivo physiological parameters. In this sense, Kopf-Bolanz et al. (2012) validated an in vitro digestion model of pasteurized milk using RP-HPLC–ESI-IT by the comparison of results to human physiological data from studies related to the intestinal peptide transport, digestion of emulsified triglycerides and starch digestibility. In this study, the results were found to be consistent since the developed method gave macronutrients degradation values similar to those found in human. Altogether, there are some common traits resulting from these in vitro static digestion studies. β-LG and α-lactalbumin are more resistant to digestion than other milk proteins (Dupont, Mandalari, Molle, Jardin, Léonil, et al., 2010; Picariello et al., 2010). There are some regions that also appear to be resistant like those containing phosphorylated fragments as proved by García-Nebot et al. (2010), previously reported in other studies (Hirayama, Toyota, Hidaka, & Naito, 1992). Likewise, hydrophobic and Pro-rich regions resist digestion, in agreement with previous observations (Hausch, Shan, Santiago, Gray, & Khosla, 2002), while neutral and basic amino acids are rapidly hydrolyzed. Some of these Pro-rich domains are well conserved between species, and interestingly, some sequences reported as bioactive are included in these regions.

3.2. Modifications during absorption

In the intestinal epithelium, the absorptive enterocytes and the goblet cells are the two main cellular types. The apical side of the enterocytes is characterized by a brush border which contains several enzymes and which increases the surface for nutrient absorption. The goblet cells secrete mucus, which covers the apical membrane of intestinal cells and partially limits molecule absorption (Meaney & O'Driscoll, 1999). Any food peptide in the intestinal lumen must transverse this environment prior to absorption. Peptidomics has emerged as an

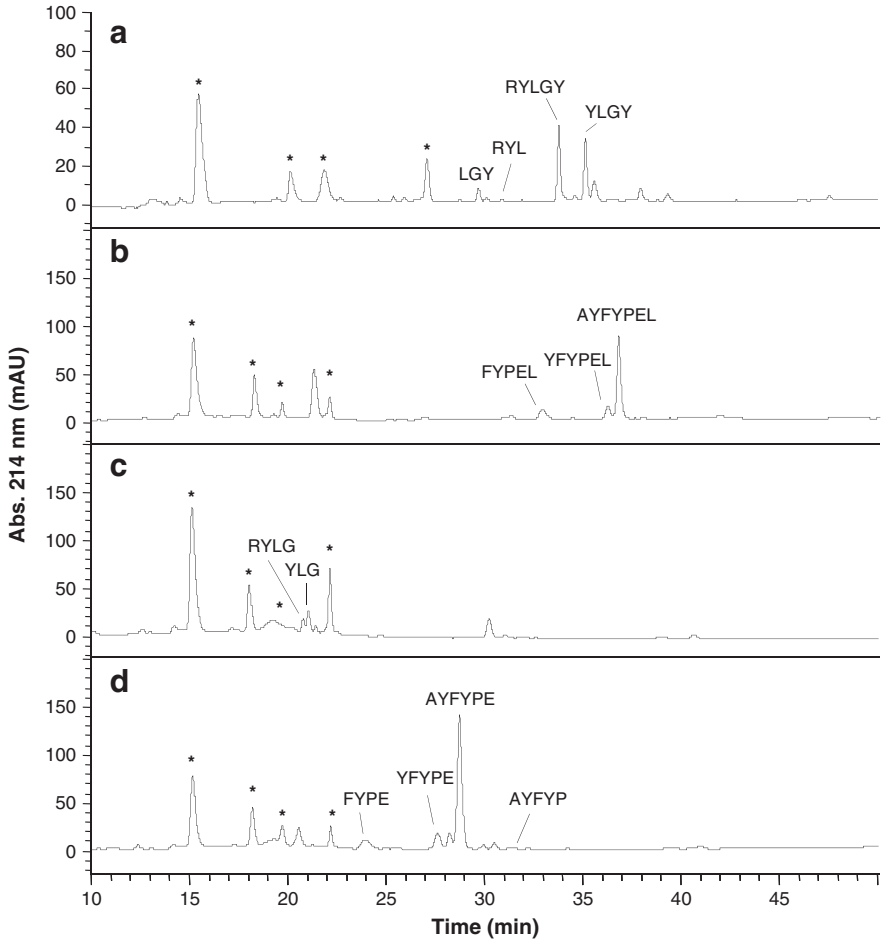

Fig. 3. Transepithelial absorption across the Caco-2 monolayer of intact peptides and derived fragments released by intestinal peptidases: a) RYLGY, b) AYFYPEL, c) RYLG, and d) AYFYPE. Asterisk indicates peaks found in basolateral chamber of cell cultures models without adding peptides.
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indispensable tool in the monitoring of eventual transformations of the food peptides in this environment.

Models physiologically close to human intestinal absorption are, in ascendant order, cell line models, excised tissues mounted on Ussing chamber (animal, ex vivo), and intestinal perfused loops (animal, in situ).

3.2.1. Peptidomics in cell line models

Cell line models offer a suitable alternative for in vivo animal testing. Their main advantage is their simplicity, interlaboratory repeatability, and large-scale testing capacity. Due to their reliability they have become an indispensable tool for the elucidation of intestinal absorption mechanisms. Most of the current intestinal cell line models are using transformed cell lines, among them the Caco-2 model being the most widely used (Langerholc et al., 2011).

For stability experiments, the digestion can be continued by a brush border phase, in order to mimic the first contact with the intestinal wall. For oligopeptides, it is known that susceptibility to brush border peptidases controls the apical-to-basolateral transepithelial transport rate (Shimizu, Tsunogai, & Arai, 1997). In the case of absorption of the antihypertensive β-CN peptide LHLPLP, using Caco-2 cell layers, it was shown that the hexapeptide was partly hydrolyzed by brush border peptidases that cleaved the peptide bond between Leu and His, releasing the pentapeptide HLPLP, which undergoes a rapid transport to the basolateral chamber. The unequivocal determination of this short form by RP-HPLC–ESI-IT confirmed that the peptide must first be hydrolyzed by peptidases before absorption occurs. As no other sequences were detected, it was suggested that HLPLP is probably the minimum active form of the peptide (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008). The presence of Pro within the sequence can have a protective effect from the action of peptidases. This was also the explanation for the survival of a relatively long CN fragment, the 17-residues peptide f(193–209) from β-CN, which was not significantly hydrolyzed by brush border exopeptidases, as shown on its quantification in the apical solutions of a Caco-2 monolayer using RP-HPLC–ESI with triple quadrupole (QqQ) as mass analyzer (Regazzo et al., 2010).

The use of Caco-2 cell model has some limitations, such as the absence of a mucus layer, to be tighter compared to human small intestine, low expression of uptake transporters, and over-expression of P-glycoprotein. The additional presence of mucin-secreting goblet cells in co-cultures with Caco-2 might modulate the tightness of the monolayer and form a mucus gel layer which mimics physiological conditions (Hilgendorf et al., 2000). Using a co-culture 75% Caco-2/25% HT29-MTX, the antihypertensive peptides from α_{s1}-CN RYLGY, AYFYPEL, and two derived fragments, RYLG and AYFYPE, identified after treatment with pepsin and Corolase PP™ showed that they can survive the action of intestinal peptidases, cross the mucus layer, and be absorbed intact through the intestinal epithelium, because they were detected in the basolateral solution (Fig. 3). The use of a powerful identification technique, RP-HPLC-ESI-IT, showed also that released fragments from these peptides could be transepithelially absorbed and they could, therefore, exert physiological effects (Contreras, Sancho, Recio, & Mills, 2012).

A recent work has characterized the entire panel of peptides produced from CN and whey proteins that survive in vitro sequential

gastro-pancreatic digestion (pepsin followed by a mixture of trypsin, chymotrypsin, elastase and carboxypeptidase) and translocate across the Caco-2 cell monolayer (Picariello et al., 2013). The combination of RP-HPLC in conventional and microscale flow and MS analyzers (MALDI-TOF and ESI-QTOF) together with the use of synthetic peptides, as the gastrointestinal-resistant model peptides, permitted a reliable peptide monitoring. The presence of some of the previously described bioactive peptides of milk on the basolateral side of the Caco-2 monolayer was negligible. For instance, the β-CMs were not detected, although it has been reported that synthetic human β-CMs can translocate across Caco-2 monolayers (Iwan et al., 2008). By contrast, some CPPs from the regions 104–119 of α_{s1}-CN and 33–52 of β-CN were shown to translocate across the intestinal epithelium model. Besides, resistant regions from β-LG f(40–60) and f(125–135) were also detected in considerable amounts.

With the aim to construct a structural diverse database for dipeptides, assessing their intestinal stability, permeability to Caco-2 cells and ACE-inhibitory activity, QSAR modeling was applied to a total of 228 synthetic dipeptides. Quantification of dipeptides derived from digestion and transport experiments was performed with a multiple reaction ion monitoring (MRM) method on a QqQ combined with previous HILIC separation. No significant correlation models were found for intestinal permeability with peptide structure. However, the intestinal stability of 12 peptides was predicted (Foltz, Van Buren, Klaffke, & Duchateau, 2009).

3.2.2. Peptidomics in ex vivo and in situ models

The Ussing chamber technique utilizes small intestinal tissue sheets that are mounted between two (luminal and serosal)buffer containing reservoirs with supply of gases to mimic physiological conditions. The test compound is added to either the mucosal or the serosal side of the tissue to study transport in the absorptive or secretory direction, respectively.

By using segments of rabbit distal ileum mounted on Ussing chamber, the transfer of the β-CN derived opioid peptide morphiceptin (YPFP-NH_2) was monitored. The observed peptide degradation was followed by RP-HPLC, and therefore no information about the released fragments was possible (Mahé, Tomé, Dumontier, & Desjeux, 1989). In contrast, using rat intestinal segments, the apparent permeability of the antihypertensive β-CN peptides VPP and IPP has been selectively calculated in the cell media by the use of a MRM method (Foltz et al., 2008). The authors showed that the values of Peyer's patches for VPP were more than three times greater than those of jejunal and duodenal segments, which can be due to lower peptidase activity and a leakier epithelium.

In situ perfusion of intestinal segments of rodents (rats or rabbits) is frequently used to study the permeability and absorption kinetics of drugs. The main advantage of in situ models is the integration of the dynamic components of the mesenteric blood circulation, the mucus layer, and all the other factors present in the intestinal content (Antunes, Andrade, Ferreira, Nielsen, & Sarmento, 2013).

By using a ligated segment of rat duodenum, Bouhallab et al. (1999) demonstrated that the CPP from β-CN f(1–25), whether to iron complexed or not, is hydrolyzed by the digestive enzymes including proteases/peptidases and phosphatases during duodenal transit. Each HPLC peak contained several ions corresponding to endogenous components of the lumen but QqQ MS permitted to attribute signals to β-CN f(1–25)-derived peptides. At least five peptidic bonds of β-CN f(1–25) were cleaved and successive dephosphorylation of the β-CN f(15–25) was observed. This pointed out the need to know the relationship between the positive role of this CPP in iron availability and its susceptibility to digestive enzymes. Subsequently, Ani-Kibangou et al. (2005) demonstrated that the inhibition of alkaline phosphatase improved the absorption of CPP-bound iron.

Comparison of the three absorption models, the Caco-2 cells, Ussing chamber with ex vivo different rat intestinal segments, and in situ intestinal perfusion model in rat has been performed for the transport of IPP and VPP, (Foltz et al., 2008). The intestinal permeability of both peptides augmented with increasing physiological relevance of the model. Therefore, the previous observation that Caco-2 cell model underestimates intestinal peptide permeability was supported by the 5 to 20-fold higher permeability across intact excised intestinal tissues and in the closed perfused loop model.

3.3. Absorption, distribution, metabolism, and excretion (ADME)

Once the stability of peptides to digestion, and the permeability by cellular models have been evaluated, ADME studies need to be conducted, in order to determine the course of potential biologically active peptides in the organism. The absorption of the dipeptide VY into human circulatory blood has been reported (Matsui et al., 2002). The plasma levels increased with dosage and after single oral administration of a VY-containing drink. The maximum absorption occurred at 2 h postprandial and the elimination half-time of VY was estimated at 3.1 h.

The application of MS-based techniques has permitted the development of selective and sensitive methods for the quantification of peptides and monitor small changes in plasma. This is the case of an atmospheric pressure ionization MRM method to quantify 17 ACE-inhibitory peptides (2, 3, and 5 residues) in human plasma samples collected after ingestion of a peptide-enriched drink. The limit of detection achieved was reported to be 0.01 ng mL^{-1}, and the quantification limit was estimated between 0.05 and 0.2 ng mL^{-1} (van Platerink, Janssen, Horsten, & Haverkamp, 2006). Other authors have also applied a MRM method to assess the bioavailability of IPP and seven other ACE-inhibitory peptides contained in a peptide-enriched beverage, and the influence of meal intake on the bioavailability of IPP (Foltz et al., 2007). The maximum peptide concentration in plasma after ingestion of the peptide-enriched beverage was 897 ± 157 pM. When the beverage was consumed after a meal, the maximum concentration of IPP compared to the placebo treatment was higher than that obtained in the fasted state. van der Pijl, Kies, Ten Have, Duchateau, and Deutz (2008) reported the kinetics of Pro-rich tripeptides (IPP, LPP, and VPP) in pig, administrated intravenously or intragastrically. This study revealed that, in the first case, the elimination half-times were significantly different between the three peptides, being higher for IPP than those obtained for the others. However, no difference in elimination half-time was found after intragastric administration of peptides. The maximum concentration detected was 10 nM and the absolute absorption was estimated to be 0.1% when administrated with saline solution. However, the absorption and elimination half-times were maximally 5 and 15 min respectively, suggesting an acute effect of the peptides under these conditions.

4. Peptidomics for bioactive peptide monitoring

4.1. Peptide occurrence in dairy products

Many commercial dairy products have been shown to contain reported bioactive peptides, which are mainly released during fermentation with starter cultures, ripening, and other manufacturing processes (e.g., thermal treatment or storage steps) (Hernández-Ledesma et al., 2011). Furthermore, peptides can be found in the milk as result of the natural proteolysis of milk proteins. Some peptidomic studies are conducted to monitor specific peptides of known activity while others are intended to provide a panel of identified peptides, among which bioactive sequences can be recognized.

Dallas et al. (2013) recently identified over 300 peptides in human milk by nanoLC–ESI-QTOF and verified that a large group of them showed sequence overlap with reported antimicrobial and immunomodulatory peptides. Also, MALDI-TOF and nanoLC–ESI-linear IT were used to analyze the peptidome profile of drying-off cow milk and study the in situ proteolysis (Ho et al., 2010). Among 202 identified peptides, five of them have been reported to possess bioactivity, i.e., opioid,

ACE-inhibitory, or immunomodulatory activity. Likewise, the occurrence of the opioid peptide β-CM-7 was identified and quantified (14 μg L^{-1}) in commercial milk by using an optimized nano-ESI-IT method (Juan-García, Font, Juan, & Picó, 2009).

Cheese constitutes one of the major sources of bioactive peptides among commercial dairy products due to the proteolytic activities developed on milk proteins during cheese ripening by rennet, microbial flora, and natural milk proteases and peptidases. López-Expósito, Amigo, and Recio (2012) recently reviewed the healthy role of cheeses since their remarkable content in bioactive peptides, especially antihypertensive, CPP, and opioid peptides. For instance, the ACE-inhibitory peptides VPP and IPP were found in several commercially available cheeses at physiological relevant concentrations by using linear IT (Bütikofer, Meyer, Sieber, Walther, & Wechsler, 2008; Bütikofer, Meyer, Sieber, & Wechsler, 2007). Similarly, Ong and Shah (2008) determined the release of several ACE-inhibitory peptides in Cheddar cheeses made with different starter cultures by the use of RP-HPLC in conjunction with MALDI-TOF. Moreover, peptides with ACE-inhibitory and antihypertensive activities were detected by RP-HPLC–ESI-IT in Manchego cheese (Gómez-Ruiz, Ramos, & Recio, 2004b). In a Mexican unripened cheese variety but manufactured with specific strains of lactic acid bacteria, ACE-inhibitory peptides were also identified through the analysis of the water extract (<3 kDa) by nanoLC–ESI-IT (Torres-Llanez et al., 2011). On the other hand, large CPPs were found in semi-hard Herrgård cheese by performing RP-HPLC–ESI-IT analysis after IEX separation (Ardö, Lilbæk, Kristiansen, Zakora, & Otte, Shalaby, Zakora, Pripp, & El-Shabrawy (2007). Regarding opioid peptides, De Noni and Cattaneo (2010) quantified β-CM-7 in different cheese varieties by using RP-HPLC–ESI-IT and they detected concentrations of 0.15 mg kg^{-1} in Brie cheese.

Fermented milks, yogurts, and kefir are another important group of commercial dairy products that can include bioactive peptides in their composition due to the milk-protein proteolysis by microbial fermentation (Muro Urista, Álvarez Fernández, Riera Rodriguez, Arana Cuenca, & Téllez Jurado, 2011). For instance, Hernández-Ledesma, Miralles, Amigo, Ramos, and Recio (2005) identified three reported potent ACE-inhibitory peptides, VPP, NIPPLTQTPV, and RY, in a commercial fermented milk after performing RP-HPLC–ESI-IT analysis of certain active fractions. Besides, some identified peptides showed sequences with a high similarity to other described ACE-inhibitory, immunomodulatory, and antioxidant peptides. In another study, Quirós, Hernández-Ledesma, Ramos, Amigo, and Recio (2005) described the occurrence of two ACE-inhibitory peptides, PYVRYL and LVYPFTGPIPN, in a commercial kefir made from caprine milk following the peptide identification by analogous MS analysis. Interestingly, Jarmołowska and Krawczuk (2012) detected the opioid peptides β-CM-7, lactoferroxin A, casoxin 6, and casoxin C in two commercially available yogurts and one kefir. In this case, ELISA assays were employed instead of MS analysis. On the other hand, the application of microLC–ESI-TOF to the peptidome analysis of a functional antihypertensive yogurt demonstrated, in addition to ACE-inhibitory and antihypertensive peptides, the occurrence of other bioactive peptides with different activities as antioxidant, antibacterial, opioid or antithrombotic (Kunda et al., 2012).

4.2. Peptide stability during industrial processing

The effect of processing may influence the final activity of the peptides in real food systems. The final extent of amino acid modifications and changes in bioactivity will largely depend on the composition of the food matrix in which the peptide is present, the processing conditions, and the peptide structure (López-Fandiño, Otte, & van Camp, 2006). However, little is known about the effects of processing and storage on bioactive peptides incorporated into foods. Recently, some studies have reported for specific bioactive peptides their limited commercial utilization in fermented foods due to their partial or total degradation along fermentation process depending on the peptide sequence, the strain, and pH. Paul and Somkuti (2009) studied the degradation of the antimicrobial (RRWQWRMKKLG) and antihypertensive (FFVAPFPEVFGK) peptides, derived from lactoferrin and α_{s1}-CN, respectively, in the presence of the yogurt starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The monitoring by RP-HPLC in conjunction with MALDI-TOF showed their higher resistance at pH 4.5 compared to that at pH 7. Likewise, the degradation of the antimicrobial peptides casocidin and isracidin was followed in conditions that mimic yogurt fermentation (Somkuti & Paul, 2010). The results indicate that the peptide addition should take place at the end of fermentation or by blending into drinkable yogurt-like products.

For a scaled up application of bioactive peptides, the resistance to the industrial conditions of manufacturing, packaging, and storage has to be assessed. Contreras, Sevilla, et al. (2011) monitored the antihypertensive peptides α_{s1}-CN f(90–94) and f(143–149), RYLGY and AYFYPEL, respectively, for scaling up the production of an antihypertensive casein hydrolysate. The identification and quantification of these two peptides was performed by RP-HPLC–ESI-IT, and it was found that they were stable during the processes of atomization, homogenization, and pasteurization.

In cheese, the ripening time can have an important effect in the concentration of bioactive peptides. The concentration of the antihypertensive peptides IPP and VPP has been monitored during ripening of different Swiss cheese varieties by using RP-HPLC and linear IT with triple MS detection (Meyer, Bütikofer, Walther, Wechsler, & Sieber, 2009). In a wider study, Sforza et al. (2012) provided a detailed peptidomic study on the evolution of the oligopeptide fraction in Parmigiano–Reggiano cheese from curd to 24 months aging, making possible to discriminate cheeses according to their aging time. Packaging technology and storage time have been considered in a recent study where the peptide profile was monitored during the shelf life at 4 °C of cheeses packaged using two different technologies, vacuum packaging and modified-atmosphere packaging (Sánchez-Rivera, Recio, Ramos, & Gómez-Ruiz, 2013). Semi-quantitative analysis of peptides (RP-HPLC–ESI-IT) revealed some differences between different packaging technologies but a common trend in the evolution of the peptides during storage was observed: differences were more pronounced at longer storage times (90 d) and peptide evolution during storage was similar for both packaging techniques

Protein lactosylation is a modification of milk proteins by the Maillard reaction, a non-enzymatic glycation in which the carbonyl group of reducing sugars, such as lactose, primarily reacts with the ε-amino group of Lys residues leading to the formation of lactulosyl-lysine. The modification leads to a decrease of milk nutritional value as it reduces the bioavailability of the essential amino acid Lys. MALDI-TOF has been applied for investigating protein lactosylation in heat treated milk samples (Siciliano, Mazzeo, Arena, Renzone, & Scaloni, 2013). Likewise, peptide lactosylation in milk powder stored for 4 weeks was analyzed by a MRM method using a hybrid QqQ/Linear IT mass spectrometer (Le, Deeth, Bhandari, Alewood, & Holland, 2013). The possibility to quantify peptide lactosylation allows to follow peptide degradation during storage, which is important when determining the shelf life of a functional food based on bioactive peptides.

5. Future prospects

In the field of bioactive peptides, peptidomics is a discipline of choice because peptides are the concerned molecules. Liquid chromatography and capillary electrophoresis will continue to progress through the miniaturization of the components to provide, besides the suitable MS instrumentation, increasingly better analytical tools for unequivocal peptide determination. Meanwhile, bioinformatic tools still have to be developed focused on this research area. For peptides absorbed that can reach blood circulation, their course in the organism has to be followed to determine their metabolism and kinetic in the organism. The correlation studies between the observed effects and the concentrations of the peptides in body fluids and even in the target organs will be progressively considered. Besides, the in vivo formation of derived

peptides, that might exert bioactivity, and the search for the minimum active fragment merit attention. In the cases where peptide bioactivity is mediated in the gut lumen or through receptors on the intestinal cell wall, only peptide stability in the intestinal environment would be necessary. To date, such data have been scarcely reported, and they refer to ACE-inhibitory peptides, which are the most extensively studied. In the field of food digestion, the analysis of digest peptidomes will provide data about cleavage traits according to enzyme activities which will help to understand this physiological path that every food matrix has to undergo. Of special interest in this context is the correlation between in vivo digestion data and those obtained in in vitro models. In addition, the identification of new biomarkers based on peptidomics constitutes a novel research line that should be encouraged. Due to the possible interaction of peptides with other food components or their degradation, a careful monitoring of bioactive peptide stability during processing or storage is mandatory in view of possible formation of toxic or allergenic substances and also to maintain the desired dosage, for the optimal exploitation of this valuable resource.

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2. RESULTS

2.1 Chapter I

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Dairy *Debaryomyces hanseni* strains produce the antihypertensive casein-derived peptides LHLPLP and HLPLP

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Abstract:

The ability of dairy *Debaryomyces hansenii, Kluyveromyces lactis* and *Kluyveromyces marxianus* strains to release the casein-derived antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP was examined. Yeast strains were grown in medium with casein as sole nitrogen source and the yeast casein hydrolysates (CSHs) were analysed by HPLC-MS/MS to search for the six antihypertensive sequences. Only LHLPLP and HLPLP were identified in CSHs and exclusively in *D. hansenii* Dh1 and Dh14 hydrolysates in which both antihypertensive sequences represented approximately 6 (CSH Dh1) and 10% (CSH Dh14) of total peptide content. In addition, a complete analysis of selected CSHs by HPLC-MS/MS allowed the identification of 35 (Dh1) and 46 (Dh14) additional peptides, which could also contribute to the observed *in vitro* ACE inhibitory potency of both hydrolysates (Dh1, IC50 = 13.6 ± 1.8 µg/mL; Dh14, IC50 = 17.5 ± 2.1 µg/mL) and might confer them multifunctional properties. Finally casein zymography revealed the presence of at least one extracellular protease with a molecular mass of about 50 kDa. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of *D. hansenii* strains to produce antihypertensive casein-derived peptides.

Keywords: Dairy yeasts, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, casein-derived antihypertensive peptides.

Abbreviations

ACE: angiotensin I-converting enzyme; CSH: casein hydrolysate; GRAS: generally recognized as safe; LAB: lactic acid bacteria; RP-HPLC-MS/MS: reversed phase-high performance liquid chromatography tandem mass spectrometry; RP-HPLC-UV-MS: reversed-phase high performance liquid chromatography-UV and mass spectrometry; SHR: spontaneously hypertensive rat.

1. Introduction

Casein is an excellent substrate to produce peptides with angiotensin I converting enzyme (ACE)-inhibitory and antihypertensive effects (Otte, Shalaby, Zakora, Pripp, & El-Shabrawy 2007; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Basically, processing of milk proteins with food grade proteolytic preparations or fermentation of milk with lactic acid bacteria (LAB) proteolytic starters have been employed to release antihypertensive peptides. Both approaches have conducted to the development of commercial products based on milk proteins with antihypertensive effects in humans. In fact, one of the most popular functional foods contain the casein-derived ACE61 inhibitory tripeptides VPP and IPP, which can be obtained by means of either milk fermentation (Nakamura, Yamamoto, Sakai & Takano, 1995) or enzymatic hydrolysis using microbial proteases (Mizuno, Nishimura, Matsuura, Gotou & Yamamoto, 2004). We have previously identified casein-derived sequences with antihypertensive activity using both methods. Two novel peptides of sequences RYLGY and AYFYPEL were identified from a peptic casein hydrolysate (Contreras, Carrón, Montero, Ramos, & Recio, 2009.). Both exerted *in vitro* inhibitory effects on ACE activity (IC_{50} values of 0.71 and 6.58 μM, respectively) and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs) at 5 mg/kg of body weight. With respect to the fermentation strategy, the antihypertensive sequence LHLPLP was identified as one of the major peptides responsible for the ACE-inhibitory and antihypertensive effects of fermented milk produced by *Enterococcus faecalis* (Muguerza et al., 2006; Quirós et al., 2007). This sequence showed an IC_{50} value of 5.5 ± 0.4 μM against ACE activity and exhibited potent antihypertensive effect

at a dose of 2 mg/kg (Quirós et al., 2007). Further characterization suggested the pentapeptide HLPLP as the minimum active form responsible for the antihypertensive effect of LHLPLP (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008.). Since the pathogenic potential of some *Enterococcus* strains (Franz, Holzapfel, & Stiles, 1999) may hamper their use in the food industry, production of HLPLP by enzymatic hydrolysis with food-grade enzymes was also optimized (Quirós, Hernández-Ledesma, Ramos, Martín Álvarez, & Recio, 2012).

Recently, we have shown the ability of some proteolytic dairy yeast strains belonging to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* species to generate casein-derived antihypertensive hydrolysates (García-Tejedor, Padilla, Salom, Belloch, & Manzanares, 2013). With the aim of further characterize these non-conventional dairy yeasts as feasible GRAS (Generally Recognized As Safe) microorganisms for the production of antihypertensive peptides, here we screen up to thirty-three of these strains on the basis of their ability to release previously identified casein derived antihypertensive sequences (RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and IPP) obtained either by enzymatic hydrolysis or bacterial fermentation. In addition, the presence of extracellular caseinolytic activity in selected strains is evaluated. Finally, quantification of the aforementioned sequences and identification of new main casein-derived peptides in the selected yeast casein hydrolysates are carried out, and their potential bioactivities in terms of ACE inhibition and other functional effects are discussed.

2. Materials and Methods

2.1. Materials

Casein (Promilk 85) was obtained from Ingredia (Arrax Cedex, France). ACE from porcine kidney, bicinchoninic acid protein assay kit, Hammarsten casein and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain). The fluorogenicsubstrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Coomassie Brilliant Blue G-250, 30% acrylamide/bis solution 29:1 and Precision Plus Protein Dual Color standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Yeast strains and growth conditions

Thirty-three yeast strains belonging to *D. hansenii* (Dh1-Dh23), *K. lactis* (Kl1-Kl8) and *K. marxianus* (Km1-Km2) species isolated from artisanal ewes' and goats' milk cheeses (Padilla, Manzanares & Belloch, 2014) were used in this study. Yeast strains were maintained on GPYA medium (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar, pH 5.5).

2.3. Preparation of casein hydrolysates

Stock solution of casein was sterilized by autoclaving at 121℃, 15 min. Casein medium (2% casein, 2% glucose) was inoculated with 10^6 cells/ml from pre-cultured strains on GPY (GPYA without agar) and incubated at 28℃ and 100 rpm in an orbital shaker for 6 days. At the end of the incubation period yeast cells

were sedimented by centrifugation (3220 x g, 10 min), and the supernatants ultrafiltered using Amicon Ultra 10K centrifugal filter devices (Millipore Corporation, Billerica, MA, USA). Supernatant ultrafiltrates enriched in peptides of molecular weight lower than 10 kDa were considered as casein hydrolysates (CSHs). Supernatant concentrates (> 10 kDa) were used for zymography experiments as specified further.

Protein concentration was estimated by the bicinchoninic acid method using bovine serum albumin as standard (Ruiz-Giménez et al., 2012).

2.4. In vitro assay of ACE-inhibitory activity

In vitro ACE-inhibitory activity of CSHs was measured using the fluorescent method described by Sentandreu & Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate o-aminobenzoyl-Gly p-nitro-Phe-Pro by the action of ACE. The IC_{50} value of CSHs was defined as the protein concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by non-linear regression of the experimental data to a four parameter logistic curve using the software package SigmaPlot v 10.0 (SPSS Inc., Chicago, IL, USA).

2.5. Casein zymography

Zymography was performed according to Folio, Ritt, Alexandre, & Remize (2008) with minor modifications. Briefly, samples (casein supernatant concentrates) in loading buffer without β-mercaptoethanol were loaded onto a 10% (w/v) SDS/PAGE gel (Laemmli, 1970) co-polymerized with 0.12% (w/v) Hammarsten casein. After electrophoresis and brief washing in ultrapure water,

the gel was washed for 2 h on a rotary shaker with 2.5% (v/v) Triton X-152 100. Afterwards the gel was washed twice with reaction buffer (50 mM citrate phosphate buffer, pH 6) and then incubated overnight in reaction buffer at room temperature. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 for 1 h and then de-stained. Protease activity bands were visualized as white bands on a dark blue background.

2.6. Synthesis and quantification of peptides HLPLP and LHLPLP

The peptides HLPLP f(134-138) and LHLPLP f(133-138) from β-casein, were synthesized in-house using the fluorenyl-methoxy-carbonyl chloride (Fmoc) solid-phase method with a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany). Peptide purity was calculated by reversed phase high performance liquid chromatography-UV and mass spectrometry (RP-HPLC-UV-MS). Six-point calibration curves of the pure peptides HLPLP and LHLPLP (from 1.95 to 62.5 µg/mL) were prepared in Milli-Q deionized water. Duplicate injections were done for each point on the calibration curve. The quantification of the peptides in each CSH was performed by the extraction of their characteristic ions.

2.7. Peptide sequencing by reversed phase-high-performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS/MS).

The analyses of CSHs by RP-HPLC-MS/MS were performed on an Agilent HPLC (Agilent Technologies, Waldbronn, Germany) system followed by on-line MS/MS analysis on a quadrupole ion trap instrument (Esquire 3000, Bruker Daltonik GmbH, Bremen, Germany) as previously described (Sánchez-Rivera,

Diezhandino, Gómez-Ruiz, Fresno, Miralles, & Recio, 2014). Chromatographic separations were performed with a Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The flow rate was 0.2 mL/min and the injection volume 50 μL. Peptides were eluted with a linear gradient from 0% to 45% of solvent B (acetonitrile:formic acid 0.1%) and 55% solvent A (water:formic acid 0.1%) in 120 min. The target mass was set at *mass-to-charge* ratio (*m/z*) 750, and the mass acquisition ranged from 200 to 2000 *m/z*.

2.8. Statistical data analysis

IC_{50} values of CSHs were compared using Student's t-test. Differences with *P*-values < 0.05 were considered significant. Data statistical analysis was performed using the GraphPad Prism 4 software (GraphPad Software Inc, La Jolla, CA, USA).

3. Results and discussion

In the present study, 33 CSHs generated by *D. hansenii* (CSH Dh1- Dh23) *K. lactis* (CSH Kl1-Kl8) and *K. marxianus* (CSH Km1-Km2) strains isolated from artisanal cheeses (Padilla et al., 2014) were analysed by HPLC MS/MS to search for the previously identified antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP. Peptides RYLGY, AYFYPEL, VPP and IPP were not identified in any of the CSHs. However, peptide HLPLP and its precursor sequence LHLPLP were released from casein after growth of *D. hansenii* strains Dh1 and Dh14. Figure 1 shows the UV chromatogram and the extracted ions corresponding to both peptides.

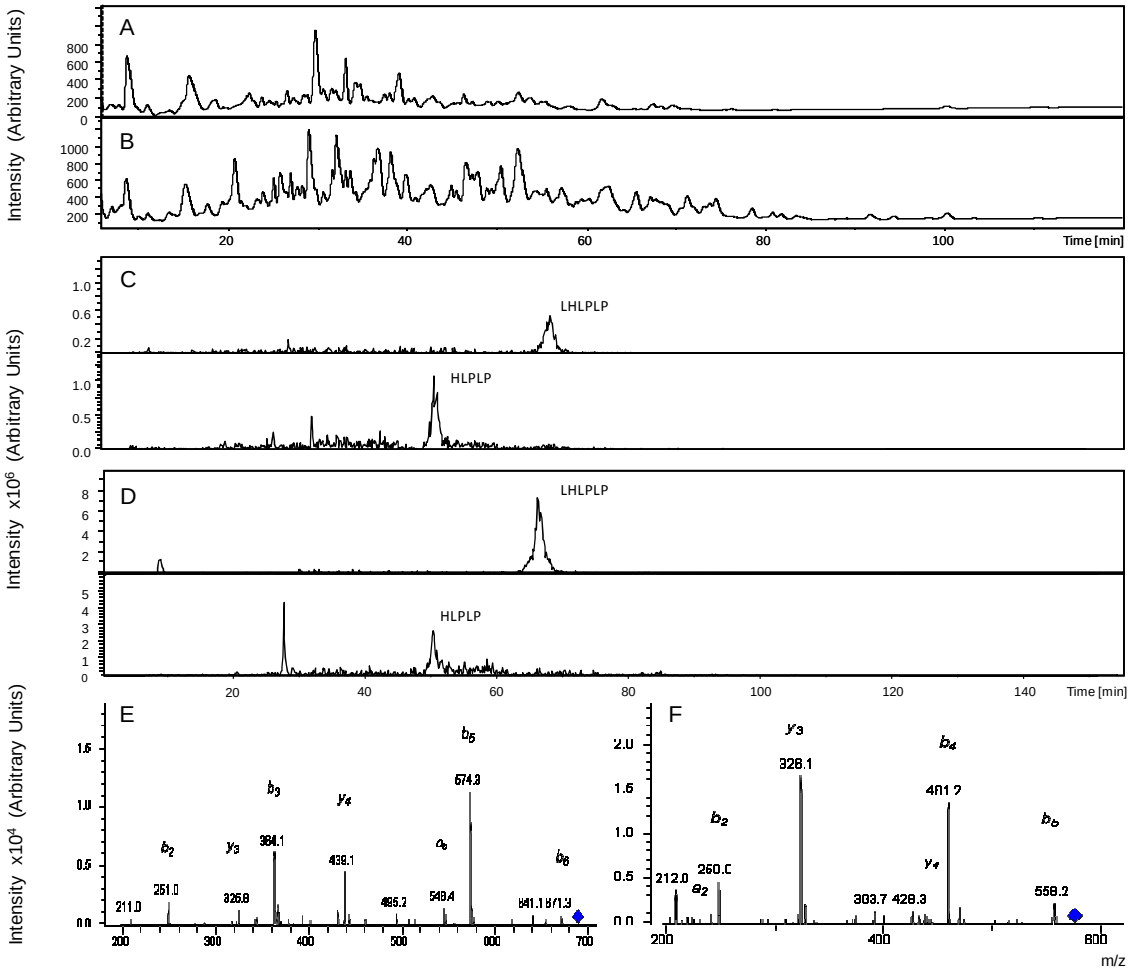

Figure 1. UV chromatogram of the casein hydrolysate Dh1 (A) and Dh14 (B). Extracted Ion chromatogram (EIC) of the peptides LHLPLP and HLPLP in Dh1 (C) and Dh14 (D). Fragmentation pattern of LHLPLP (E) and of HLPLP (F), with mass-to-charge ratios (m/z) of 689.5 and 576.3, respectively.

Quantification of HLPLP and LHLPLP in CSH Dh1 and Dh14 was carried out using linear regressions obtained by HPLC-MS/MS. As shown in Table 1, highest total content was obtained in CSH Dh14. However both hydrolysates contained similar quantities of the pentapeptide. These antihypertensive peptides represented approximately 6 and 10% of total peptide content in Dh1 and Dh14 hydrolysates, respectively. No significant differences were found between the ACE inhibitory potencies of both *D. hansenii* CSHs, which showed IC_{50} values of

13.6 ± 1.8 µg/mL (Dh1) and 17.5 ± 2.1 µg/mL (Dh14). ACE inhibition was slightly higher than the previously reported values of milk fermented by *E. faecalis* (34-59 µg/mL; Muguerza et al., 2006) but lower than that showed by a pepsin casein hydrolysate (5.68 µg/mL; Contreras et al., 2009).

Table 1. Content of peptides LHLPLP and HLPLP in casein hydrolysate (CSH) Dh1 and Dh14.

CSH	LHLPLP (µg/mg)	HLPLP (µg/mg)	Total (µg/mg)
Dh1	15.6	43.5	59.1
Dh14	52.7	46.4	99.1

During milk fermentation, proteolysis by LAB has been studied in detail. The key enzyme in this process is a cell-envelope proteinase involved in the first step of casein degradation (Savijoki, Ingmer, & Varmanen, 2006). By contrast, although yeast caseinolytic activity is well documented few reports deal with the characterization of the extracellular proteolytic system. With regards to *D. hansenii*, Kumura, Takagaki, Sone, Tsukahara, Tanaka, & Shimazaki (2002) reported that a cell-wall associated protease acted on β-casein whereas α_S-casein degradation was attributed to an intracellular protease released by cell lysis. However, extracellular proteolytic activity on both α_S- and β-casein for *D. hansenii* strains isolated from cheese has been described (Gardini et al., 2006). Here we conducted a casein zymography to detect extracellular proteases in the supernatants of the two selected *D. hansenii strains*. On casein substrate,

extracellular proteolytic activities were detected (Figure 2). Analysis of Dh14 supernatant revealed the presence of one hydrolysis band which corresponded to a molecular mass of about 50 kDa. The same band was observed in Dh1 supernatant, in which a second band of slightly higher molecular mass was also detected. Band intensities for both supenatants were not related to the amount of total protein loaded (Dh1, 4.8 µg; Dh14, 4.4 µg). Although increasing amounts of Dh14 total protein were loaded onto SDS/PAGE gels for protease detection no other clear activity bands were observed (data not shown). These results might suggest a different proteolytic profile for both strains in the conditions tested. Future work should include purification of the extracellular *D. hansenii* enzymes and determination of its biochemical properties.

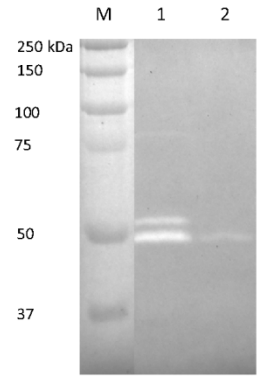

Figure 2. Casein zymography of *D. hansenii* Dh1 and Dh14 extracellular proteolytic activities. Lane M, molecular weight markers (Precision Plus Protein Dual Color standards, Bio-Rad); lane 1, Dh1 culture supernatant concentrate (4.8 µg); lane 2, Dh14 culture supernatant concentrate (4.4 µg).

Over the last two decades, a large number of casein-derived peptides obtained by fermentation and enzymatic hydrolysis with ACE-inhibitory potency and proven antihypertensive effects in SHRs have been identified (Martínez-Maqueda et al., 2012). Although LHLPLP and HLPLP might contribute to the

inhibitory potency of *D. hansenii* CSHs, other sequences generated from casein hydrolysis might also be responsible for the observed inhibitory effects. This prompted us to carry out a complete analysis of CSH Dh1 and Dh14 by HPLC MS/MS, which allowed the identification of 35 and 46 additional peptides (Table 2 and 3), respectively.

Table 2. Identification of peptides contained in *D.hansenii* casein hydrolysate (CSH) Dh1.

Ion for MS/MS (m/z)[a]	Observed mass[b]	Theoretical mass	Protein fragment	Identified sequence[c]
806,388	805,372	805,401	α_{S1}-CN f(24-30)	FVAPFPE
530,179	529,172	529,29	α_{S1}-CN f(25-29)	**VAPFP**
659,312	658,304	658,333	α_{S1}-CN f(25-30)	**VAPFPE**
608,373	607,366	607,333	α_{S1}-CN f(32-36)	FGKEK
618,394	617,387	617,302	α_{S1}-CN f(35-39)	EKVNE
632,362	631,354	631,318	α_{S1}-CN f(81-85)	IQKED
502,15	501,143	501,28	α_{S1}-CN f(95-98)	LEQL
468,196	467,189	467,249	α_{S1}-CN f(127-130)	IHAQ
602,321	601,313	601,307	α_{S1}-CN f(138-142)	VNQEL
680,309	679,302	679,297	α_{S1}-CN f(159-164)	YPSGAW
517,184	516,177	516,233	α_{S1}-CN f(160-164)	PSGAW
777,435	776,428	776,407	α_{S1}-CN f(167-173)	VPLGTQY
581,257	580,25	580,286	α_{S1}-CN f(169-173)	**LGTQY**
555,344	554,337	554,27	α_{S1}-CN f(181-185)	**DIPNP**
519,164	518,156	518,234	α_{S1}-CN f(186-190)	IGSEN
602,321	601,313	601,253	α_{S2}-CN f(4-8)	MEHVS
1000,532	999,525	999,539	α_{S2}-CN f(100-108)	YQGPIVLNP
837,459	836,452	836,476	α_{S2}-CN f(101-108)	QGPIVLNP
698,394	697,386	697,401	α_{S2}-CN f(116-122)	**AVPITPT**
528,248	527,24	527,296	α_{S2}-CN f(118-122)	**PITPT**
720,417	719,409	719,422	α_{S2}-CN f(198-203)	TKVIPY
628,27	627,263	627,323	β-CN f(6-11)	LNVPGE
748,265	747,258	747,304	β-CN f(38-43)	QQQTED
622,309	621,302	621,316	β-CN f(59-63)	VYPFP
439,026	438,018	438,284	β-CN f(74-77)	**IPPL**
668,38	667,373	667,39	β-CN f(74-79)	IPPLTQ
732,347	731,34	731,385	β-CN f(113-118)	KYPVEP
690,282	689,275	689,323	β-CN f(127-132)	LTDVEN
577,164	576,157	576,239	β-CN f(128-132)	TDVEN
689,449	688,442	688,427	β-CN f(133-138)	LHLPLP
576,304	575,297	575,343	β-CN f(134-138)	**HLPLP**
649,265	648,258	648,28	β-CN f(144-148)	MHQPH
805,351	804,343	804,384	β-CN f(155-161)	**VMFPPQS**
780,457	779,45	779,491	β-CN f(170-176)	VLPVPQK
536,148	535,14	535,228	β-CN f(193-196)	YQEP
633,35	632,343	632,353	k-CN f(26-30)	IPIQY
663,318	662,311	662,339	k-CN f(43-47)	YQQKP

[a]Charge of precursor ion: 1; [b]Calculated monoisotopic mass; [c]Main peptides peptides are labeled in bold. Casein (CN)

Table 3. Identification of peptides contained in *D.hansenii* casein hydrolysate (CSH) Dh14.

Ion for MS/MS (m/z)[a]	Observed mass[b]	Theoretical mass	Protein fragment	Identified sequence[c]
805.399	805,378	805,401	α_{S1}-CN f(24-30)	**FVAPFPE**
676,407	675,400	675,359	α_{S1}-CN f(29-34)	PEVFGK
707,446	706,439	706,401	α_{S1}-CN f(31-36)	VFGKEK
763.321	762,322	762,358	α_{S1}-CN f(54-59)	MEDIKQ
732.394	631,328	631,318	α_{S1}-CN f(55-59)	EDIKQ
903,472	902,465	902,453	α_{S1}-CN f(135-142)	MIGVNQEL
517,171	516,164	516,233	α_{S1}-CN f(160-164)	PSGAW
789,371	788,634	788,370	α_{S1}-CN f(179-185)	FSDIPNP
730.385	729,314	729,329	α_{S1}-CN f(184-190)	NPIGSEN
675.257	674,322	674,324	β-CN f(1-5)	RELEE
649,284	627,306	627,323	β-CN f(6-11)	LNVPGE
633,295	632,288	632,277	β-CN f(38-42)	QQQTE
748,284	747,277	747,304	β-CN f(38-43)	QQQTED
875.371	876,305	876,346	β-CN f(38-44)	QQQTEDE
632.355	631,335	631,318	β-CN f(44-48)	ELQDK
681,303	680,296	680,313	β-CN f(52-57)	FAQTQS
622,309	621,302	621,316	β-CN f(59-63)	VYPFP
668,416	667,409	667,390	β-CN f(74-79)	IPPLTQ
769.342	768,386	768,438	β-CN f(74-80)	IPPLTQT
866,472	865,465	865,491	β-CN f(74-81)	**IPPLTQTP**
753,385	752,378	752,407	β-CN f(75-81)	PPLTQTP
689.313	688,395	688,412	β-CN f(95-100)	VSKVKE
760,456	759,449	759,449	β-CN f(95-101)	VSKVKEA
577,327	576,267	576,294	β-CN f(98-102)	VKEAM
651,384	650,376	650,343	β-CN f(111-115)	FPKYP
732.274	731,364	731,385	β-CN f(113-118)	KYPVEP
611.329	610,267	610,260	β-CN f(119-123)	FTESQ
698,269	697,262	697,292	β-CN f(119-124)	FTESQS
904,446	903,439	903,455	β-CN f(125-132)	LTLTDVEN
689.313	688,392	688,427	β-CN **f(133-138)**	**LHLPLP**
802,505	801,498	801,511	β-CN f(133-139)	LHLPLPL
1130,667	1129,660	1129,686	β-CN f(133-142)	LHLPLPLLQS
576,334	575,327	575,343	β-CN **f(134-138)**	**HLPLP**
793,521	792,514	792,511	β-CN f(135-141)	LPLPLLQ
880,515	879,508	879,543	β-CN f(135-142)	LPLPLLQS
670,420	669,413	669,406	β-CN f(137-142)	LPLLQS
533,184	532,177	532,265	β-CN f(140-143)	LQSW
649.329	648,277	648,280	β-CN f(144-148)	MHQPH
805.402	804,348	804,384	β-CN f(155-161)	**VMFPPQS**
649,327	648,320	648,344	β-CN f(164-169)	SLSQSK
543,270	542,262	542,343	β-CN f(168-172)	SKVLP
559,253	558,246	558,284	β-CN f(185-189)	**MPIQA**
762,385	761,378	761,396	β-CN f(191-196)	LLYQEP
649,318	648,310	648,312	β-CN f(192-196)	LYQEP
826,445	825,438	825,450	β-CN f(199-206)	**GPVRGPFP**
939,500	938,493	938,534	β-CN f(199-207)	**GPVRGPFPI**
658,370	657,363	657,370	k-CN f(119-124)	IPTINT
757,362	756,355	756,365	k-CN f(155-161)	**SPPEINT**

[a]Charge of precursor ion: 1; [b]Calculated monoisotopic mass; [c]Main peptides peptides are labeled in bold. Casein(CN)

The most abundant peptides in each hydrolysate are given in bold. As can be seen in Table 2, most of the peptides present in CSH Dh1, derived from hydrolysis of α_{S1}-CN (15 fragments) and β-CN (14 fragments) whereas only 6 and 2 peptides corresponded to α_{S2}-CN and *κ*-CN fragments, respectively. By contrast, in CSH Dh14 most of the peptides (37 fragments) derived from β-CN. In addition 9 fragments from α_{S1}-CN and 2 from *κ*-CN were also identified, while none of the fragments derived from α_{S2}-CN. Finally, only 11 of the peptides identified, including the two antihypertensive sequences LHLPLP and HLPLP, were common for both *D. hansenii* strains, which indicates strain-specific proteolysis in accordance with the results obtained in the zymogram and also as described for the *Streptococcus thermophilus* cell envelope proteinase (Miclo et al., 2012).

Sequences LHLPLP and HLPLP were also included in several longer fragments identified in CSH Dh14, such as LHLPLPL, HLPLPL and LHLPLPLLQS. The heptapeptide LHLPLPL, with an IC_{50} value of 425 ± 44 μM against ACE, was also identified in *E. faecalis* fermented milk (Quirós et al., 2007). Another peptide (β-CN f(58-76), LVYPFPGPIPNSLPQNIPP; IC_{50} = 5.2 ± 0.3 μM) identified in *E. faecalis* fermented milk caused also significant decrease of the systolic blood pressure in SHRs (Quirós et al., 2007). Part of thi sequence (VYPFP) was also identified in both *D. hansenii* CSHs. On the other hand, although neither VPP nor IPP were found in *D. hansenii* CSHs, sequences containing the latter at the N-terminal end were identified (IPPL, IPPLTQ, IPPLTQT and IPPLTQTP). Whether these newly identified sequences could also contribute to the ACE-inhibitory activity of CSH Dh1 and Dh14 deserves further characterization studies. Moreover, the role of gastrointestinal digestion to

generate active fragments from longer sequences should also be taken into account, although degradation of active fragments can also occur. More than 60% of the identified sequences have at least one Pro residue. Particularly, Pro in the C-terminal and antepenultimate position has been recognised as one of the most favourable residues for peptide binding to the active site of ACE (Rohrbach, Willians, & Rolstad, 1981). Interestingly, in addition to the antihypertensive sequences LHLPLP and HLPLP, peptides VAPFP, DIPNP, VYPFP, FSDIPNP and GPVRPFP with a Pro residue in bothpositions might be promising candidates to inhibit ACE activity. Finally, some other sequences identified in CSH Dh1 and Dh14 were previously characterised as ACE-inhibitory and antioxidant peptides (sequence YQEP in Dh1; Silva, Pihlanto, & Malcata, 2006) or having lipoxygenase inhibitory properties (VLPVPQK in Dh1; Rival, Fornaroli, Boeriu, & Wichers, 2001). Also some sequences identified here share structure homology with fragments able to inhibit prolyl-peptidases of human colon cells (FVAPFPE, VAPPFP and VAPFPE in Dh1; Juillerat-Jeanneret, Robert, & Juillerat, 2011) or having immune-modulating and ACE-inhibitory properties (GPVRGPFPI and GPVRGPFP in Dh14; Hayes, Stanton, FitzGerald, & Ross, 2007; Hernández-Ledesma, Amigo, Ramos, & Recio, 2004). The multifunctional properties and the resulting health benefits of dairy protein hydrolysates have been pointed out by several studies (reviewed in Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). To what extent *D. hansenii* CSHs might exert different functional effects requires further research.

Our results point to *D. hansenii* strains Dh1 and Dh14 as feasible GRAS microorganisms with strain-specific caseinolytic systems for the production of the antihypertensive sequences HLPLPL and HLPLP. These two peptides could at

least in part be responsible for the ACE-inhibitory properties of both *D. hansenii* CSHs, although data reported here suggest that other potential bioactive sequences are also produced. *D. hansenii,* one of the predominant yeast species in all type of cheeses, plays important roles in cheese making and further investigation is needed to exploit its biotechnological potential.

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2.2 Chapter II

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Short communication: Peptide profiling in cheeses packed using different technologies

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ABSTRACT

Peptides released during the shelf life of cheeses packaged using 2 different technologies, vacuum packaging (VP) and modified-atmosphere packaging (MAP), were identified by on-line reverse phase-HPLC-tandem mass spectrometry. A total of 22 peptides from the N-terminal domain of α_{S1}-casein (CN) and 26 from β-CN were identified, the latter more evenly distributed over the whole sequence. Peptides were monitored during the shelf life of these cheeses when stored at 4°C, revealing that the peptide profile changed significantly with the storage time. Qualitative differences between VP and MAP cheeses were only found for 3 α_{S1}-CN peptides, which were absent in MAP cheeses. Semiquantitative analysis of peptides revealed some differences between cheeses packaged using different technologies. However, evolution of peptides during storage followed a common trend in both types of cheeses. In addition, the presence of certain peptides, which had been previously described because of their potential bioactivity, is illustrated. For instance, some of the identified peptides had been previously reported as antihypertensive peptides, such as peptide α_{S1}-CN (1–9) or β-CN f(201–209).
Key words: cheese, vacuum packaging, modified-atmosphere packaging, cheese bioactive peptide

Short Communication

Proteolysis is a phenomena occurring during cheese ripening comprised of numerous biochemical reactions that contribute to the flavor and texture of the final product. In the primary proteolysis, indigenous milk enzymes and those present in the coagulant play an important role. During secondary proteolysis, a great variety of peptides are released from the milk CN fraction by proteolytic enzymes which mainly belong to microorganisms that participate in cheese manufacturing (primary and secondary starters, as well as adventitious microflora; Fox, 1989).

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In recent decades, food packaging has undergone major technological development, partly in response to consumer demand for preservative-free food and in control-packaging methods to preserve quality and food safety. Changes in consumer preferences have affected buying habits and a growing interest in sliced and ready-to-eat products has been observed. These types of goods have a high value of convenience, but are also susceptible to physical and chemical changes. Vacuum packaging (**VP**) of cheese retards molds growth; however, the product may undergo changes in color, flavor, and texture (Romani et al., 2002) or show excessive surface humidity due to the migration of water from the inside to the surface (Pantaleão et al., 2007). Therefore, VP is not suitable for all kinds of cheeses, as it may lead to some structural and visual changes in the product. Consequently, alternative methods of packaging have been proposed, such as modified-atmosphere packaging (**MAP**). This technique appears suitable for cheese packaging, taking into account physicochemical and microbiological criteria (Dermiki et al., 2008), especially when using a gas mixture combination of 30% CO_2 to 70% N_2, which is able to extend the shelf life in terms of microbiological stability, keeping the sensory characteristics of Provolone cheese (Favati et al., 2007) or Greek whey cheese (Papaioannou et al., 2007) intact.

When cheese is packed to extend its shelf life, the effect of packaging on the ripening process should be evaluated. Some studies focused on the evaluation of proteolysis of VP cheese during ripening have been carried out using total nitrogen and ripening index (Tarakci and Kucukoner, 2006). The application of reverse phase-HPLC analysis (Sousa et al., 2001; Poveda et al., 2003) or HPLC-tandem mass spectrometry (**HPLC-MS/MS**; Piraino et al., 2007) has been proposed as a reliable tool to evaluate proteolysis in cheese. However, to our knowledge no comparative studies have been carried out on peptide profile changes occurring along the shelf life of cheese subjected to different packaging techniques.

In this study, HPLC-MS/MS detection has been used to identify the peptides released and evaluate the peptidic profile changes during the shelf life of cheeses packaged using 2 different technologies. Based on previ-

ous studies, the presence, origin, and potential bioactivities of these peptidic sequences are also discussed.

Semihard cheeses samples, provided by a national dairy manufacturer, were made out of mixed pasteurized sheep, cow, and goat milk with 2 different packaging systems (VP for cheese wedges and MAP for sliced cheeses). Prior to cheese packaging, the curd was processed using different molds, depending on the final format, and subsequently ripened for 20 d under controlled temperature and humidity conditions

Cheese samples were received immediately after manufacturing and stored at 4°C for the duration of the study: 0, 30, 60, and 90 d for the MAP samples, and 0, 30, 60, 90, and 150 d for the VP samples. In both cases the maximum number of storage days refers to the shelf-life of the respective cheese type.

Water-soluble extracts were obtained at each selected time according to the method described by Gómez-Ruiz et al. (2002). Supernatants were ultrafiltered on a 3,000 Da cut-off ultrafiltration membrane (Pall Corporation, Ann Arbor, MI). The 3,000 Da permeates were freeze-dried and kept at −20°C until their analysis.

Reverse phase-HPLC-MS/MS analyses of the permeates <3,000 Da were carried out on an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) connected on line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). A Mediterranea Sea18 15 × 0.21 cm column was used in the experiments (Teknokroma, Barcelona, Spain). Auto MS(n) analyses used a signal threshold of 10,000, a voltage ramp from 0.35 to 1.4 V for the fragmentation of precursor ions, and an isolation width of 4.0 m/z. The estimated amount of peptides (in arbitrary units) in each sample was calculated by extracting their corresponding characteristic ions (molecular ion or doubly charged ion, when present); duplicate samples were prepared for each time and package condition, and were individually analyzed.

The peptide profile of the different cheeses was studied by evaluating both the UV spectra and the mass spectra after analysis by liquid chromatography. Essentially no differences in the UV spectra were found between the 2 types of packaging along the storage times (results not shown). A more detailed MS analysis revealed some differences in the peptide profile of cheeses packaged with both systems. A total of 48 peptides were identified, 22 sequences belonging to α_{S1}-CN and 26 sequences to β-CN. No peptides were detected from α_{s2}-CN or κ-CN. This can be linked to their lower content compared with α_{S1}- or β-CN. In addition, the fragment incorporated to the curd derived from κ-CN, para-κ-CN, has been described to be rather resistant to proteolysis. As cheeses were manufactured with different types of milk some of the sequences could belong to different species (cow, goat, or sheep). Figure 1 shows the α_{S1}-CN-derived peptides identified in VP and MAP cheeses, their presence during the storage time, and their intensity obtained from the HPLC-MS analysis at 0 d of MAP cheese. Most of the identified peptides from α_{S1}-CN were released from the N-terminal segment. The peptides that showed the highest intensities at 0 d corresponded to α_{S1}-CN f(1–16), f(17–23), and f(24–32), although many other peptides that also belong to these 3 regions were identified. In cheese, chymosin rapidly cleaves at Phe_{23}-Phe_{24} in cow milk and Phe_{23}-Val_{24} in sheep and goat milk, giving 2 major fragments as a result: α_{S1}-CN f(1–23) and f(24–199) (McSweeney and Fox, 1993). Further hydrolysis by cell envelope-associated proteinases and endopeptidases of starter and nonstarter bacteria release several peptides from the fragment α_{S1}-CN f(1–23). As shown in Figure 1, the same sequences were identified regardless the packaging method used; the only exceptions were the α_{S1}-CN peptides RPKHPIK, LPQEVLN, and PFPEVF, which were absent in MAP cheeses. Additional differences between the 2 packaging methods were also notable at the quantitative level. For example, the evolution of some of the most abundant α_{S1}-CN peptides during the storage time in VP and MAP cheeses is shown in Figure 2. In some cases, higher peptide intensities during storage time were detected in VP cheeses compared with MAP cheeses, especially at longer storage times (90 d). This is the case of the peptides α_{S1}-CN f(24–32) and α_{S1}-CN f(25–32; Figure 2). However, some peptides reached higher concentration in MAP cheeses compared with VP cheeses, as can be observed for the peptide α_{S1}-CN f(1–16). Concerning their evolution during the storage time, some peptides [α_{S1}-CN f(17–23), α_{S1}-CN f(24–32), and α_{S1}-CN f(25–32)] behaved similarly regardless the packaging technology, whereas others followed different patterns [α_{S1}-CN f(1–9) and α_{S1}-CN f(17–22)].

In contrast to α_{S1}-CN, peptides from β-CN were more evenly identified over the whole sequence. For β-CN peptides, no qualitative differences were found between VP and MAP cheeses (Figure 3). Those peptides that reached the highest concentrations in the water-soluble extract corresponded to β-CN f(1–6), f(44–52), f(45–52), and f(47–52; Figure 4). It is notable that, in general, for β-CN peptides differences were more pronounced at longer storage times (90 d) and peptide evolution during storage was similar for both packaging techniques. At 90 d, peptides β-CN f(7–14) and f(44–52) reached higher levels in VP cheeses than MAP cheeses, but the opposite was found for β-CN f(46–52), f(47–52), and f(74–82).

Some of the peptides identified in the water-soluble extracts of these cheeses have been previously described because of their potential to exert biological activities. For instance, α_{S1}-CN f(1–9) with sequence

Calculated Mass	Protein Fragment	Identified Sequence	VP	MAP
874.5	α_s1-CN f(1-7), c, s	RPKHPIK	4, 5	-
1139.6	α_s1-CN f(1-9), c, s	RPKHPIKHQ	1, 2, 3, 4, 5	1, 2, 3, 4
1534.9	α_s1-CN f(1-13), c	RPKHPIKHQGLPQ	1, 2, 3, 4, 5	1, 2, 3, 4
1876.1	α_s1-CN f(1-16), c	RPKHPIKHQGLPQEVL	1, 2, 3, 4, 5	1, 2, 3, 4
1991.1	α_s1-CN f(1-17), c	RPKHPIKHQGLPQEVLN	1, 2, 3, 4, 5	1, 2, 3, 4
754.4	α_s1-CN f(10-16), c	GLPQEVL	1, 2, 3, 4, 5	1, 2, 3, 4
811.4	α_s1-CN f(11-17), c	LPQEVLN	2, 3, 5	-
813.4	α_s1-CN f(15-27), c, g, s	VLNENLL	1, 2, 3, 4, 5	1, 2, 3, 4
601.3	α_s1-CN f(17-21), c, g, s	NENLL	1, 2, 3, 4, 5	1, 2, 3, 4
757.4	α_s1-CN f(17-22), c, g, s	NENLLR	1, 2, 3, 4, 5	1, 2, 3, 4
904.5	α_s1-CN f(17-23), s, c, g	NENLLRF	1, 2, 3, 4, 5	1, 2, 3, 4
790.4	α_s1-CN f(18-23), c, s, g	ENLLRF	1, 2, 3, 5	1, 2, 3, 4
1150.6	α_s1-CN f(23-32), g, s	FVVAPFPEVF	1, 2, 3, 4, 5	1
757.4	α_s1-CN f(24-30), g, s	VVAPFPE	1, 2, 3, 4, 5	1, 2, 3, 4
805.4	α_s1-CN f(24-30), c	FVAPFPE	2, 3, 4, 5	4
1003.5	α_s1-CN f(24-32), g, s	VVAPFPEVF	1, 2, 3, 4, 5	1, 2, 3, 4
1051.5	α_s1-CN (24-32), c	FVAPFPEVF	1, 2, 3, 4, 5	1, 2, 3, 4
658.3	α_s1-CN f(25-30), c, g, s	VAPFPE	1, 2, 3, 4, 5	1, 2, 3, 4
904.5	α_s1-CN f(25-32), s, c, g	VAPFPEVF	1, 2, 3, 4, 5	1, 2, 3, 4
805.4	α_s1-CN f(26-32), c	APFPEVF	1, 2, 3, 4, 5	1, 2, 3, 4
734.3	α_s1-CN f(27-32), c, g, s	PFPEVF	4, 5	-
1014.5	α_s1-CN f(33-40), g, s	RKENINEL	1, 2, 3, 4, 5	1, 2, 3, 4

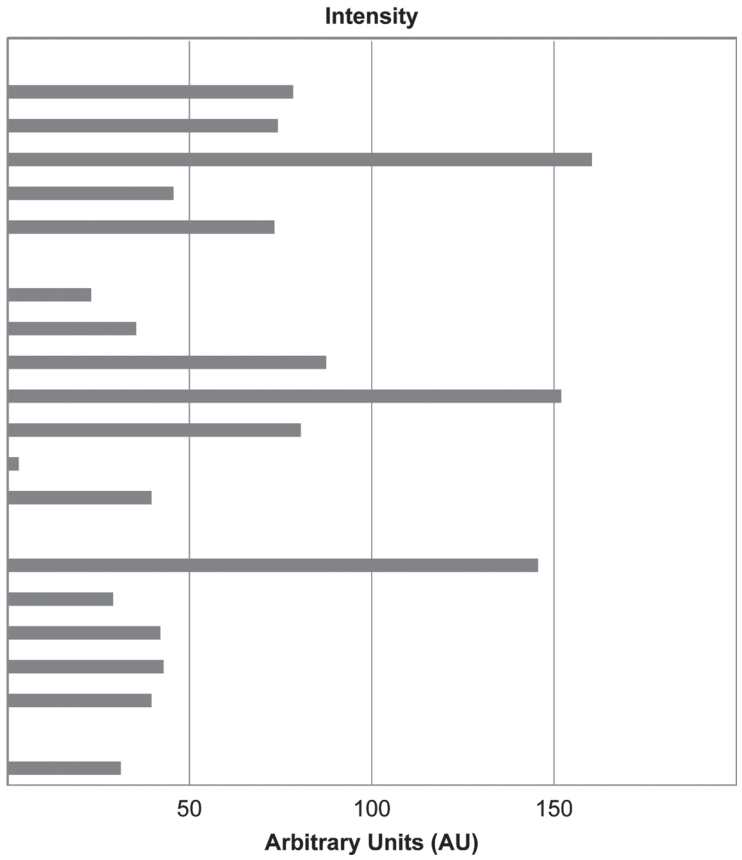

Figure 1. Peptides derived from α_{S1}-CN identified in the water-soluble extract of semihard cheeses vacuum packaged (VP) and modified-atmosphere packaged (MAP). Peptides were identified by HPLC-tandem mass spectrometry analysis, and the intensity of the peptides of MAP cheeses at 0 d is represented on the right (value $\times$ 10^7 units). Protein fragments come from cow (c), sheep (s), and goat (g) milk. The first column of the table indicates calculated monoisotopic mass, and the numbers for VP and MAP indicate the presence of peptides at 0 (1), 30 (2), 60 (3), 90 (4), and 150 (5) d.

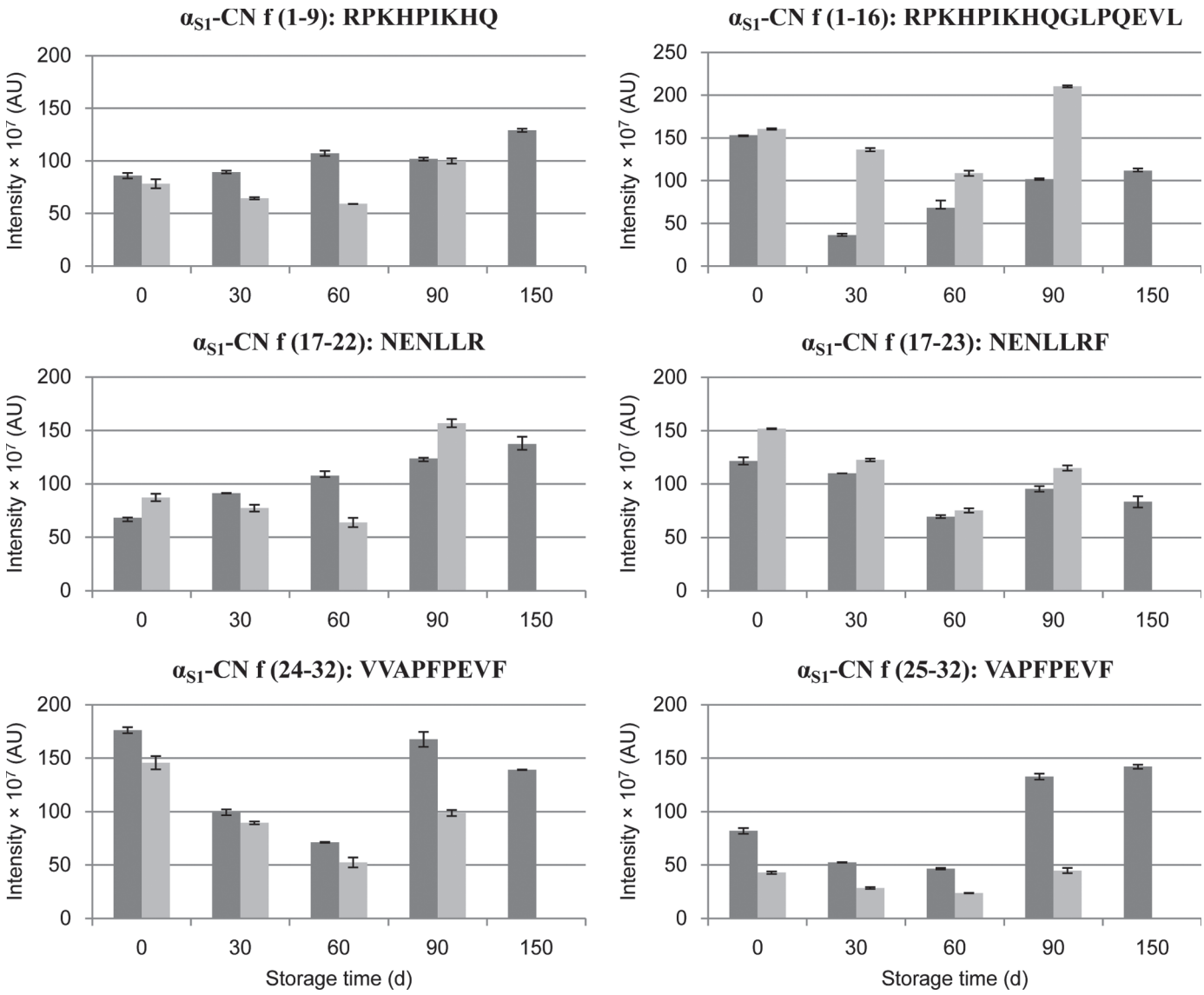

Figure 2. Relative amount of α_{S1}-CN-derived peptides found in the 3,000 Da permeate from the water-soluble extracts of vacuum-packaged (dark gray bars) and modified-atmosphere-packaged (light gray bars) cheeses stored at 4°C during their shelf life (150 and 90 d, respectively). Amount of peptide is expressed as intensity (arbitrary units) of the signal obtained from the HPLC-tandem mass spectrometry analysis. Each value corresponds to the average of a duplicate sample.

RPKHPIKHQ had been identified and isolated in other cheeses, such as 8 mo-ripened Gouda cheese (Saito et al., 2000), Festivo cheese (Ryhänen et al., 2001), and Scandinavian cheeses (Lund and Ardö, 2004). A potent angiotensin-converting enzyme (**ACE**)-inhibitory activity had been reported for this peptide ($IC_{50} = 13.4\ \mu M$), although it showed weak antihypertensive activity in rats (Saito et al., 2000). Similarly, other peptides that possess ACE-inhibitory activity are α_{S1}-CN f(18–23), ENLLRF, with $IC_{50} = 82.4 \pm 9\ \mu M$ (Quirós et al., 2005), and α_{S1}-CN f(25–32), VAPFPEVF, which shows moderate ACE-inhibitory activity (Contreras et al., 2009). Another related identified fragment was the peptide α_{S1}-CN f(24–32), FVAPFPEVEF, which had been reported with both antimicrobial activity (Rizzello et al., 2005) and ACE-inhibitory activity (Ong et al., 2007). A decapeptide derived from the α_{S1}-CN N-terminal region, α_{S1}-CN f(3–13), has demonstrated antioxidant activity and stimulatory activity of adiponectin secretion in adipose cell culture (Higurashi et al., 2007). It has to be highlighted that this sequence is included in several of the most abundant peptides in Figure 1.

One of the most abundant peptides, β-CN f(1–6), RELEEL, had been previously identified in fermented milk produced by *Lactobacillus helveticus* CP790, showing moderate ACE-inhibitory and antihypertensive

Calculated Mass	Protein Fragment	Identified Sequence	VP	MAP
674.3	β-CN f(1-5), c	RELEE	1, 2, 3, 4, 5	1, 2, 3, 4, 5
802.4	β-CN f(1-6), g, s	REQEEL	1, 2, 3, 4, 5	1, 2, 3, 4, 5
787.4	β-CN f(1-6), c	RELEEL	1, 2, 3, 4, 5	1, 2, 3, 4, 5
916.4	β-CN f(1-7), g, s	REQEELN	1, 2, 3, 4, 5	1, 2, 3, 4, 5
855.4	β-CN f(7-14), c	NVPGEIVE	1, 2, 3, 4, 5	1, 2, 3, 4, 5
661.4	β-CN f(19-23), c, g, s	NLLRF	1, 3	1, 2, 3, 4, 5
1125.6	β-CN f(44-52), c, s, g	ELQDKIHPF	1, 2, 3, 4, 5	1, 2, 3, 4, 5
996.5	β-CN f (45-52), c, s, g	LQDKIHPF	1, 2, 3, 4, 5	1, 2, 3, 4, 5
883.4	β-CN f(46-52), c, g, s	QDKIHPF	1, 2, 3, 4, 5	1, 2, 3, 4, 5
755.4	β-CN f(47-52), s	DKIHPF	1, 2, 3, 4, 5	1, 2, 3, 4, 5
1004.5	β-CN f(60-68), g, s	YPFTGPIPN	1, 2, 3, 4, 5	1, 2, 3, 4, 5
964.5	β-CN f(74-82), c	IPPLTQTPV	1, 2, 3, 4, 5	1, 2, 3, 4, 5
755.4	β-CN f(94-100), g, s	GVPKVKE	1, 2, 3, 4, 5	1, 2, 3, 4, 5
801.5	β-CN f(133-139), c, g, s	LHLPLPL	1, 2, 3, 4, 5	1, 2, 3, 4, 5
856.5	β-CN f(94-101), c, g	GVPKVKET	1, 2, 3, 4, 5	1, 2, 3, 4, 5
816.5	β-CN f(94-101), c	GVSKVKEA	1, 2, 3, 4, 5	1, 2, 3, 4, 5
987.5	β-CN f(94-102), s, g	GVPKVKETM	1	1, 2, 3, 4, 5
1000.6	β-CN f(97-105), c	KVKEAMAPK	1, 2, 3, 4, 5	1, 2, 3, 4, 5
830.4	β-CN f(99-105), g, s	QKFALPQ	1, 2, 3, 4, 5	1, 2, 3, 4, 5
658.3	β-CN f(164-169), s	SLSQPK	1, 2, 3, 4, 5	1, 2, 3, 4, 5
779.5	β-CN f(169-175), s, c	KVLPVPQ	1, 2, 3, 4, 5	1, 2, 3, 4
1125.6	β-CN f(173-182), c	VPQKAVPYPQ	1, 2, 3, 4, 5	1, 2, 3, 4, 5
801.4	β-CN f(176-182), c	KAVPYPQ	1, 2, 3, 5	1, 2, 3
1150.7	β-CN f(197-207), c/ f(195-205), s	VLGPVRGPFPI	1, 2, 3	1, 2, 3, 4, 5
996.6	β-CN f(201-209), c/ f(199-207), s, g	VRGPFPIIV-VRGPFPILV	1, 2, 3	1, 2, 3, 4, 5
1150.7	β-CN f(197-207), g, s/ f(199-209), c	GPVRGPFPILV-GPVRGPFPIIV	1, 2, 3, 4, 5	1, 2, 3, 4, 5

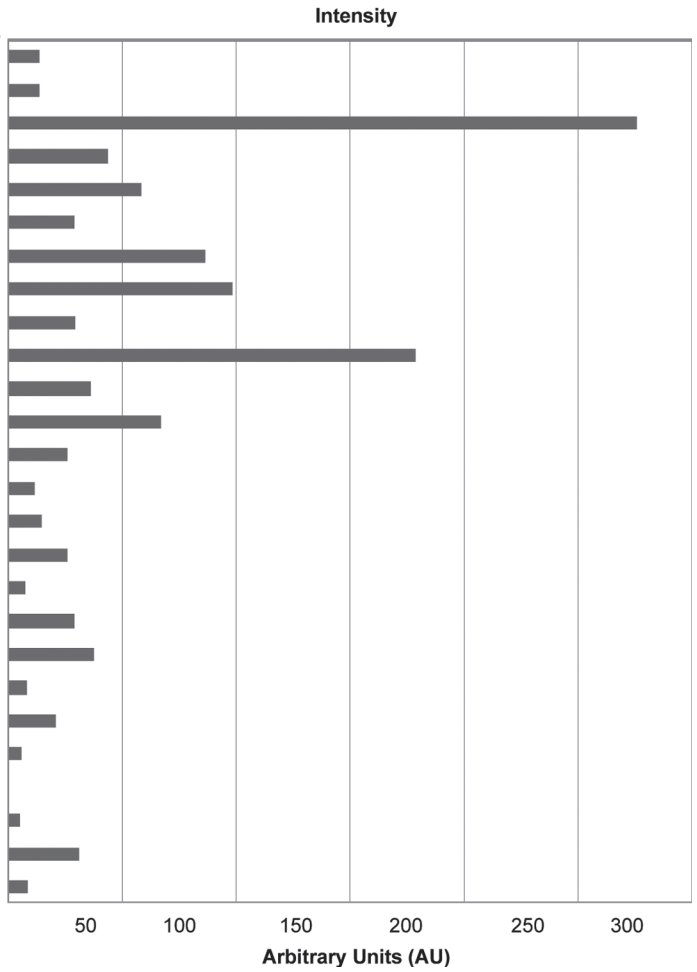

Figure 3. Peptides derived from β-CN identified in the water-soluble extract of semi-hard cheeses vacuum packaged (VP) and modified-atmosphere packaged (MAP). Peptides were identified by HPLC-tandem mass spectrometry analysis, and the intensity of the peptides of MAP cheeses at 0 d is represented on the right (value $\times$ 10^7 units). Protein fragments come from cow (c), sheep (s), and goat (g) milk. The first column of the table indicates calculated monoisotopic mass, and the numbers for VP and MAP indicate the presence of peptides at 0 (1), 30 (2), 60 (3), 90 (4), and 150 (5) d.

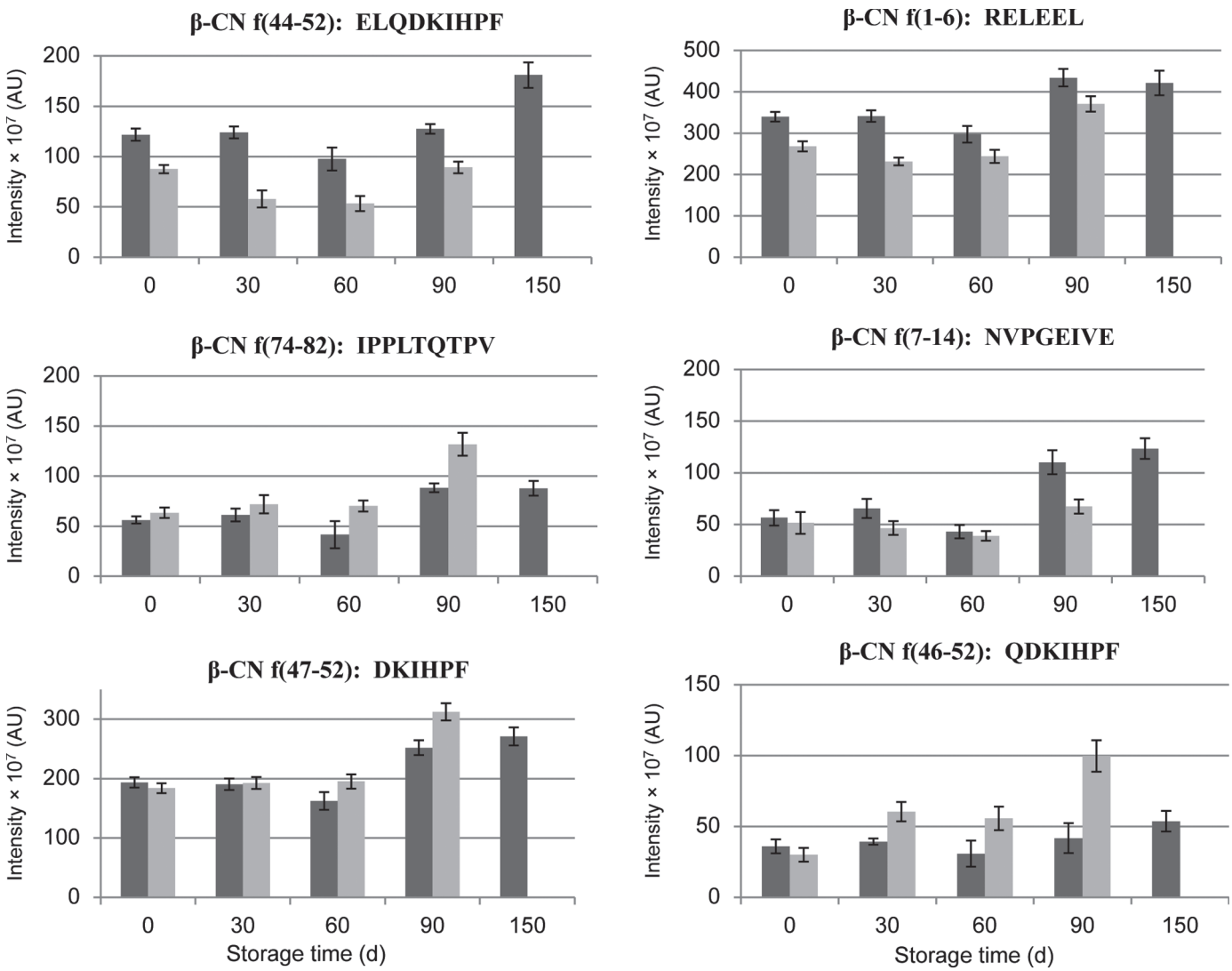

Figure 4. Relative amount of β-CN-derived peptides found in the 3,000 Da permeate from the water-soluble extracts of vacuum-packaged (dark gray bars) and modified-atmosphere-packaged (light gray bars) cheeses stored at 4°C during their shelf life (150 and 90 d, respectively). Amount of peptide is expressed as intensity (arbitrary units) of the signal obtained from the HPLC-tandem mass spectrometry analysis. Each value corresponds to the average of a duplicate sample.

activity (Maeno et al., 1996). Specifically important in terms of bioactivity is the presence of the β-CN peptide LHLPLPL [f(133–139)], a well-conserved sequence in many species. Although this peptide shows only moderate ACE-inhibitory activity (IC_{50} = 432.7 μM; Miguel et al., 2006), it can be a precursor of the potent antihypertensive peptide LHLPLP (Quirós et al., 2007). Likewise, the peptide with sequence YPFTGPIPN [β-CN f(60–68)] showed a potent ACE-inhibitory activity (IC_{50} = 14.8 μM) (Saito et al., 2000), and was isolated in Manchego cheese (Gómez-Ruiz et al., 2004). Other identified peptides from β-CN reported as moderate ACE-inhibitors are β-CN f(69–75) with sequence KVLPVPQ (although with significant antihypertensive activity in rats; Maeno et al., 1996) and peptide DKIHPF [f(47–52)], identified in several Spanish cheeses (Gómez-Ruiz et al., 2004, 2006). Likewise, peptide VRGPFPIIV [β-CN f(201–209)] showed a significant effect on the reduction of blood pressure in rats despite possessing only moderate ACE-inhibitory activity (Miguel et al., 2006).

In summary, 48 peptides were identified in the water-soluble extracts of VP and MAP cheeses. It has been described that product composition, processing, storage conditions (time and temperature), and packaging contribute to the residual cheese-ripening processes observed during storage. The influence of 2 factors, storage time and packaging, were evaluated in this

study. During storage time at 4°C it was observed that the peptide profile changed under both packaging technologies. The variation mainly referred to the relative amount of the different peptides, as most of the peptidic sequences identified were the same regardless of packaging technique and storage time. Some exceptions were the α_{S1}-CN peptides f(1–7), f(11–17), and f(27–32) that were only identified in VP cheeses. In contrast, the same β-CN peptides were identified in VP and MP cheeses. Comparing both packaging systems, a nonconstant pattern was observed concerning the relative amounts of peptides during the storage time. Looking at the longest common storage time (90 d), peptides such as α_{S1}-CN f(24–32), f(25–32), and β-CN (44–52) reached higher relative levels in VP cheeses compared with MAP cheeses, whereas the opposite was observed for other peptides [α_{S1}-CN f(1–16), β-CN f(46–52–32), and β-CN (74–82)]. In addition, though all β-CN peptides showed a similar trend during storage time in both types of cheeses, the behavior of α_{S1}-CN peptides in VP and MP cheeses varied depending on their sequence. The identification of these minor differences between VP and MAP cheeses merits further research to better understand their correlation with the organoleptic properties of cheeses packaged with different technologies. Among the identified peptides, several of them have been described as bioactive sequences showing different bioactivities. These bioactivities should be also further evaluated using in vivo studies to corroborate the potential of these cheeses.

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2.3 Chapter III

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Research Article

Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion

It is increasingly evident that digestion can affect the biological activity of cheese by the release of new active peptides from their precursors or, on the contrary, giving rise to fragments without activity. The characterization of the peptidome of a Spanish blue cheese, Valdeón, has been conducted before and after gastrointestinal digestion, and the digests have been compared to those obtained from pasteurized skimmed milk powder (SMP) using a bioinformatics platform. Peptidomic profiling of digests revealed several regions that are especially resistant to digestion (among them β-casein 60–93, 128–140, and 193–209). Some of them correspond to well-conserved regions between species (human, cow, sheep, and goat) and include peptide sequences with reported bioactivity. The great peptide homology found between both digests, cheese and SMP, suggests that the gastrointestinal digestion could bring closer the profile of products with different proteolytic state. Although most of the biologically active peptides found in cheese after digestion were also present in SMP digest, there were some exceptions that can be attributed to the absence of the relevant precursor peptide before digestion.

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Active peptide / Gastrointestinal digestion / Peptidomics / MS/MS / Valdeón cheese
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1 Introduction

Peptides in cheese are not only important for the organoleptic characteristics of this product but they might also contribute to its high biological value. An important number of medium and low molecular weight peptides resulting from casein during cheese ripening have been identified. Many of these peptides can interact with receptors at the gastrointestinal tract (opioid receptors), facilitate mineral absorption as the caseinophosphopeptides (CPPs), or be absorbed and reach the blood stream where they can exert a biological effect, for instance, the antihypertensive activity [1,2]. These peptides are released by residual coagulant, indigenous milk enzymes, starter, and nonstarter adventitious microflora; and, sometimes, enzymes from secondary flora (e.g. from *Penicillium* sp. in mould-ripened cheeses) [3]. Upon human ingestion, gastrointestinal enzymes come into play. There are several studies on cheese digestion using human gastrointestinal enzymes under physiological conditions [4–6]. These in vitro simulations are intended to be consistent with the in vivo digestion studies [7, 8]. It is increasingly evident that digestion can affect the biological activity, by the release of new active peptides from their precursors or, on the contrary, giving rise to fragments with less or no activity. This brings up the question about the effect that this physiological process could have on the peptide profile of food matrices containing the same proteins but in a different proteolytic state. In order to shed some light on this issue, we have characterized the peptidome of the Spanish fat, blue-veined cheese. This cheese is made out of cow`s milk or a mixture of cow, sheep, and/or goat's milk, in a region called Valdeón (León, Spain). Its authenticity is guaranteed by a Protected Geographical Indication since 2003. The peptide profile of Valdeón cheese has been checked before and after simulated gastrointestinal digestion. The digestion products have been compared to those obtained from pasteurized skimmed milk powder (SMP), a

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Abbreviations: **ACE**, angiotensin I-converting enzyme; **CPP**, caseinophosphopeptide; **IC_{50}**, the half maximal inhibitory concentration; **SMP**, skimmed milk powder; **WSE**, water-soluble extract

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matrix where milk proteins are mainly intact, subjected to the same enzymatic digestion.

2 Materials and methods

2.1 Samples

Two samples of different batches of 60 days old cheese, made out of pasteurized cow and goat's milk, were analyzed. One corresponded to the winter cheese production (batch D) and the other to the summer production (batch A), according to the standard methods established by the Regulation Council. The protein in dry matter contents of cheese (Kjeldahl standard method, International Dairy Federation) were 36.2 and 35.7%, respectively. A sample of SMP (protein content was 40.0%) purchased at a local market was also used.

2.2 Preparation of samples

The water-soluble extracts (WSE) of Valdeon cheese were obtained as described by Gómez-Ruiz et al. [4]. Afterwards, the WSEs were ultrafiltrated using membranes Centripore Amino Ultra of 3000 Da (Millipore, MA, USA). The WSE and ultrafiltrates were frozen at –20°C until analysis.

In order to perform CPPs determination, an enrichment step by selective precipitation was carried out, as previously described [9]. Briefly, this was performed by adding $CaCl_2$ at 1% w/v (40 mol $CaCl_2$/mol casein) and ethanol, 99.8% v/v to a final concentration of 50% v/v. The resulting precipitate was collected by centrifugation at 12 000 × *g* for 10 min at 10°C, resolubilized in Milli-Q® water, subsequently freeze-dried and stored at –20°C until use.

2.3 Simulation of the in vitro static gastrointestinal digestion

A representative sample of cheese (1 g) and water were mixed (0.5%, p/v) and homogenized using an ULTRATURRAX T25 Basic S25N-18G (IKA® Werke, Germany). The SMP was reconstituted with water (10%) and homogenized. The simulated gastrointestinal digestions were carried out in triplicate by the method previously described [10]. The samples were dissolved (13 mg/mL of protein) in simulated gastric fluid (35 mM NaCl) at pH 2, preheated at 37°C. Porcine pepsin at an enzyme/substrate ratio of 1:20 w/w (182 units/mg) at 37°C during 1 h, in the presence of phosphatidylcholine (P3841; Sigma) vesicles was used. The in vitro duodenal digestion was carried out on the resulting product from stomach digestion adjusted to pH 7 by using 1M $CaCl_2$, 0.25 M Bis-Tris pH 6.5, and 0.125 M bile salts equimolar mixture of sodium taurocholate (Sigma) and glicodeoxycholic acid (Sigma). Trypsin (EC 232–650–8, Sigma; 40 units/mg protein), Chymotrypsin (EC 232–671–2, Sigma; 0.5 units/mg protein), porcine pancreatic lipase (EC 232–619–9, Sigma; 28.9 units/mg protein), and colipase (EC 259–490–12, Sigma; enzyme: substrate ratio 1:895 w/w) were diluted in 35 mM NaCl adjusted to pH 7 and added to the mixture. The reaction was stopped by adding Pefabloc® SC (Fluka 76307) at 1 mM final concentration.

2.4 SDS-page

Samples were diluted at a concentration of 2 mg/mL of protein in a buffer containing 2% w/v SDS, 62.5 mM Tris HCl pH 6.8, 10% v/v glycerol, 5% v/v β-mercaptoethanol were heated for 5 min at 95°C and loaded on 12% Bis-tris polyacrylamide gels (Criterion_XT, Bio-Rad, CA, USA). Electrophoretic separations were run at 150 V in a Criterion cell using XT-MES running buffer. Gels were stained with Coomassie Blue (BioSafe Coomassie G-250 Stain, Bio-Rad).

2.5 Analysis by reversed phase-HPLC-MS/MS

Reversed phase-HPLC-MS/MS analyses of WSE and digests from cheese and SMP samples were carried out as described by Sánchez-Rivera et al. [11]. The column used was Waters (XBridge™ BEH 300 C18 5 μm, 4.6 × 250 mm; Waters, MA, USA), the injection volume 50 μL, and the flow set at 0.8 mL/min. The peptides were eluted with a linear gradient from 0 to 45% of solvent B (acetonitrile/trifluoroacetic acid 0.027%) and 55% of solvent A (water/trifluoroacetic acid 0.037%) in 60 min. Two runs were performed per digestion triplicate in order to set up the method for different target mass: 600 *m/z* (permeate < 3 kDa, WSE and SMP) and 1200 *m/z* (WSE and SMP). Spectra were recorded over the mass/charge *m/z* range 100–3000.

In the case of samples enriched in CPPs, the analyses were carried out using a Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The injection volume was 50 μL and the flow rate 0.2 mL/min. A linear gradient from 0 to 45% of solvent B (acetonitrile/formic acid 0.1%) and 55% of solvent A (water/formic acid 0.1%) in 120 min was used. In these analyses, the target mass was set at 750 *m/z* and 1500 *m/z*.

Data processing was done by using Data Analysis™ (version 4.0; Bruker Daltoniks, Germany). The peptide sequencing was performed by MASCOT, using a homemade database that includes the cow's and goat's milk proteins and main genetic variants thereof. The matched MS/MS spectra were interpreted by using BioTools version 3.2 and the comparative peptidome analysis by means of the bioinformatics platform Protein Scape 3.0, both from Bruker Daltoniks. Peptides present in at least one replicate have been included in data sets.

3 Results and discussion

3.1 Electrophoretic profile of WSE of the cheeses and SMP

Figure 1A shows the SDS-PAGE separation of Valdeón cheese (batches D and A) and their gastrointestinal simulations in

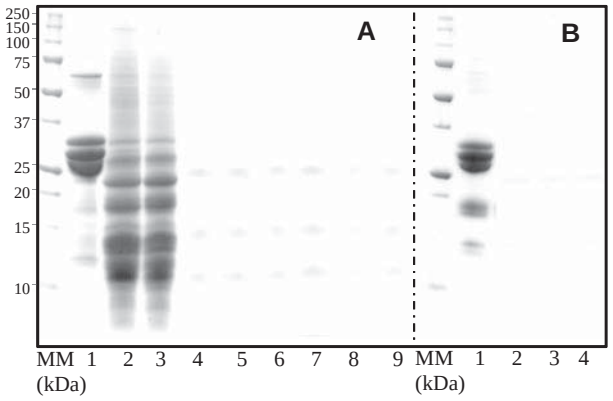

Figure 1. (A) Bis-Tris SDS-PAGE electrophoretic run of commercial casein (lane 1), WSE of Valdeón cheese batch D (lane 2), batch A (lane 3) and digests of cheese in triplicate from batch D (lanes 4–6) and batch A (lanes 7–9). (B) Bis-Tris SDS-PAGE electrophoretic run of SMP control sample before digestion (lane 1), digests of SMP in triplicate (lanes 2–4). MM = molecular marker.

triplicate. Figure 1B corresponds to PAGE-SDS of SMP digest in triplicate. Despite the extensive proteolysis that naturally occurs in Valdeón cheese, as a blue-mould cheese, there were still bands corresponding to caseins in the cheese before digestion (WSE). Similar protein patterns could be observed for both batches. After gastrointestinal digestion these bands were barely visible. The bands that remained detectable with MWs of approximately 24, 14, and 12 kDa were also found after the electrophoretic separation of the gastrointestinal fluid in a blank (i.e. digestion without sample, data not shown) and therefore, they could correspond to the enzymes employed or fragments thereof, since the MW of trypsin and chymotrypsin are 23.3 and 24 kDa, respectively.

3.2 Peptide profile of Valdeón cheese

The identified peptides (121) in the total WSE and the 3 kDa fraction of the two different cheese batches before digestion are listed in Supporting Information Table 1. Peptides identified in at least one batch have been included, since 75% of the peptides were found in both. Those peptides originating from the most abundant casein fractions (β- and α_{s1}-casein, from cow, and/or goat) dominated the cheese peptidome although a few peptides derived from α_{s2}-casein and κ-casein were also detected. The analysis of the 3 kDa permeate fraction allowed to narrow the identification of 64 peptides between 500–1000 Da, that contained less than 7 residues. The blue cheese undergoes extensive proteolysis due to the action of enzymes from different origins as mentioned before, with the main contribution from the mould culture, *Penicillium roqueforti*. This leads to the larger number of different peptides produced in blue cheese compared to semihard cheeses [12].

Figure 2 shows the peptides released from β- and α_{s1}-casein, where bovine and/or caprine origin is indicated. A distributed pattern of peptides was found within the β-casein sequence, covering most of its sequence (78% protein coverage) (Fig. 2A). The N-terminal part of the protein, where the phosphorylated serines are located, was source of a high amount of peptides, whose detection was enhanced by the selective precipitation employed. A total of 19 CPPs were identified from β-casein. Among these, 5 contained more than one phosphorylated serine and the other 13 were identified in the region comprised between the residues 30–46 of the β-casein sequence, and were monophosphorylated. From these, four arose from the 29–30 cleavage site (f30–36/37/39/42), which is characteristic of aspartyl-proteinases action, secreted by *P. roquefortii* [13, 14]. In accordance to our results, numerous phosphorylated peptides from the N-terminal region of β-casein had been also reported in Parmiggiano cheese [15]. Similarly, various peptides from the C-terminal region of this protein have been found in Manchego [4], Parmiggiano [15], and Cheddar cheeses [16], among others. Some other regions that gave rise to a notable number of peptides were the ones containing residues 60–70, 80–90, 110–120, 130–140, and the C-terminal part of the β-casein sequence. Some of the identified regions include previously reported biologically active sequences like opioid peptides: fragment (f) 60–66 (β-casomorphin 7) and f114–119 (neocasomorphin) [17] or angiotensin I-converting enzyme (ACE) inhibitors, like f47–52 (DKIHPF), which had been previously found in Manchego cheese [4, 18]. Other identified peptides, like β-casein f169–175 had been previously reported to show low in vitro ACE-inhibitory activity, whose half maximal inhibitory concentration ($IC_{50} > 1000$) was high, but it exerted a high antihypertensive activity in spontaneously hypertensive rats after oral administration [19].

The sequence coverage of α_{s1}-casein reached 52% (Fig. 2B). Among others, 17 CPPs were identified of which four of them arose from region 109–118 and were monophosphorylated. CPPs containing two or more phosphorylated residues were also found, as it is the case of f62–73. Likewise, 12 of the CPPs from α_{s1}-casein were diphosphorylated and belonged to the domain comprised between residues 39 and 55. In complex peptide mixtures the detection of phosphorylated forms is impaired, since the nonphosphorylated peptides are more easily ionized. To overcome this difficulty, instead of a selective precipitation, other authors have conducted a selective isolation on TiO_2 micro-columns when analyzing complex samples from milk proteins digestion [20]. On the other hand, the region comprising the residues from 83 to 91 was also source of a noteworthy number of peptides, 8 identified sequences. Similarly, in Manchego cheese, numerous peptides between residues 83 and 95 were found [4], while in Parmiggiano cheese, peptides α_{s1}-casein f80–114, f83–114, and f85–114 were identified [15]. Among the identified peptides from α_{s1}-casein, the sequence f157–164, with ACE-inhibitory activity (IC_{50} 98 μM), had been previously found in fermented casein with different lactic acid starters, and after hydrolysis with pepsin and trypsin [21].

Figure 2. Identified peptides in Valdeón cheese before digestion from (A) β-casein and (B) α_{S1}-casein. Peptides belonging exclusively to goat's milk are represented at the bottom, differing residues squared. Sequences in bold: peptides unique from cow's milk. *S*: phosphorylated serine.

Table 1. Identified peptides with reported bioactivity found before and/or after simulated digestion of Valdeón cheese

Fragment	Before digestion	After digestion	Activity
α_{s1}-Casein f(143–149)[a)]		AYFYPEL	Antihypertensive [34]/Mucin production [35]
α_{s1}-Casein f(144–149)[a)]		YFYPEL[c)]	Antioxidant [31, 33]/Mucin production [35]
α_{s1}-Casein f(157–164)[a) b)]	DAYPSGAW	DAYPSGAW[c)]	IACE [21]
β-Casein f(47–52)[a) b)]	DKIHPF	DKIHPF	IACE [4, 18]
β-Casein f(60–68)[a)]	YPFPGPIPN		IACE/Antihypertensive [48]
β-Casein f(98–105)[a)]		VKEAMAPK[c)]	IACE [32]
β-Casein f(108–113)[a) b)]		EMPFPK[c)]	IACE [21]
β-Casein f(114–119)[a) b)]	YPVEPF	YPVEPF[c)]	Opioid [17]
β-Casein f(133–138)[a) b)]		LHLPLP	Antihypertensive [29]
β-Casein f(133–139)[a) b)]		LHLPLPL[c)]	IACE [29]
β-Casein f(132–140)[a)]		NLHLPLPLL	IACE [30]
β-Casein f(130–140)[a)]		VENLHLPLPLL	IACE [30]
β-Casein f(169–175)[a) b)]	KVLPVPQ		Antihypertensive [19]
β-Casein f(193–198)[a)]/f(191–196)[b)]		YQEPVL	IACE [48]
κ-Casein f(18–24)[a)]		FSDKIAK	Antibacterial, IACE [37]

a) Peptide sequence is present only in cow's milk.
b) Peptide sequence is present in goat's milk.
c) Found also in skim milk powder digests.

3.3 Identification of peptides after simulation of gastrointestinal digestion of Valdeón cheese

A total of 139 peptides were found after Valdeón cheese digestion (Supporting Information Table 2). There was not a great difference in the number of peptides found before digestion (121 peptides), due to the proteolysis naturally occurring in this cheese [22–24]. However, a careful analysis, using a bioinformatics platform (ProteinScape), revealed some differences in the peptide profile before and after gastrointestinal digestion. A total of 28 peptides found in the digested cheese samples were already present before digestion that demonstrates their resistance to the gastrointestinal enzymes, and resulted in a total peptide homology of 12.1%. Indicative examples of resistant peptides to digestion from β-casein are GPFPIIV [f203–209 (cow's milk)/f201–207 (goat's milk)], YPVEPF f114–119, and DKIHPF f47–52. The last sequence has been often found after simulated digestion of different dairy products, including in vivo studies [8]. However, the two first peptides have been found only in cheese and not in simulated digests of other dairy products, which could indicate that they are generated by the fermentation process [4, 25, 26]. Regarding the α_{s1}-casein resistant peptides, the sequences f83–89 (KEDVPSE), f84–90 (EDVPSER), and f83–90 (KEDVPSER) survived digestion. These results are in agreement with previous reports that found several peptides comprised between residues 80–91 in casein subjected to simulated gastrointestinal digestion [20]. Moreover, α_{s1}-casein f80–88 (HIQKEDVPS), f80–89 (HIQKEDVPSE), f80–90 (HIQKEDVPSER), f80–91 (HIQKEDVPSERY), and f81–89 (IQKEDVPSE) have been identified in the jejunum of healthy humans who ingested casein [8]. Another resistant sequence corresponded to an ACE-inhibitory peptide, f157–164 DAYPSGAW, which had been also detected in an in vivo study of the evacuation of casein peptides in calf stomach [27].

In addition, new peptides formed during digestion were identified. An example of new sequences generated during digestion is the β-casein f133–138 (LHLPLP), a potent antihypertensive peptide [28], whose precursor peptide f128–138, was present in cheese before digestion (Table 1). Therefore, it is important to highlight that the active form of the antihypertensive peptide, which produced a decrease in the systolic blood pressure of spontaneously hypertensive rats of 25.3 mm of Hg [29], was released during cheese-simulated digestion but not during SMP digestion. Another new peptide arose from digestion, β-casein f132–140, NLHLPLPLL, had been previously identified in sodium caseinate hydrolysates produced by *Lb. helveticus* NCC2765, and described as ACE-inhibitor, showing an IC_{50} value of 15 μM [30]. Both peptides belong to a highly conserved β-casein region in different mammals, which has led to numerous and different combination of peptides that have been found in digests from human milk [31] and cows' milk proteins both in vitro [20, 26] and in vivo [8], by using different MS combinations: nanoLC-QTOF, HPLC-ion trap, or MALDI-TOF. Another new released sequence from β-casein was f98–105, EAMAPK, previously described as antioxidant [32]. Similarly, the β-casein f108–113, EMPFPK, to whom ACE-inhibitory activity has been attributed [21], was only identified after digestion (Table 1). Furthermore, this peptide has been also found in human effluents after ingestion of casein [8]. From the α_{s1}-casein sequence, peptides f144–149 (AYFYPEL) and f143–149 (YFYPEL) were generated during digestion. Both peptides have been previously found to exert antioxidant activity [31, 33], and mucin secretory activity in HT29-MTX cells [35]. In addition, the second one has demonstrated potent antihypertensive activity [34] (Table 1). Both peptides are known to be released after hydrolysis of casein with pepsin [36]. The sequence f143–149 (AYFYPEL) has been also found in human [7] and calf stomach [27] during in vivo

digestion studies. Although before simulated digestion, no peptides were found beyond position 164, after digestion at least 10 fragments from the C-terminal part of the protein were identified. The formation of these peptides might be produced by the action of gastric pepsin, since α_{s1}-casein f165–169 has been found in calf stomach after casein ingestion [27]. κ-Casein gave rise to newly released peptides during in vitro digestion, as for instance, f18–24. This peptide shows ACE-inhibitory activity (IC_{50} 113.6 μM) and antibacterial activity [37].

A total of 39 CPPs could be identified in cheese digests. The majority of these peptides (30) were monophosphorylated. Similar results were reported in digestion studies of Beaufort cheese [6] and casein [20]. From these identified CPPs, 16 arose from β-casein, 14 of which were monophosphorylated and belonged to the region corresponding to residues 30–46. Moreover, eight of these peptides survived digestion, since they were already present before digestion (f30–37/42, f32–37/42/43, and f33–37/42/43). Interestingly, the common trait of these eight peptides after digestion is the presence of ^{30}Ile ^{32}Lys, and ^{33}Phe at the N-terminal ending but the absence of ^{31}Glu. Moreover, the digestion process led to the formation of four additional peptides from this domain containing N-terminal Phe^{33} (f33–39/44/45/46), which can be generated by pepsin [38]. Some identified CPPs showed two or more phosphorylated residues. Among them, the peptides β-casein f7–25 and also α_{s2}-casein f5–15, displayed the cluster sequence SpSpSpEE, which could provide mineral-binding properties [39]. α_{s1}-Casein gave rise to 14 CPPs after digestion, seven of them being diphosphorylated, while the other seven were monophosphorylated, and belonged to the domains 40–55 and 108–119, respectively. No CPPs were found from α_{s1}-casein 60–70, where the phosphorylated cluster is located. Despite the sequence C- or N-terminal changes caused by the digestive enzymes activity, no change in the phosphorylated residues was observed for the identified CPPs upon cheese digestion.

3.4 Comparison of peptides released after simulation of gastrointestinal digestion of Valdeón cheese and skimmed milk powder

In order to assess if the same peptides can be generated with the ingestion of foods with a very different proteolytic state, the peptide profile of the digested cheese was compared with that found after gastrointestinal digestion of SMP (Supporting Information Table 3). The analysis revealed a difference in the number of peptides found in SMP (84 peptides) and cheese (122 peptides, leaving out those from goat origin). Taking into account that no undigested casein was found neither in Valdeón cheese nor SMP (Fig. 1), this difference was attributed to the high grade of proteolysis of Valdeón cheese. The proteolysis in cheese creates a large number of cleavage sites exposed to enzymes leading to the generation of a higher number of different peptides. Despite of this, good sequence coverage was reached in the peptide analysis of both, Valdeón cheese and SMP digests. In the case of β-casein, protein coverage was slightly higher in cheese (87%) than in milk powder (80.4%).

The exhaustive comparative analysis revealed differences in peptide identity between the SMP and cheese digests. From the casein fraction, 36 peptides were found to be common for both matrices, and a total peptide homology of 19% was achieved. β-Casein contributed the most to this issue, since 25 of the common peptides were generated from this protein. In contrast, as described above, the percentage of homology in cheese (before and after digestion) was 12.1%. The higher peptide homology between digested cheese and the SMP digest suggests that the digestion process reduces the differences in the peptidome of both matrices. Figures 3 and 4 show the peptides that have arisen from β-casein and α_{s1}-casein, respectively, after digestion of Valdeón cheese and SMP. In β-casein, various peptides belonging to the region 128–140 displayed C-terminal Leu residues, which could be due to the chymotrypsin activity in accordance to previous studies [40]. This domain seemed to be particularly resistant to hydrolysis during cheese ripening or digestion. For instance, the nonactive fragments f133–139 (LHLPLPL) and f134–139 (HLPLPL) were found in both digested matrices. Dupont et al. [41] also reported the resistance of this region and the presence of those two peptides after in vitro digestion of β-casein. Although the high number of peptides from this highly conserved region is remarkable, the potent antihypertensive peptide, LHLPLP f133–138 could not be found in milk digests. It seems that the presence of a certain precursor peptide is needed for the release of the active peptide during digestion [42]. However, recently this peptide was identified in the jejunum of humans who consumed casein [8]. It remains to be investigated if this peptide could be released in vivo in a sufficient amount to exert an antihypertensive effect, since in most clinical studies where milk or fermented milk is used as control, no antihypertensive effects were observed [43–45]. Similarly, bovine α_{s1}-casein f144–149 (YFYPEL), whose antioxidant activity has been cited above, was identified in SMP together with the related fragment 145–149 (FYPEL), but the antihypertensive sequence f143–149 (AYFYPEL) was not found. Nevertheless, the last sequence was found in human gastric samples after ingestion of milk or yogurt [7]. It would be of great interest to know the amount of these peptides after human digestion to correlate their activity with their presence in gastrointestinal effluents.

Other peptides that were found in both digests are the β-casein sequences VKEAMAPK f98–105, EMPFPK f108–113, reported as ACE-inhibitors [21, 33] and YPVEPF f114–119, reported as opioid [17]. The β-casein region 106–119, that gave rise to the latter peptides, together with the domains 60–93 and 133–140 seem to be very resistant to gastrointestinal digestion in both SMP and cheese, probably due to the presence of Pro in their sequences [46]. These results are in agreement with those found in casein fractions and β-casein digests [20, 41]. Moreover, the resistance of the phosphorylated regions of caseins to digestion has been proved in these

Figure 3. Identified peptides in Valdeón cheese (A) and SMP (B) from β-casein after digestion. Peptides belonging exclusively to goat's milk are represented at the bottom, differing residues squared. *S*: phosphorylated serine.

Figure 4. Identified peptides in Valdeón cheese (A) and SMP (B) arising from α_{S1}-casein after digestion. Peptides belonging exclusively to goat's milk are represented at the bottom, differing residues squared. *S*: phosphorylated serine.

samples. In Valdeón cheese, the β-casein region 30–46 appeared to be highly resistant, leading to the formation of 14 CPPs in digests. Picariello et al. [20] also reported the presence of numerous CPPs from this region and specifically found the sequences f33–42 and f33–43 after in vitro digestion of casein. In SMP, a total of 18 CPPs were identified. Ten of them were found in β-casein, only three of those being monophosphorylated. However, the phosphorylated region 5–25 appeared to be more resistant to digestion, giving rise to seven peptides, among them, two sequences containing the phosphoseryl cluster, i.e. f12–25 and f6–25. The phosphoseryl cluster was conserved in both SMP f12–25, f6–25, and cheese f7–25, as a common trait resulting from digestion. This is consistent with the presence of β-casein f7–25, f2–25, and f1–24 in lumen contents of rats perfused with β-casein f1–25 [47]. This behavior could help to elucidate and better understand the cleavage patterns and breakdown of milk proteins and peptide release during the gastrointestinal process in the small intestine.

4 Concluding remarks

The peptide profile of Valdeón cheese showed the presence of some ACE inhibitory and opioid peptides. After gastrointestinal simulation, a higher number of bioactive peptides, including antihypertensive, antioxidant, intestinal mucin-secretor, and antibacterial were found. However, not all these sequences could be detected after digestion of SMP. The exceptions could be due to peptide precursor differences that result in a distinct peptide profile after digestion. On the other hand, the peptidomic profiling of digests reveals several regions that are especially resistant to gastrointestinal digestion in SMP and cheese, mainly in β-casein 60–93, 128–140, and 193–209 but also in β-casein 30–46, and α_{s1}-casein 40–55 and 109–118, where the phosphoserines are located. Indeed, no changes in the phorphorylated residues were observed after simulated digestion of cheese. Some of these resistant regions are well conserved between species in dairy proteins. Interestingly, numerous sequences with reported biological activity belong to them. The higher peptide homology between digested cheese and the SMP digest compared to that found in cheese before and after digestion, confirms that the digestion process could bring closer the profile of certain regions, even if they have different grade of proteolysis before digestion. The remaining differences in the cleavage pattern during digestion could also have other health implications, like metabolic or toxicological ones (exposure of potential epitopes). Kinetics of peptide release could provide further information in this sense.

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2.4 Chapter IV

(Manuscript)

Peptide mapping during dynamic gastric digestion of heated and unheated skimmed milk powder

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ABSTRACT

This study aims at the evaluation of the impact of heat treatment on the hydrolysis kinetics of milk proteins and on the peptide release during dynamic gastric digestion. SDS-PAGE and ELISA techniques were employed to assess the hydrolysis of proteins over time of digestion. The evolution of the peptidome generated through dynamic digestion of heated and non-heated milk, was studied at different times, by using MS-based techniques (ion trap and MALDI-TOF/TOF) preceded by liquid chromatography. The homology values between samples, point out the importance of the exposure time to enzyme on the identity of peptides, causing an increase of 14% in peptide homology from 50 min to the end of digestion. Peptide homology at the end of digestion (48%), confirmed the impact of heat treatment on the identity of peptides generated from the two milk samples during digestion, despite their identical initial protein content and being the same matrix in both cases. Heat treatment produced an increase of the resistance in the casein fraction. However, β-Lg was found to be more susceptible to hydrolysis. Although differences on the pattern of peptide release were found between both samples, also some common traits after digestion were found. Resistant regions could be confirmed through the analyses, such as the region comprised between the residues 76-93 of β-casein, where several binding epitopes are included. Also, β-casein regions 126-140 and 190-209 were found to be resistant.

Keywords: dynamic digestion / Peptidomics/ mass spectrometry/ heat treatment

1. Introduction

The study of digestion process has gained importance in the last years, since the release of products through this physiological event may have subsequent health implications. The evaluation of the peptides generated during digestion may be relevant to understand this process itself, in respect of protein behavior upon digestion, but also for nutritional, pharmacological and toxicological concerns. The formation of peptides derived from milk proteins digestion has been recently reviewed (Sanchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014a), pointing out the importance of MS-based techniques on the study of digestion process, applied to either targeted or untargeted products. Several *in vitro* static digestion models have been developed and widely used over the years (Guerra et al., 2012; Hur, Lim, Decker, & McClemens, 2011; Kopf-Bolanz et al., 2012). These models intended to mimic physiological conditions, however, they lack of mechanisms to control the sequential secretion of enzymes, the removal of digestion products, the appropriate mixing and the continuous changes in pH. Therefore, dynamic digestion models, either monocompartmental or bi- and multicompartmental ones, have been proposed to be more realistic with respect to physiological parameters (Guerra et al., 2012; Ménard et al., 2014; Wickham, Faulks, & Mills, 2009). Gastric phase plays an important role in digestion, since the peptides produced by pepsin are delivered to small intestine, where they can either interact in situ with gastrointestinal receptors or be absorbed, mainly in duodenum and upper jejunum (Shimizu et al., 2010; Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011).

However, it is increasingly evident that food processing can influence the behavior of proteins towards digestion, and therefore it may affect the release of

peptides. Milk products are often thermally-treated in food industry in order to enlarge their shelf-life, or to modify their functional properties (Guo, Fox, Flynn, & Kindstedt, 1996). Food processing has an impact on structural and nutritional characteristics of proteins that could result in a loss of amino acid bioavailability (Guo, Flynn, & fox, 1999; Finot, Deutsch, & Bujard, 1981). The modifications that proteins usually undergo through these technological processes include unfolding and protein aggregation. The formation of heat-induced aggregates of casein and whey protein has been reported (Patel, Singh, Anema, Creamer, 2006; Jean, Renan, Famelart, Guyomarc'h, 2006). Likewise, modifications on β-Lactoglobulin (β-Lg) structure upon heat treatment have been evaluated under different conditions, for instance pH, ionic strength, protein concentration (Loveday, Wang, Rao, Anema & Singh, 2012; Aymard, Durand & Nicolai, 1996; Iametti, Cairoli, Gragory & Bonomi, 1995). Nevertheless the extent to which proteins are altered during food processing depends on the type of process (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). These changes may have further health implications, for instance, a distinct effect on the allergenic response (Shandilya, Kapila, Haq, Kapila & Kansal, 2013), and also on the formation of bioactive peptides through digestion (Meisel et al., 1998). The heat-induced changes that food proteins undergo have been reported to influence their stability to *in vitro* static digestion of bovine and caprine milk (Almaas et al., 2006), of infant formula (Dupont, Boutrou, Ménard, Jardin, Tanguy, et al., 2010a), or even influence the kinetics of protein digestion in mini-pigs (Barbé et al., 2013). Although it is reported that β-Lg is resistant to pepsin digestion (Miranda, & Pelissier, 1983), the increase of β-Lg susceptibility to hydrolysis by this enzyme has been studied under different conditions, such as heat treatment at low pH, presence of alcohols, esterification

and heating under high pressure (Bateman, Ye, & Singh, 2010; Dalgalarrondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995; Chobert, Briand, Grinberg, & Haertle, 1995; Zeece, Huppert, & Kelly, 2008). In this regard, the study of the peptidomic profile generated through dynamic digestion of milk products subjected to different heat treatments can help to understand the protein behavior under gastric digestion. Therefore, the aim of this work is to evaluate the impact of heat treatment on the hydrolysis kinetics of milk proteins and the distinct effect of this process on the evolution of the resulting peptidome generated at different times of gastric dynamic digestion, using a combination of two mass spectrometry analyzers (Ion trap and MALDI TOF/TOF).

3. Materials y methods

3.1 Samples

Two milk samples (unheated and heat treated skim milk) were used for the experiments. They were prepared using a skimmed milk powder as described by Barbé et al. (2013). The powder was reconstituted in distilled water to reach a final concentration of 50 g/L of protein. Heat treatment (90ºC, 10 min) was applied to the reconstituted liquid to obtain the heat treated milk sample. The unheated milk did not undergo any treatment after reconstitution of the powder. For digestion process, 200 mL of each sample (unheated and heated milk) were used.

3.2 Dynamic digestion

A dynamic digester available at STLO (INRA Rennes, France) was used to perform digestions on the two samples (unheated and heated milk) in triplicate. The digester was previously described by Ménard et al. (2014). The half–time of gastric emptying used in the present study was set at 191 and 283 min for unheated and heated milk, respectively, as estimated by Barbé et al. (2013) during a digestion study conducted in mini-pigs fed these milks. The beta (β) parameter for the Elashoff equation that controlled the gastric emptying was set at 0.8. The pH curve followed during digestions was controlled by the software (Ménard et al., 2014) using the pH data previously reported (Minekus, Marteau, Havenaar, & Huis in Veld, 1995). The enzyme used to perform digestions was porcine pepsin (P-6887, Sigma), at 1000 Units/mL (Chiang, Croom, Chuang, Chiou, & Yu, 2008) of simulated gastric fluid (SGF) (NaCl 150mM). The flow of SGF into the gastric compartment was set at 0.5 mL/min as previously described (Minekus et al., 1995). The sampling was done at 4, 10, 20, 50, 105, 165, 225, 315 and 405 min.

3.3 SDS-PAGE:

SDS-PAGE analyses were performed using 4-12% Bis-Tris polyacrilamide precast gels (1.5 mm x 15 wells; NuPAGE Novex, Invitrogen). The triplicates of digestion at different times, mentioned above, were included in electrophoretic analyses. In addition, each milk sample before digestion was also analyzed in each SDS-PAGE run. These analyses were carried out as described by Bouzerzour et al. (2012). The molecular marker used for the experiments was Mark 12 Unstained Standard NuPAGE 4-12% (Invitrogen). The image analyses

of the gels were performed using Image scanner III (GE Health-care Europe GbmH, Velizy-Villacoublay, France). Densitometry analyses of the gel images were carried out using. The relative quantification of the β-lactoglobulin (β-Lg) through digestion process was performed by measuring the colored area volume of the band on the SDS-PAGE gel image. Both milk samples before digestion, included in each SDS-PAGE experiment, were used to establish the initial amount of protein. The volume of their colored area was measured and considered as 100%. The hydrolysis of this protein through digestion was estimated by referring the colored areas at different digestion times to that of the initial amount.

3.4 ELISA

Inhibition ELISA was performed on the triplicates of digestion from each sample (unheated and heat treated milk) at different times of digestion (0, 4, 10, 20, 50, 105, 165, 205, 315 and 405 min). ELISA was carried out as previously described (Dupont, Mandalari, Molle, Jardin, Role-Répécaud, et al., 2010b) using caseins-specific polyclonal antibodies to estimate the residual immunoreactivity of this protein during digestion process. Samples were first homogenized with a thurrax (Ultra Thurrax T8 IKA, Fischer Scientific, 20,000 tr/min-5 min). Each digestion time, mentioned above, was analyzed in triplicate.

3.5 LC-MS/MS-based analysis by ion trap and MALDI TOF/TOF

The triplicates of digestion from unheated and heated milk were individually analyzed by the two MS-based techniques: RP-HPLC-ion trap and nanoLC-MALDI-TOF/TOF at three digestion times (4, 50 and 405 min).

The RP-HPLC-MS/MS analyses of samples were carried out on a HPLC-ion trap described by Sanchez-Rivera Recio, Ramos, and Gómez-Ruiz (2013). The column used for the experiments was a Mediterranea Sea18 15 cm × 0.21 cm (Teknocroma, Barcelona, Spain). The voltage ramp went from 0.35 V up to 1.4 V for the fragmentation of precursor ions and an isolation width of 4.0 *m/z*. The samples were eluted at 0.2 mL/min. A linear gradient was used from 0 to 45% of solvent B (trifluoroacetic acid-TFA 0.027% in acetonitrile) and 55% of solvent A (TFA 0.037% in water) in 120 min.

The samples were also analyzed by a nano-LC (Easy-nLC II; Bruker Daltonik, GmbH Bremen, Germany) coupled to a fraction collector (Proteineer fc II; Bruker Daltonik) and finally by MALDI-TOF/TOF (Autoflex speed; Bruker Daltonik). The column used was a pepMap 100 column, C18 3µm of particle size, 75 µm x 15 cm (Dionex Acclaim pepMap 100; Thermo Scientific). The elution of peptides was carried out with a linear gradient from 0 to 40% of solvent A (water/formic acid 0.1%) and 60% of solvent B (acetonitrile/formic acid 0.1%) in 90 min. The flow was set at 300 nL/min and the injection volume was 18 µL. The fraction collector gathered the sample from the nano-LC and deposed a drop every 15 sec on a prespotted Anchorchip α-Cyano-4-hydroxycinnamic acid (HCCA) plate (set for proteomics II; PAC-II 384 well plate; Bruker Daltonics). Later on, the plate was washed with 10 mM monohydrated ammonium phosphate in aqueous solution at 0.1% of TFA, to perform the MS/MS analyses off-line using MALDI-TOF-TOF. The mass range was set from 500-3500 m/z using an exclusion list containing the peaks from the matrix to avoid interferences.

Data processing was performed by using Data AnalysisTM (version 4.0; Bruker Daltoniks). The peptide sequencing was done by MASCOT, using a homemade database; and also by BioTools (version 3.2), using both, complete homemade database and individual homemade databases including all the milk proteins with the main genetic variants, by multiple protein search. The matched MS/MS spectra were interpreted by using BioTools (3.2). The comparison of the peptidomes between the samples and the evolution of the peptide profile over time was carried out by the bioinformatic platform ProteinScape (version 3.0), both from Bruker Daltoniks.

3.6 Statistical analyses

Two-way analysis of variance (ANOVA) was applied to ELISA and densitometry data using Graphpad Prism 5.0 software. The effect of time and the heat treatment on the hydrolysis of proteins were evaluated. The mean values are presented with the standard error of mean (SEM), and the effects were found significant at $p \leq 0.001$. Data were expressed as the percentage (%) of remaining protein over time of digestion with regard to the initial value (before digestion).

4. Results and discussion

4.1 *Hydrolysis of milk proteins by the action of pepsin*

Figure 1 shows the electrophoretic profile of unheated and heat treated milk at different times of digestion (0, 4, 10, 20, 50, 105, 165, 205, 315 and 405 min). In the case of the unheated milk (Figure 1A), it is observed that the casein fraction (32 kDa) is rapidly hydrolyzed compared to the heated one. At 4 min, the electrophoretic bands that corresponded to casein showed a drastic decrease in

the non-heated sample compared to the heated one, and at 10 min of digestion the casein bands are barely visible in unheated milk. This rapid degradation of casein by the action of pepsin is also in accordance with other results reported after gastric digestion of caseins (Picariello et al., 2010), or β-casein using adult and infant *in vitro* digestion models (Dupont, Mandalari, Molle, Jardin, Léonil, et al., 2010c) and of raw milk (Dupont et al., 2010b). However, in heated milk, the bands corresponding to the casein fraction still remained visible up to 50 min of digestion time. This suggests that food processing, such as heat treatment, has an influence on the kinetics of protein hydrolysis, in accordance to other studies (Almaas et al., 2006).

As it can be observed in Figure 1A, the bands corresponding to β-Lg (18 kDa) were visible until the end point of gastric digestion in unheated milk, although a slight decrease of the intact protein was observed at 315 and 405 min. Similar results were found by other authors after the gastric digestion of whey proteins (Picariello et al., 2010) or β-Lg (Dupont et al., 2010b). However, in the heated milk, at 50 min, the action of pepsin caused a pronounced decrease in the band corresponding to β-Lg (Figure 1B). This protein was completely degraded during gastric digestion. Although this protein has been reported to be resistant to the action of pepsin under gastric conditions (Miranda & Pelissier, 1983), it has also been shown that certain conditions such as heat treatment and the application of heat either at low pH or under high pressure can make the protein more susceptible to digestion (Peram, Loveday Ye, & Singh, 2013; Bateman et al., 2010; Zeece et al., 2008). It has to be taken into account, that under our conditions, a faster gastric emptying was established for unheated versus heated milk and therefore, the protein was exposed to the action of the enzyme shorter

time in the case of the non-heated milk. The remaining amount of β-Lg was estimated by using densitometry (figure 2A). At 105 min of digestion 70 % β-Lg remained in unheated milk while in heat treated milk only 35% of β-Lg was detected at the same time.

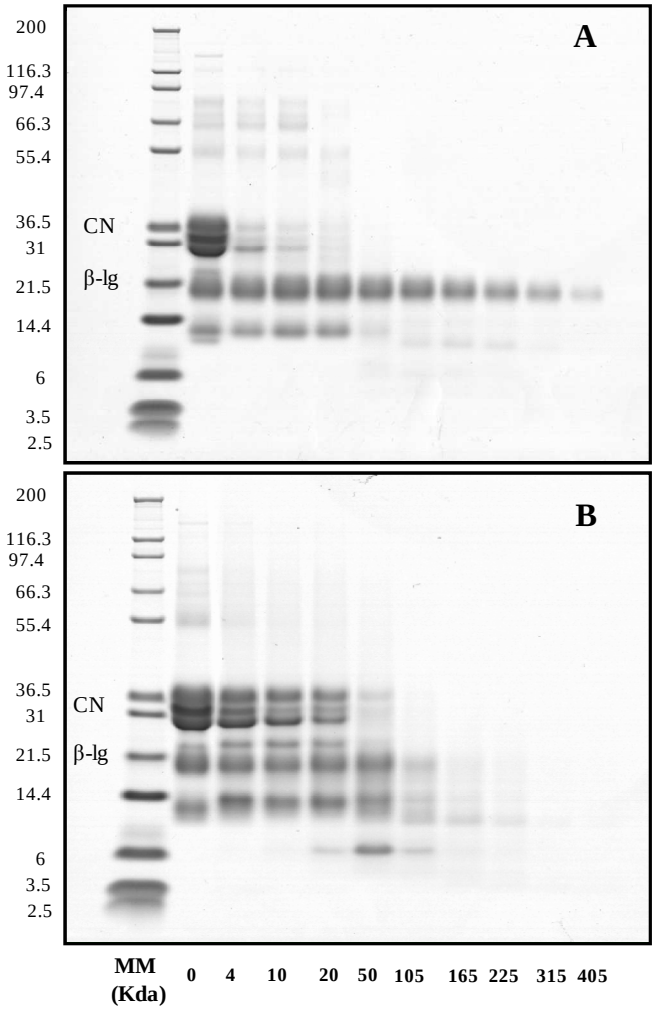

Figure 1. SDS-PAGE electrophoretic run of unheated (1A) and heat treated milk (1B). Lane numbers represent the time of digestion (min). MM= molecular marker.

Hydrolysis of milk proteins was also followed by ELISA using caseins-specific polyclonal antibodies (figure 2B). Again, it was confirmed the difference on caseins hydrolysis kinetics between heated and non-heated milk. These differences were more evident at early digestion times. After 4 min digestion, the

gastric digestion had caused an 80% decrease of the signal for casein in unheated milk while the decrease was just of 8% in heat treated milk. The resistance of casein to the action of pepsin in heated milk may be explained by the formation of aggregates of caseins with whey proteins during heat treatment of milk (Patel et al., 2006; Jean et al., 2006; Guyomarc'h, Law, & Dalgleish, 2003).

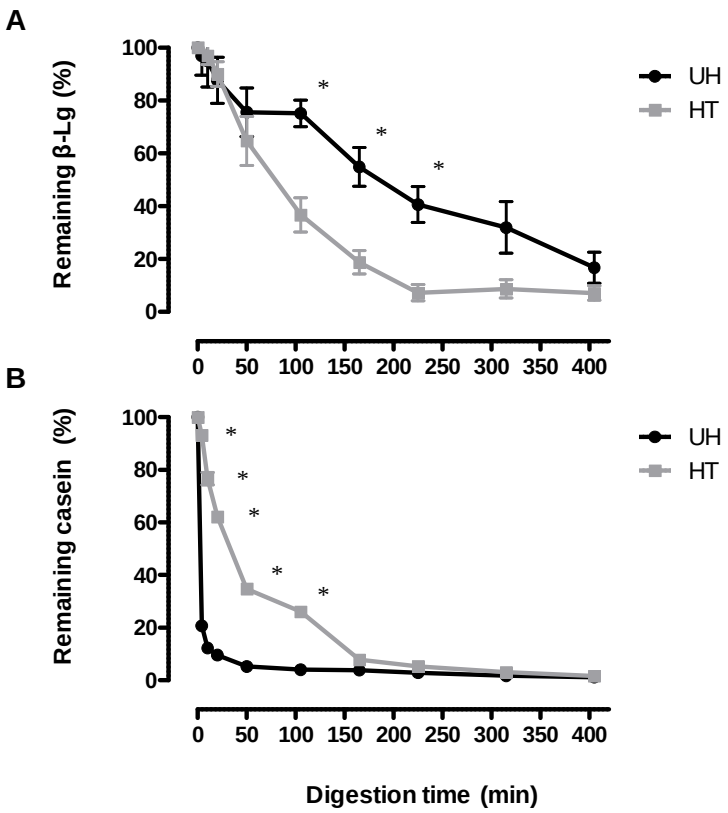

Figure 2. Evolution of β-Lg (2A) and caseins (2B) over time of dynamic gastric digestion. Mean values ± SEM (n=3) are expressed as the percentage (%) of the remaining protein in respect of the initial value (before digestion). Significant differences between UH (unheated milk) and HT (heat treated milk) at the same time of digestion were found at p≤0.001 (*).

4.2 *Evolution of peptidomic profile of digests*

Overall, sequence coverage was found to increase over time of digestion, as more peptides could be identified, since they are gradually smaller and fit better to the selected target mass during the analyses. At the end point of digestion the sequence coverage reached for the major proteins was 80% and 76% for β-casein and α_{S1}-casein, respectively, in the case of heated milk; and 83% and 83% for β-casein and α_{S1}-casein, respectively, in the case of unheated milk. In general, more peptides were identified from the casein fraction in unheated milk compared to the heat treated one, and also a better sequence coverage was reached in the first case, suggesting that the heat treatment could influence the release and/or peptide identification under the same analyses conditions. Partly, this could be due to different phenomena that occur during the heat treatment, i.e., lactosylation, glycosylation, as well as protein-protein interaction, that represent an additional difficulty for peptide sequencing by MS.

A study of peptide homology was carried out in digestion samples from heated and non-heated milk. These results show that the peptide homology between samples increased with time of digestion. The total peptide homology in samples at 4 and 50 min was found to be 34%. However, at 405 min this value increased up to 48%. These results point out the difference in the peptide release in both samples at early times of digestion (4 min and 50 min); and suggest that digestion process could bring closer the peptidome of the resulting products in both samples after 50 min, since at this point, the pattern of peptide release started to be similar in heated and non-heated milk. Nevertheless, the peptide homology of the two samples only reached 48% at the end of the gastric

digestion, in spite of being the same matrices, with the same initial protein content and composition.

Figure 3 shows the peptides from β-casein identified in non-heated milk (3A) and heated milk (3B) at 4, 50 and 405 min of digestion. At 4 min, in both samples, most of the peptides belonged to the C-terminal region comprised between residues 190-209, suggesting that the hydrolysis starts at this part of the protein. Peptides from this region were more abundant in unheated milk (14 peptides) compared to the heated sample (seven peptides), although some of these were common in both samples. Among them, f193-209 (YQEPVLGPVRGPFPIIV), was also found in the stomach contents of calves (Yvon & Pelissier, 1987). In addition, f191-209, which was found only heated milk, was recently reported to show moderate IgE binding (Benedé et al., 2014). On the contrary, sequence f1-11 (RELEELNVPGE) was only found in unheated milk. Interestingly, the same sequence with Ile at C-terminal end, f1-12 (RELEELNVPGEI) was found in the stomach of humans after milk ingestion (Chabance et al., 1998). Moreover, three large fragments were identified in heated milk containing 20, 21 and 22 residues (f143-163, f91-112, f141-163, respectively), whereas in the unheated one, smaller peptides were found from these regions, i.e., f143-156 and f94-105. The latter sequence (GVSKVKEAMAPK) was also identified in stomach of humans after 20 min of yogurt ingestion (Chabance et al., 1998). Moreover, the region 125-142 gave rise to several peptides in non-heated milk (five peptides), while in the heated treated sample only one was detected. No peptides from N-terminal phosphorylated region of the protein were identified. It is known that in complex samples detection of phosphorylated peptides detection is impaired by the presence of the

non-phosphorylated ones (Picariello et al., 2010). At 50 min, the C-terminal part of the protein still dominated the peptidome. Among them, f193-209 could still be found in both samples, accordingly to other studies (Yvon & Pelissier, 1987), and also the IgE binding peptide f191-209. Picariello et al. (2010) reported high signal in peptides generated from this region after gastrointestinal digestion of caseins. However, also other regions containing residues 1-14, 45-58 and 75-107 gave rise to several peptides in unheated milk digests (with 3, 6, 12 peptides, respectively). Among these sequences, f1-11 remained intact (present before digestion). In addition, 11 peptides from region 128-140 were identified i.e DVENLHLPLPLL, VENLHLPLPLL, NLHLPLPLL, LHLPLPLL among others. With respect to these domains (45-58, 75-107 and 128-140), similar patterns were observed in heated milk, although it was noteworthy the absence of peptides from region comprised between residues 1-14. Also, another exception was found, the f59-80 (VYPFPGPIHNSLPQNIPPLTQT) was only present in the heat treated sample, suggesting that this region in unheated milk might not have been released or, more likely, it could have been already digested into smaller sequences. At the end of digestion (405 min) a total of 68 peptides were identified in unheated and 60 in heated milk. The C-terminal part of the protein was not the main source of peptides. Although the pattern of peptide release at this point was similar in both samples, some differences were found. The N-terminal part of the protein, gave rise to several new peptides in heated milk samples which had not been found at earlier times of digestion (f1-5, f1-6, f1-11, f6-11). Among them, f1-6 (RELEEL) was found in humans effluents after ingestion of caseins (Boutrou et al., 2013). By the contrary, in unheated milk, this part of the protein was hydrolyzed into several peptides, and, at 405 min, only f1-

5 (RELEE) and f6-11 (LNVPGE) could be identified, noting the absence of f1-11. This suggests that the N-terminal part of the protein could be faster hydrolyzed in non-heated compared to heated milk. Moreover, numerous peptides from C-terminal part of the protein could be still identified in both, non-heated (16 peptides) and heated milk (17 peptides), among them f193-209, which was identified at all digestion times.

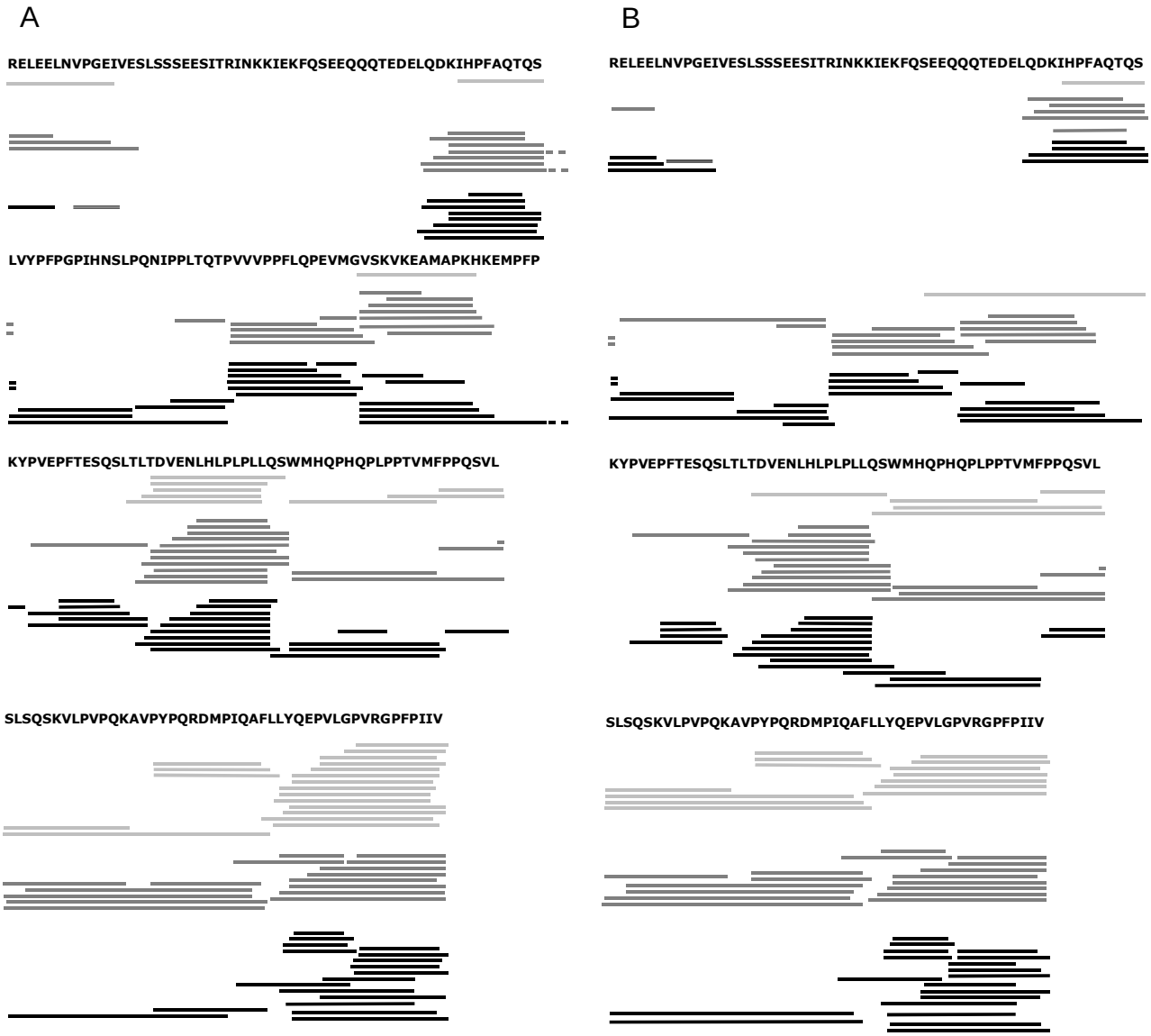

Figure 3. Peptidomic profile from β-casein after dynamic digestion of unheated (3A) and heat treated milk (3B). The lines in light gray indicate 4 min of digestion, dark gray lines 50 min, and black lines the end point of digestion (405 min).

Nevertheless, other regions also seemed to be resistant. As an example of this, two sequences f164-189, SLSQSKVLPVPQKAVPYPQRDMPIQA (2876 Da) and f164-188, SLSQSKVLPVPQKAVPYPQRDMPIQ (2805 Da), containing 34 and 33 residues, respectively, remained undigested in heated samples. This is also the case of region 76-93, whose hydrophobicity at pH 3 may have influenced the resistance of this area to pepsinolysis (Dupont et al., 2010c). Interestingly, this was also reported as a resistant region after digestion of raw, pasteurized, sterilized milks and yogurt using infant digestion model (Dupont et al., 2010b). Likewise, many peptides from this domain were identified in piglets after ingestion of infant formula (Bouzerzour et al., 2012). Moreover, similar results were reported after gastrointestinal digestion of other dairy products, such as blue cheese (Sánchez-Rivera, Diezhandino, Gómez-Ruiz, Fresno, Miralles, et al., 2014b). Similarly, peptides from region 128-140 were highly abundant in both unheated and heated milk, showing a common trait in both cases. All of these sequences except one displayed Leu^{140} at C-terminal position. Recently, NLHLPLPLL f132-140, was identified in effluents of humans after casein ingestion (Boutrou et al., 2013). Probably, the resistance of these regions (76-93 and 128-140) was also determined by the high content of proline residues in both cases (Vanhoof, Goossens, De Meester, Hendriks, & Scharpe, 1995; Hausch, Shan, Santiago, Gray, & Khosla, 2002).

Figure 4 shows the identified peptides from α_{S1}-casein in unheated (4A) and heat treated milk (4B) at different times of digestion (4, 50 and 405 min). The hydrolysis of this protein started from the N-terminal part of the protein (region 1-39), and therefore determined the peptide release at early times of digestion. Large fragments from this part of the protein were abundant at 4 min of digestion

in both samples. Nevertheless, the sequences present in heated milk (i.e f1-14/16/18/20/21/23, f3-20, f12/17/19-23) were larger than those found in the unheated one (i.e f1-9/12/14/16/18/20, 15/16/17/19-23). The antibacterial peptide Isracidin, f1-23 (RPKHPIKHQGLPQEVLNENLLRF) (table 1), was also found in the stomach of calves after ingestion of casein and skimmed milk (Yvon & Pelissier, 1987). Several sequences containing N-terminal Phe_{24} (five peptides) were found in both digests from heated and non-heated milk, among them, f24-34 (FVAPFPEVFGK), being already present before digestion in both samples (data not shown). The presence of this peptide before digestion could be attributed to plasmin, since α_{S1}-casein is susceptible to its action (McSweeney, Olson, Fox, Healy, & Hojurp, 1993), and concretely peptide bond Lys^{34}-Glu^{35} was one of its specific sites of cleavage (LeBars, & Gripon, 1993). In both milk samples, the release of N-terminal peptides during digestion occurred from regions 1-23 and 24-39. This confirms the high susceptibility of the bond Phe^{23}-Phe^{24}. No peptides were found from region 114-164, which is the phosphorylated domain of this protein. At the end point of digestion (405 min), the identification of peptides through the whole protein sequence was more homogeneous. Peptides from region 56-62, were identified for the first time in 405 min in unheated and heat treated milk. Likewise, new peptides were identified from domain 121-143 only at the end point of digestion. In addition, the number of peptides identified in non-heated samples from regions 80-190 and 180-199 was twice those found in heated milk. Among them, f180-191 only found in unheated milk, and was also detected in the stomach of humans after the ingestion of yogurt (Chabance et al., 1998).

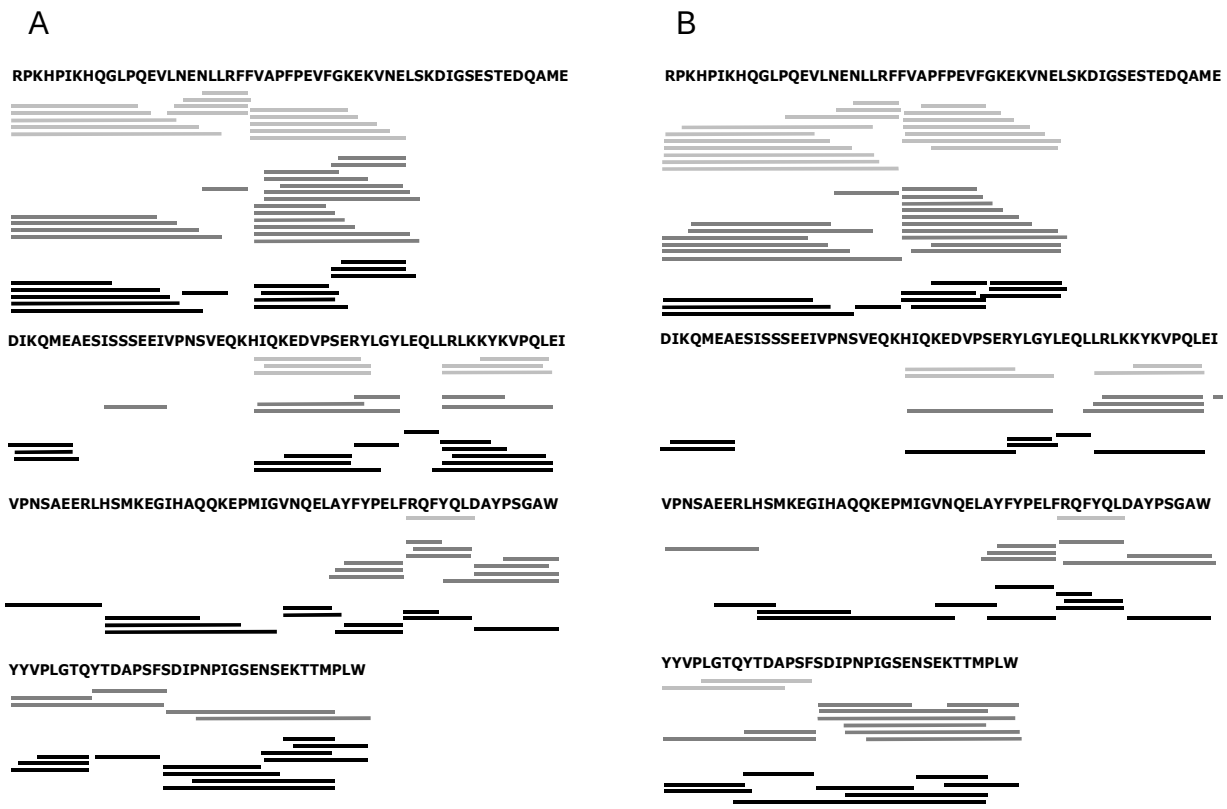

Figure 4. Peptidomic profile from α_{s1}-casein after dynamic digestion of unheated (3A) and heat treated milk (3B). The lines in light gray indicate 4 min of digestion, dark gray lines 50 min, and black lines the end point of digestion (405 min).

Peptides from α_{S2}-casein and κ-casein were identified in heat treated and non-heated milk digests at different times of digestion. The sequences identified from this protein represented a 14% and a 16%, respectively, of the total peptides, since these proteins are less abundant than β-casein and α_{S1}-casein. It has been also proposed a higher resistance of α_{S2}-casein and κ-casein to *in vitro* digestion compared to other caseins (Dupont et al., 2010a). Main differences between heated and non-heated samples at the end of the digestion were found. For instance, in heat treated milk, α_{S2}-casein gave rise to five sequences from region 99-123, two of them had 23 (2718 Da) and 24 residues (2831 Da) but no

peptides from this region were detectable in the non-heated samples. This suggests that in unheated milk this region could have been hydrolyzed into smaller sequences, since peptides from that region had been identified at 50 min. Moreover, in unheated milk, two peptides from region 151-163 (TKLTEEEKNRLNF and EEKNRLNF) were found. Interestingly, only one peptide was found from C-terminal part of the protein in non-heated milk, whereas four sequences were detected in heat treated samples, two of them f183-206 and f183-207 contained 23 and 24 residues, respectively. Both peptides showed antibacterial activity (Table 1) (Recio, & visser, 1999).

Among the κ-casein sequences identified at the end point (405 min), six peptides from C-terminal region (139-169) could be found in heat treated milk and five in the unheated one. These results and the presence of f106-169 at 4 and 50 min, point out the resistance of this area to digestion, in agreement with other reported results after casein digestion (Dupont et al., 2010a).

No peptides from β-Lg were found at 4 min of digestion nor in heated neither in unheated milk. In the present work, this protein was found to be rapidly hydrolyzed in heated milk, and be resistant in the unheated one. After 405 min, several peptides from region 75-82 were found in heated, but also in non-heated milk, in agreement with the slight decrease of the intact protein observed at 315 min (Figure 1A). In addition, peptides from the domain comprised between residues 95-149 were highly abundant at this point in both samples (seven and nine peptides in heat treated and unheated milk, respectively). Among these sequences identified in unheated milk, it is worthy to highlight that 4 of them (f123-130/131/132/133), were included in the region 125-135, which was reported to be especially resistant to digestion (Picariello et al., 2010).

To summarize, Table 1 shows the identified peptides physiologically relevant either concerning their potential bioactivity, their previous *in vivo* detection after dairy products ingestion or with allergenic implications as previously reported IgE epitopes. Different and common peptides could be observed in the two samples after digestion. Nevertheless, despite their similarities found in the sequence identity, Table 1 also shows their differences in regard to their time of release. In addition, the MS-based techniques, besides the identification of the peptides, allowed the observation of their behavior in terms of intensity (relative abundance) during digestion. Some differences in this sense were noticed between the two samples. For instance, several biologically active sequences from α_{s1}-casein as DAYPSGAW (f157-164), YFYPEL (f144-149), and AYFYPEL (f143-149), among others, were found to be common for both samples, however, they showed differences in intensity between unheated and heated milk (data not shown).

Table 1. Identified peptides after gastric digestion of heated and unheat treated milk with previously reported bioactivity, and the time at which they were identified in each sample. Their presence in vivo in the gastrointestinal track within effluents gathered from humans or animals has been indicated.

Sequence	Protein fragment	Sample	In vivo	Described activity	Reference
AYFYPEL	α_{s1}-CN f143-149	HT (50, 405); UH (50, 405)	Human[1]; Calf[3]	Antihypertensive	*Contreras, Carrón, Montero, Ramos, & Recio, 2009*
DAYPSGAW	α_{s1}-CN f157-164	HT (50, 405); UH (50, 405)	Calf[3]	ACE- inhibitor	*Pihlanto-Leppälä, Rokka, & Korhonen, 1998*
NLHLPLPLL	β-CN f132-140	HT (50, 405); UH (50, 405)	Human[2]	ACE- inhibitor	*Robert, Razaname, Mutter & Juillerat, 2004*
RPKHPIKHQGLPQEVLNENLLRF	α_{s1}-CN f1-23	HT (4, 50)	Calf[3]	Antibacterial	*Lahov, & Regelson, 1996*
YFYPEL	α_{s1}-CN f144-149	HT (50, 405); UH(50, 405)	Human[1]	Antioxidant ; MSE	*Suetsuna, Ukeda, & Och, 2000; Martínez-Maqueda, Miralles, Ramos, & Recio, 2013.*
RYLGY	α_{s1}-CN f90-94	HT (405); UH (50, 405)		Antihypertensive	*Contreras et al., 2009*
RYLGYL	α_{s1}-CN f90-95	HT (405)		Opioid	*Loukas, Varoucha, Zioidrou, Streaty, & Klee, 1983*
VAPFPEVF	α_{s1}-CN f25-32	HT (405); UH (50, 405)	Human[1 ; 2]	ACE- inhibitor	*Contreras et al., 2009*
FVAPFPEVF	α_{S1}-CN f24-32	HT (50, 405); UH (50, 405)	Human[2]	ACE- inhibitor; antimicrobial	Ong, Henriksson, & Shah, 2007; Rizzelo et al., 2005
LLYQEPVLGPVRGPFPIIV	β-CN f191-209	HT (4, 50); UH (50)		ACE- inhibitor; IgE binding	*Yamamoto, Akino, &Takano, 1994; Benedé et al., 2014*
VVVPPFLQPEVM	β-CN f82 - 93	UH (405)		IgE binding	*Benedé et al., 2014*
YAKPVA	k-CN f61-66	HT (405)		Antihypertensive	*Miguel, Gómez-Ruiz, Recio, & Aleixandre, 2010*
HPHPHLSF	k-CN f98-105	HT (4)		Antihypertensive	*Miguel et al., 2010*
VYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-CN f183-207	HT (405)	Mini-pig[4]	Antibacterial	*Recio, & Visser, 1999*
VYQHQKAMKPWIQPKTKVIPYVRY	α_{S2}-CN f183-206	HT (405)	Mini-pig[4]	Antibacterial	*Recio, & Visser, 1999*
YQEPVLGPVRGPFPIIV	β-CN f193-209	HT (4, 50, 405); UH (4, 50, 405)	Calf[3]; Mini-pig[4]; Calves[5]	ACE- inhibitor; Immunomodulator	*Ong et al., 2007; Coste et al., 1992*
LYQEPVLGPVRGPFPIIV	β-CN f192-209	HT (4, 50); UH (4, 50)	Mini-pig[4]	Immunomodulator	*Coste et al., 1992*
PYVRYL	α_{S2}-CN f202-207	HT (4, 50, 405); UH (50)		Antihypertensive	*Recio et al., 2006*
SRYPSY	k-CN f33-38	HT (50, 405); UH (405)		Opioid	*Yoshikawa, Tani, Yoshimura, & Chiba, 1986*

[1] Chabance et al., 1998 ; [2] Boutrou et al., 2013 ; [3] Yvon & Pelissier, 1987; [4]Barbé et al., 2014 ; [5]Scanff et al., 1992

ACE : Angiotensin I-converting enzyme ; HT : Heat treated milk ; UH : Unheated milk ; MSE : Mucin secretion and expression.

4. Conclusions

The impact of the heat treatment on the peptidome released from milk proteins during gastric dynamic digestion was evaluated. The homology values between samples, point out the importance of the exposure time to enzyme on the identity of peptides, causing an increase of 14% in peptide homology from 50 min to the end of digestion. Nevertheless, the peptide homology at the end of digestion only reached 48%. That confirms the impact of heat treatment on the identity of peptides generated from the two milk samples during digestion, despite their identical initial protein content and composition, and being the same matrix in both cases. Heat treatment produced an increase of the resistance in casein fraction. However, β-Lg showed to be more susceptible to hydrolysis.

The MS analyses revealed differences between the heat and unheated milk in the pattern of peptide release and in the identity of the sequences, which may have been induced by the heat treatment. These differences could have implications in health, in regard to bioactivity or formation/resistance of potential epitopes. Nevertheless, also some common traits after digestion could be found. Resistant regions could be confirmed through the analyses, such as the region comprised between the residues 76-93 of β-casein, where several binding epitopes are included. Also, β-casein regions 126-140 and 190-209 were found to be resistant. The differences found on the peptide release could have distinct further nutritional and physiological implications, since the gastric emptying could deliver to the duodenum the peptides that have faster hydrolysis rate, i.e. α_{s1}-casein f25-32 (VAPFPEVF), α_{s1}-casein f90-94 (RYLGY) or β-casein f 94-105 (GVSKVKEAMAPK), β-casein f132-140 (NLHLPLPLL), α_{s1}-casein f144-149 (YFYPEL) among others.

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2.5 Chapter V

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Bioavailability and Kinetics of the Antihypertensive Casein-Derived Peptide HLPLP in Rats

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Run title: Bioavailability of an antihypertensive peptide

ABSTRACT: The aim of this study was to investigate the oral bioavailability and kinetics of the milk casein-derived peptide HLPLP, which had previously demonstrated antihypertensive effect in spontaneously hypertensive rats. HLPLP disposition after single intravenous (4 mg/kg body weight) and oral (40 mg/kg body weight) doses was studied in rats. Plasma concentrations of HLPLP [β-casein fragment f(134-138)], and two derived fragments found after HLPLP administration, LPLP [β-casein fragment f(135-138)] and HLPL [β-casein fragment f(134-137)] were determined by Ultrahigh Performance Liquid Chromatography (UPLC) coupled *on line* to a Q-TOF instrument. For HLPLP, the elimination half-lives ($T_{1/2\beta}$) were 7.95 min after intravenous and 11.7 min after oral administration. The volume of distribution at steady state (V_{ss} = 30.8 L/kg) suggests a considerable uptake of HLPLP into tissues. HPLPL was converted to the peptides, LPLP and HLPL. After HLPLP intravenous administration, the elimination half-lives ($T_{1/2\beta}$) for these biotransformed peptides, LPLP and HLPL, were 8.38 and 10.9 min, respectively. After oral administration, HLPLP was rapidly absorbed with an absorption half-life ($T_{1/2a}$) of 2.79 min. The oral bioavailability of HLPLP was found to be 5.18%. Our study suggested that HLPLP was rapidly absorbed and eliminated after oral administration, biotransformed into smaller fragments LPLP and HLPL, and distributed throughout the body by the circulation blood. The present pharmacokinetic information from a pre-clinical kinetic study in rats can also play an important role in designing future kinetic studies in humans for assessing HLPLP dose-response relationship

Keywords: *Antihypertensive peptide HLPLP, Oral bioavailability, Plasma disposition, Rats,*
Tandem MS.

INTRODUCTION

Research on bioactive peptides has led to the discovery of particular protein sequences that modulate physiological functions with hormone- or drug-like activity.[1] There are several peptides derived from food proteins that interact with the renin-angiotensin system and reduce arterial blood pressure. Much work has been done to assess the *in vitro* activity of food peptides on the angiotensin-I-converting enzyme (ACE). However, only in some cases the correspondence between *in vitro* and *in vivo* effects has been demonstrated.[2] Discrepancy between ACE-inhibitory and antihypertensive activity of peptides can be due to their degradation during gastrointestinal digestion, the impaired access to the target organ in a sufficient amount or because other mechanism different than ACE inhibition may be involved.[3]

Milk fermentation represents one of the strategies most commonly employed to produce peptides with biological activity. For instance, recently *Kluyveromyces marxianus* has been demonstrated to produce antihypertensive lactoferrin-derived peptides, with ACE-inhibitory activity in vivo[4]. Milk fermented using selected strains of *Enterococcus faecalis* showed acute antihypertensive effect in spontaneously hypertensive rats (SHR) after a single oral administration.[5] In following experiments, the decrease of the arterial blood pressure was demonstrated after long-term intake of fermented milk.[6] The β-casein fragment (f) (133-138), with the sequence LHLPLP, was identified as one of the major peptides responsible for the biological activity of the fermented product. In fact, it is a potent *in vitro* ACE-inhibitor (IC_{50} of 5.5 μM) and antihypertensive peptide which causes a decrease in the systolic blood pressure in SHR of 25.3 mm Hg at a dose of 2 mg/kg when orally administered.[7] Simulated gastrointestinal digestion has shown that the sequence LHLPLP is resistant to digestive enzymes,[8] but upon incubation using Caco-2 cells, it was partly hydrolysed by brush border peptidases

that cleaved the peptide bond between Leu and His, releasing the pentapeptide HLPLP. The peptide HLPLP is also resistant to in vitro gastrointestinal digestion and brush border peptidases of Caco-2 cells, being rapidly transported through the cell monolayer.[9] This peptide HLPLP has been also demonstrated to exert antihypertensive activity in SHR.[10] Later on, the production of the active pentapeptide HLPLP by enzymatic hydrolysis from caseinate was also optimized[11] as an alternative to the fermentation with *E. faecalis* strains since its use in the food industry is controversial due to their pathogenic potential.[12] Since the peptide HLPLP might be commercially attractive as an active compound, it is important to investigate its capacity to reach blood circulation.

Previous studies on oral bioavailability of selected food-derived peptides have reported their presence in plasma at pico- and nanomolar concentrations using very selective and sensitive analytical techniques.[13] van Platerink et al.[14] developed a sensitive method to detect peptides in human plasma samples with detection limits from 0.01 to 0.001 ng/mL. Although the research on the absorption of antihypertensive peptides has revealed that certain short peptides, di- and tripeptides such as VY and IPP can reach blood stream undegraded after oral administration, either in fasted or fed states, very low absorption extents were reported[15-17]. To the best of our knowledge, kinetic studies with longer peptides than tri-peptides from dietary sources have not been reported.

Because the information regarding the kinetic profile is needed in order to establish a scientific basis for efficacy in the use of any food supplement, the objective of this study was to investigate the oral bioavailability and the kinetic behaviour of the antihypertensive peptide HLPLP after its administration to rats determining HLPLP plasma concentrations using a sensitive and specific analytical method. In addition, this technique allowed us to monitor the plasma concentrations of two derived peptide

fragments, LPLP [β-casein fragment f(135-138)] and HLPL [β-casein fragment f(134-137)].

MATERIALS AND METHODS

Chemicals. The peptides HLPLP, β-casein f(134-138), HLPL, β-casein f(134-137), HLP, β-casein f(134-136), LPLP, β-casein f(135-138), LPL, β-casein f(135-137) and PLP, β-casein f(136-138) were used in this study. These peptides were prepared in-house following the solid-phase synthesis method with fluorenyl-methoxy-carbonyl (FMOC), using a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany). Their purities, verified by reversed phase-high performance liquid chromatography-ultraviolet-mass spectrometry (RP-HPLC-UV-MS) as previously described,[18] were 98.0%, 97.68%, 96.20%, 93.43% and 99.36%, respectively. Peptide PLP, with a purity of 92.94%, was purchased from SynPeptide CO, LDT (Shanghai, China). All other chemicals were of the highest quality grade and obtained from commercial sources.

Animals and experimental design. The study was undertaken in accordance with the ethics requirements and authorized by the official ethical committee of University Complutense of Madrid. Adult male Wistar rats (Charles River Inc., Margate, Kent, UK) each weighing 220-230 g of 9 weeeks of age were used. The animals were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2ºC and 50 ± 10% relative humidity) with a 12 h light/dark cycle (light from 08.00 to 20.00 h). Food (A04 rodent diet, Panlab SL, Barcelona, Spain) and water were available *ad libitum*. The rats were divided into two groups, one group of 60 animals (Group 1) received the synthetic

peptide HLPLP intravenously and the other group of 84 animals (Group 2) orally. Group 2 rats were deprived of food for 12 h before the single oral administration of 40 mg/kg body weight but they had *ad libitum* access to water. The peptide was dissolved in distilled water (10 mg/mL) and was administered by oral gavage in a volume of 1 mL peptide solution/rat of 250 g of body weight. Group 1 rats were given a single i.v. injection of 4 mg/kg body weight into the lateral tail vein (in this case a peptide solution of 4 mg/mL was prepared, and 0.25 mL peptide solution/rat of 250 g of body weight was injected). The i.v. 4 mg/kg and oral 40 mg/kg doses of peptide HLPLP were selected on the basis of preliminary experiments where both doses and routes of administration did not show any adverse effects, abnormal clinical signs, behavioral changes, body weight changes, or change in food and water consumption in the animals treated (data not shown). The oral HLPLP dose was higher than the i.v. dose due to the low absolute oral bioavailability described for other food-derived peptides in pigs, approximately 0.1%.[17] The administered oral dose is higher than that reported to cause a significant decrease in the systolic blood pressure in spontaneously hypertensive rats (SHR).[10] Five additional animals were used as control to collect blank blood samples and to prepare calibration curves from plasma control fortified with the synthetic peptides HLPLP, LPLP and HLPL.

Animals in groups 1 and 2 were killed by cervical dislocation (six animals at each time) and then exsanguination at 0, 1.5, 2, 3, 5, 8, 10, 15, 20 and 30 min after i.v. and at 0, 3, 5, 10, 15, 20, 30, 40, 45, 50, 55, 60, 65 and 70 min after oral administration of peptide HLPLP, respectively. Time points were selected based on preliminary experiments and taken into account the limit of quantification (LOQ) of the analytical method. Blood samples (1 ml) were withdrawn from jugular vein and collected in heparinized tubes. Plasma was separated by centrifugation and stored frozen at -80ºC

until analysed. Blood samples at time greater than 70 or 30 min after oral or i.v. HLPLP doses, respectively, were not collected, because preliminary experiments (data not shown) showed that at these times no levels of the synthetic peptides HLPLP, LPLP and HLPL were detected.

Analysis of plasma samples. *Plasma sample preparation.* Plasma samples from rats were prepared by adding 40 µL of an aqueous solution of 10 % of trifluoroacetic acid (TFA) to 1 mL of plasma, homogenized in a vortex and the mixture was heated at 99ºC for 2 min to precipitate proteins, as previously described.[14] Then, the samples were centrifuged (MiniSpin, Eppendorf, Hamburg, Germany) at 8500 *g* for 30 min. The supernatant was purified using mixed mode cation exchange cartridges (Bond Elut Plexa PCX, 60mg, 1mL, Agilent, Santa Clara, CA, USA). Cartridges were preconditioned with 1500 µL of methanol, and 2500 µL of Milli-Q water (Millipore, Bedford, MA, USA). The plasma sample, previously mixed (1:3 v/v) with an aqueous solution containing 2% orthophosphoric acid was loaded. The cleaning step consisted in the addition of 1500 µL of 2 % formic acid in Milli-Q water, and 2500 µL of a mixture comprised by 50% methanol and 50% acetonitrile (v/v). The elution step was carried out by adding 1 mL of ammonia at 5% (35% purity) in methanol:acetonitrile (50:50, v/v). The elution volume was afterwards totally dried by vacuum centrifugation (SpeedVac SC 200 Savant, Irving, TX, USA). For the injection, the dried samples were reconstituted with 50 µL of 0.1% formic acid.

To prepare the standard curves, the synthetic peptides HLPLP, LPLP and HLPL were diluted in Milli-Q water to final concentrations that ranged from 0.08 to 300 ng/mL, corresponding to 0.13 to 521 nmol/L, 0.18 to 682.9 nmol/L and 0.17 to 625.9 nmol/L respectively, in rat control plasma. Immediately after adding the peptide solution to the plasma, they were treated as described before for the preparation of plasma samples from rat assays. The calibration curves were injected the same day as the rat samples.

Peptide analysis by UPLC-Q-TOF. The analyses of samples were performed on an Acquity Ultrahigh Performance Liquid Chromatography (UPLC) from Waters (Milford, MA, USA), coupled to a Microtof-QII (Bruker Daltonik, Bremen, Germany). The LC-MS system was controlled by the HyStar 3.2 software (Bruker). The column employed for the analyses was an Acquity UPLC BEH 130 C_{18} of 2.1 mm × 100 mm (Waters), with a particle size of 1.7 µm. The analyses were run at 30ºC, the flow rate was 0.2 mL/min and the injection volume was 25 µL. A linear gradient was set from 0 to 45% of solvent B (acetonitrile/formic acid 0.1%) and 55% of solvent A (water/ formic acid 0.1%) in 16 min. The nebulizer pressure was set at 3 bar, the temperature of the source at 180ºC and the capillary voltage at 4.5 kV. MS *full-scan* acquisitions were first performed during UPLC runs, using a 50-1200 *m/z* range. The method developed was a pseudo-selected reaction monitoring (SRM) in which the parent ion with mass-to-charge (*m/z*): 576.343 was selected for fragmentation. The peptide HLPLP was identified by its retention time and also by its fragmentation profile. The identification of the possible derived peptide fragments, i.e., LPLP and HLPL, HLP, LPL, and PLP was done by the retention time and the extraction of the characteristic molecular ions *m/z*: 439.284, 479.290, 366.206, 342.231 and 326.200, respectively. The Q-TOF analyzer was calibrated on a daily basis in MS mode, using the *m/z* ratios of adduct ions arising from sodium formiate as reference.

Data analysis. The mean plasma concentration *versus* time after i.v. and oral administration were sequentially fitted to 1-, 2- and multiple-compartment models, using the computer program WinNonlin (Version 6.3; Pharsight Corporation, Mountain View, CA, USA). The model was determined for best fit on the basis of a smaller value for the Akaike's Information Criterion (AIC).[19] The two-compartment model was the best fit for the two animal groups. This model was used to establish pharmacokinetic characteristics.

Mean plasma concentration curves of peptide HLPLP after a single i.v. and oral administration and those of the peptides LPLP and HLPL (metabolites in plasma) after a single i.v. administration of HLPLP were fitted to the following exponential equations:

$$C = A_1 \times e^{-\alpha t} + A_2 \times e^{-\beta t} \text{ (i.v.)}$$

$$C = A_1 \times e^{-\alpha t} + A_2 \times e^{-\beta t} - A_3 \times e^{-Kat} \text{ (oral)}$$

where C is the plasma concentration of the compound; A_1 and A_2 and A_3 are mathematical coefficients (i.e. A_1 and A_2 are the plasma concentrations extrapolated to time zero of the first and second elimination phases of the compound and A_3 for the absorption phase); α is the hybrid rate constant for the distribution phase; β is the hybrid rate constant for the elimination terminal phase (i.e. α and β are the slopes of the first and second elimination phases of the compound disposition) and *t* is the time. Absorption half-life ($T_{1/2a}$), half-life of α phase ($T_{1/2\alpha}$), the half-life of β phase ($T_{1/2\beta}$), distribution rate constants for transfer of the compound from the central to the peripheral compartment (K_{12}) and from the peripheral to the central compartment (K_{21}), and the elimination rate constant (K_{10}) were calculated using standard equations as described.[20,21] After i.v. and oral administration, the area under the concentration-time curves (AUC) was calculated as follows:

$$AUC = (A_1/\alpha) + (A_2/\beta); \text{ or}$$

$$AUC = (A_1/\alpha) + (A_2/\beta) - (A_3/K_a)$$

where bioavailability (F) is $(dose_{iv} \times AUC_{oral})/(dose_{oral} \times AUC_{iv})$.

Total plasma clearance (CL) was calculated, using the following formula:

$$CL = [dose\ (mg/kg)]/AUC; \text{ or}$$

$$CL = [dose\ (mg/kg)]\ (F)/AUC$$

Mean residence time (MRT) (only for HLPLP i.v. administration) was calculated as follows:

$$MRT = [(A_1/\alpha^2) + (A_2/\beta^2)] \times (1/AUC)$$

Volume of distribution in the central compartment (V_1) (only for HLPLP i.v. administration) was determined as follows:

$$V_1 = [\text{dose (mg/kg)}]/ A_1 + A_2$$

Apparent volume of distribution in the second compartment (V_2) (only for HLPLP i.v. administration) was determined as follows:

$$V_2 = (V_1) \times (K_{12}/K_{21})$$

Volume of distribution at steady state (V_{ss}) (only for HLPLP i.v. administration) was determined as follows:

$$V_{ss} = MRT \times CL$$

Maximum drug plasma concentration (C_{max}) after oral administration and the time at which C_{max} was achieved (T_{max}) was determined directly from the concentration versus time curve.

RESULTS

Quantitative determination of peptides by UPLC-Q-TOF. In preliminary experiments, the quantification of the HLPLP peptide was attempted with a sample preparation protocol consisting in the addition of 10% TFA to 1 mL of plasma followed by heating at 99ºC for 2 min to precipitate the proteins and further centrifugation. However, this protocol did not allow determining plasma peptide concentrations below 12.5 ng/mL. Therefore, peptide purification and concentration was investigated by using different solid phase extraction (SPE) cartridges, such as, reversed phase C_{18} cartridges, mixed-mode cation, anion, and weak cation exchangers and several solvent mixtures for the elution step. The best recovery values were obtained with a cation exchanger with mixed sorbent characteristics suitable for polar and non-polar

bases. The elution solvent selected was ammonia at 5% (35% purity) in methanol:acetonitrile (50:50, v/v).

The calibration curves provided linear results over the HLPLP concentration values from 0.13 to 521 nmol/L in plasma (R^2= 0.99). **Figure 1a** shows the extracted ion chromatogram of a blank plasma sample considering the masses of the pentapeptide HLPLP and both tetrapeptides, HLPL and LPLP, where it can be shown that there are no interferences with endogenous compounds are expected. The extracted ion chromatogram of the pentapeptide after oral and i.v. administration and its fragmentation pattern are shown in **Figure 1b, 1c** and **1d**, respectively. One of the most intense fragment ions was observed at *m/z* 326.207, corresponding to *y*-type ion y_3. Other major fragment ions were detected at *m/z* 251.149 and 461.290, which corresponded to *b*-type ions b_2 and b_4, respectively.

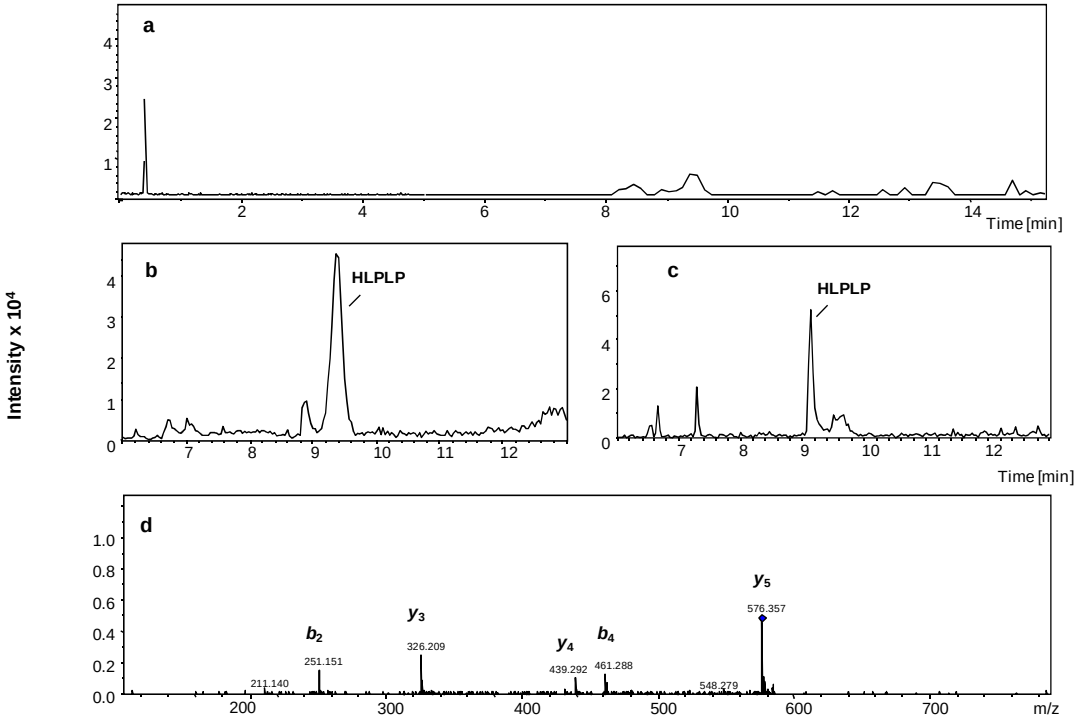

Figure 1. a) Extracted ion chromatogram (EIC) of HLPLP (*m/z* 576.343; RT 9.4), LPLP (*m/z* 439.284; RT 10.5) and HLPL (*m/z* 479.290; RT 8.4) in blank plasma. EIC of the peptide HLPLP (*m/z*: 576.343; RT 9.4 min) b) at 15 after oral administration and c) at 20 min after intravenous administration. d) Fragmentation pattern of HLPLP at 15 min after oral administration.

These fragments (y_3, b_2, and b_4) resulted from the cleavage at N-terminal proline, which often generates highly intense *b* and *y*-type fragment ions.[22]The recovery was calculated as the ratio between the concentration determined in a blank plasma sample spiked with HLPLP and further submitted to SPE purification and the concentration determined in a spiked sample after SPE purification. This was performed in triplicate using two concentrations of the peptide. The recovery values ranged from 96.9% to 98.7%. The reproducibility was estimated by the analyses of five spiked plasma extracts purified by SPE in three different days separated by 10 days, and repeatability by performing these determinations in consecutive analyses. Relative standard deviations (RSD) of the peak areas were 6% and 5% for reproducibility and repeatability, respectively. These values reflect the high performance achieved in the present study in terms of accuracy and precision. The limit of detection (LOD) was estimated to be 0.03 nmol/L, equivalent to 0.02 ng/mL, and was calculated as the concentration of peptide that gave a signal equal to 3 times the standard deviation of the blank. The LOQ was estimated at 0.17 nmol/L, equivalent to 0.10 ng/mL, by using 10 times the standard deviation of blank signal. With respect to the peptides LPLP and HLPL, the calibration curves provided linear results from 0.18 to 682.9 nmol/L (R^2= 0.98) and 0.17 to 625.9 nmol/L (R^2= 0.99), respectively. The LOD values for LPLP and HLPL were estimated at 1.13 ng/mL (0.50 nmol/L) and 0.11 ng/mL (0.22 nmol/L), respectively. The LOQ values for these peptides resulted in 1.67 ng/mL (3.71 nmol/L) and 0.36 ng/mL (0.75 nmol/L), respectively.

Pharmacokinetics of peptide HLPLP and its biotransformed peptides LPLP and HLPL.

In the plasma samples collected at time 0, i.e. before HLPLP i.v. and oral administration, no peptides HLPLP, HLPL and LPLP were detected. The two peptide

fragments (*m/z* 479.290, 439.284) corresponding to HLPL and LPLP respectively, were found in plasma from 1.5 min until 30 min after i.v. administration of HLPLP. However, after oral administration, peptide fragments were detected between 5 and 60 min although they could not be quantified at all time points. **Figure 2** shows the extracted ion chromatogram of the derived peptides, LPLP and HLPL, at different times after oral and i.v. administration, and their corresponding fragmentation patterns.

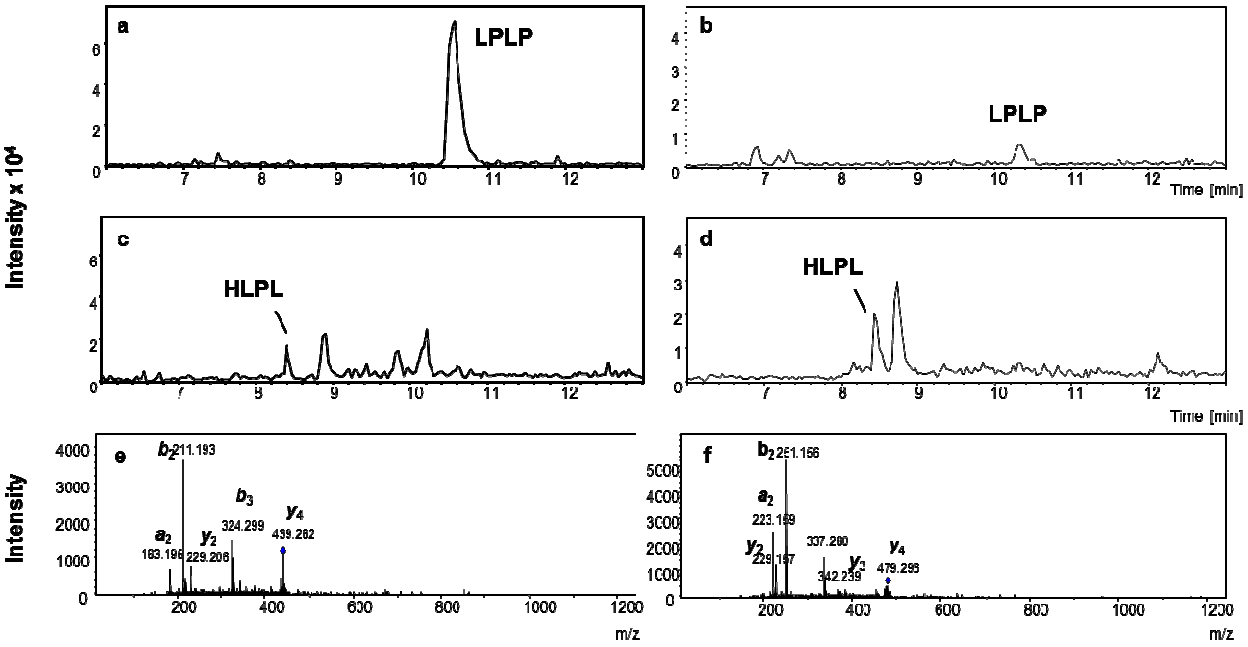

Figure 2. **a)** Extracted ion chromatogram (EIC) of LPLP (RT 10.5 min) at 2 min after intravenous administration and **b)** at 40 min after oral administration of the peptide HLPLP; **c)** EIC of HLPL (RT 8.4 min) at 2 min after intravenous administration and **d)** at 60 min after oral administration of HLPLP; fragmentation pattern of **e)** LPLP and **f)** HLPL.

Mean plasma concentrations of peptide HLPLP and its biotransformed peptides LPLP and HLPL after single HLPLP i.v. administration, and mean plasma concentrations of peptide HLPLP after single oral administration are shown in **Figure 3.** Analysis of plasma concentration-*versus*-time curves indicated a biphasic decrease after i.v. and oral

administration. Good fit of the observed data for a two-compartment open model was obtained.

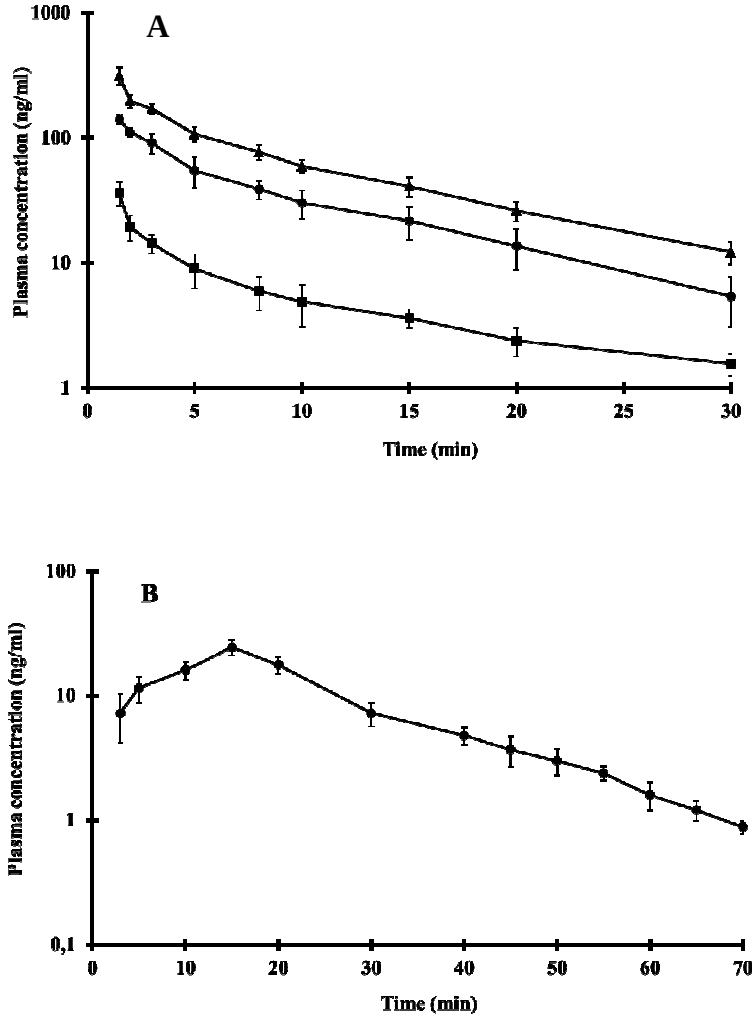

Figure 3. **(A)** Mean plasma concentrations of the peptides HLPLP (●), LPLP (▲), and HLPL (■) after single HLPLP intravenous dose of 4 mg/kg of body weight, and **(B)** mean plasma concentrations of the peptide HLPLP (●) after single oral dose of 40 mg/kg of body weight. Data represent mean ± S.D. values for six rats (i.e. six rats/time) (one-way ANOVA). Symbols without bars indicate that S.D. is within the symbols.

The values of the kinetic parameters of HLPLP and its derived peptides LPLP and HLPL after i.v. administration of HLPLP are presented in **Table 1**. Values of the relevant kinetic variables that described absorption and disposition kinetics of HLPLP after oral administration are presented in **Table 2**.

Table 1. Kinetic parameters for peptide HLPLP and its biotransformed peptides LPLP and HLPL after a single HLPLP i.v. dose of 4 mg/kg body weight in rats

Parameters[a]	HLPLP	LPLP	HLPL
A_1 (ng/mL)	189	537	91.8
A_2 (ng/mL)	75.7	141	9.60
α (1/min)	0.64	0.77	0.90
β (1/min)	0.09	0.08	0.06
$T_{1/2\alpha}$ (min)	1.08	0.90	0.77
$T_{1/2\beta}$ (min)	7.95	8.38	10.9
V_1 (L/kg)	15.1	-	-
V_2 (L/kg)	15.7	-	-
V_{ss} (L/kg)	30.8	-	-
K_{12} (1/min)	0.25	0.35	0.42
K_{21} (1/min)	0.24	0.23	0.14
K_{10} (1/min)	0.23	0.28	0.40
AUC (µg min/L)	1163	2404	253
MRT (min)	8.95	8.97	9.87
CL (L/min/kg)	3.41	-	-

[a] Kinetic parameters were calculated from the mean plasma concentration-time curves

Table 2. Kinetic parameters for peptide HLPLP after oral administration in rats

Parameters[a]	Oral dose (40 mg/kg body weight)
B_1 (ng/mL)	242
B_2 (ng/mL)	56.9
B_3 (ng/mL)	299
α (1/min)	0.29
β (1/min)	0.06
K_a (1/min)	0.25
$T_{1/2\alpha}$ (min)	2.38
$T_{1/2\beta}$ (min)	11.7
$T_{1/2a}$ (min)	2.79
AUC (µg min/L)	603
F (%)	5.18
CL (L/min/kg)	3.41
C_{max} (ng/mL)	20.1
T_{max} (min)	11.8

[a] Kinetic parameters were calculated from the mean plasma concentration-time curve

DISCUSSION

The limited information available on the absorption of antihypertensive peptides derived from food proteins makes difficult to interpret the functional properties of these bioactive peptides and to make an efficacy assessment as a new food supplement. These topics are commonly under debate for most functional ingredients.

Efficacy and safety assessment of many substances are normally extrapolated from animal data to humans. Laboratory animals, particularly rodents, are widely used for these evaluations. When several factors like differences in species sensitivity are considered, kinetic variables are of major importance and should be taken into account in extrapolation of laboratory data to humans, since changes in various kinetic parameters

are either easily determined experimentally or can be predicted with a reasonable accuracy. It is apparent that the principles of pharmacokinetics are indispensable in the overall assessment of human health risk.

Kinetic characteristics combined with dynamic patterns should be considered in the efficacy evaluation of peptide HLPLP. Pre-clinical pharmacokinetic information in experimental animals, such as the rat, is essential in designing further pharmacokinetic studies in humans which should be applied to the safe and effective use of this bioactive peptide.[23]

To the best of our knowledge, the present paper is the first report of plasma disposition of HLPLP peptide in rats using a selective analytical method in order to evaluate its pharmacokinetic profile. The LC-MS determination of the peptide was carefully optimized by selecting the parameters and analysis mode providing the highest signal-to-noise ratio. The validation parameters used show that the method is reliable and sensitive and allow an adequate characterization of the disposition of peptide HLPLP in rat plasma. In addition, this analytical method also allowed the identification and the quantification of two derived fragments**,** LPLP and HLPL, from the hydrolysis of HLPLP by the action of plasma peptidases**.** However, the presence of shorter fragments derived from the action of plasma peptidases on HLPLP cannot be excluded. The inherent limitations of the mass spectrometry techniques to identify small peptides and the complexity of the matrix may have impaired detection of shorter fragments.

This study reports the kinetics of the peptide HLPLP after a single i.v**.** (4 mg/kg body weight) and oral (40 mg/kg body weight) administration in rats. Plasma disposition of HLPLP after i.v. and oral administration in rats as well as the appearance of the peptide fragments, LPLP and HLPL after i.v. administration of HLPLP were best described by use of a two-compartment open model. Disappearance of the peptide from

plasma was characterized by an initial rapid distribution phase followed by a rapid elimination phase. van der Pijl et al.[17] reported in pigs that elimination from plasma of the proline-rich tripeptides IPP, VPP and LPP, followed a two-compartment decay process only for 18 of the 30 observations. Therefore, it was concluded that the pharmacokinetic behavior of these peptides in pig can be described with both 1- and 2- compartment model, although values for AIC for fitted peptide plasma concentrations after i.v. administration were lower using a two-compartment model.[17]

In the present study, after i.v. administration of HLPLP at 4 mg/kg, the distribution phase was rapid ($T_{1/2\alpha}$ = 1.08 min) and with a high value of V_{ss} (30.79 L/kg) which suggests that the peptide HLPLP is distributed into extravascular tissues. The elimination half-life ($T_{1/2\beta}$) calculated after i.v. administration was 7.95 min**,** much longer than that reported in pigs for peptides IPP, LPP or VPP (range $T_{1/2el}$ = 2.5 - 3.1 min).[16] The $T_{1/2\beta}$ of peptide HLPLP increased from 7.95 min after i.v. to 11.7 min after oral administration. This may indicate that the plasma disposition of HLPLP after oral administration could be conditioned by the absorption process.

Peptide HLPLP was extensively biotransformed to peptides LPLP and HLPL in rats and, after i.v. administration, represented 206% and 21.8% of the HLPLP plasma concentrations, respectively, as calculated by the ratio between the AUC for LPLP or HLPL and AUC for HLPLP. This suggests that these biotransformed peptides might contribute to the physiological activity of the peptide. At this respect, further experiments aiming the evaluation of the activity of these derived peptides are already in progress.

When orally administered, HLPLP was rapidly absorbed through the gastrointestinal tract in rats, as reflects a $T_{1/2a}$ of 2.79 min. This $T_{1/2a}$ is comparable to that previously obtained in pigs for the tripeptides IPP, LPP and VPP, where after a single

oral dose of 4 mg/kg body weight, the absorption half-life ranged from 2.0 to 4.6 min.[17] The absorption rate of the peptide HLPLP was low with a C_{max} of 20.1 ng/mL at 11.8 min (T_{max}) after oral dose of 40 mg/kg body weight. However, it has to be highlighted that, as shown after i.v. administration, HLPLP is rapidly hydrolysed by plasma peptidases into smaller fragments but they were not considered to calculate the absorption rate. In addition, it is hypothesized that other smaller fragments, tri-and di-peptides, might be released by the action of the peptidases, and due to the rapid degradation of the peptide, the effective absorption is underestimated. Nevertheless, the oral bioavailability found for HLPLP (5.18%) in rats was higher than that estimated for peptides IPP, LPP and VPP in pigs, for which approximately 0.059 - 0.077% of the dose was absorbed.[17] Probably, the low bioavailability of HLPLP observed in the present study could be due to first-pass effect (metabolism in gastrointestinal tract or liver before reaching systemic circulation.[24,25] However, the absolute bioavailability of 5.18% after oral administration of the peptide HLPLP which is not high for a small organic molecule, it is a valuable data for a peptide, given that they are subjected to extensive metabolism by peptidases before, during and after the absorption process. The bioavailability of a chemical compound is a prime consideration in formulation approaches, especially when attempting to increase bioavailability through physicochemical and technical manipulations in regard of the dosage form. Further studies in this concern should be conducted.

In summary, the present study indicates that when orally given, peptide HLPLP is absorbed and distributed throughout the body by the blood stream. The kinetic characteristics of peptide HLPLP identified in this study warrant further research on possible new ways of supplementation in food to increase its absorption and time of exposure. The present pharmacokinetic information in rats can play an important role designing future kinetic studies in humans for assessing HLPLP dose-response

relationship. In addition, this work reports an accurate analytical method for the determination of HLPLP, which also allowed the identification and quantification of some derived peptide-fragments. Although the peptapeptide reaches the blood stream, its rapid degradation in plasma affects the estimation of the kinetic parameters. This study raises as well the question about the final active form of dietary compounds, and specifically of dietary peptides, in the organism.

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The authors declare that there are no conflicts of interest

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2.6 Chapter VI

(Manuscript)

Peptide fragments from β-casein f(134-138), HLPLP, generated by the action of plasmatic peptidases retain antihypertensive activity

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ABSTRACT

Recently, the intact absorption of β-casein f(133-138), HLPLP, into rat blood circulation after oral administration has been demonstrated and several derived fragments were detected after both, intravenous and oral administration. In this study, an incubation of the penta-peptide in rat plasma was performed and the peptide fragments generated by plasmatic peptidases (HLPL, LPLP and HLP) were detected and quantified by using ultra-high performance liquid chromatography (UPLC) coupled *on line* to a Quadrupole-time-of-flight (Q-TOF). The tetra peptides (HLPL and LPLP) and the tri-peptide HLP were generated within seconds of incubation and were detected up to 60 min (HLPL), 120 min (LPLP) and 240 min (HLP). These peptide fragments and other two possible fragments (LPL and PLP) were chemically synthesized and administered to SHR to assess their antihypertensive activity. The ability to inhibit the angiotensin-converting-enzyme (ACE) was assessed on aortic rings by measuring the response to angiotensin I in presence of each peptide. All peptides showed potent antihypertensive activity and inhibition of vascular contraction elicited by angiotensin I.

Keywords: antihypertensive peptides, in vitro incubation, plasma metabolism, peptide fragments, tandem mass spectrometry, SHR.

1. Introduction

In recent years, the evaluation of changes that bioactive peptides undergo in the organism after ingestion has gained importance, since these modifications determine their fate and further health effect. Among these changes, gastrointestinal digestion and subsequent phenomena such absorption, distribution, metabolism and elimination (ADME) play a key role in the evaluation of bioactivity of peptides. During gastrointestinal digestion, food peptides can resist or can undergo hydrolysis, and this may cause a loss of their activity or, on the contrary, the release of the active sequence. For instance, the active form of the peptide β-casein f(169-175) is generated after losing the C-terminal glutamine residue during digestion (Maeno et al., 1996). Several antihypertensive peptides from an egg white peptic hydrolysate, such as RADHPFL and YAEERYPIL are degraded when subjected to simulated gastrointestinal digestion, giving rise to smaller active peptides, such as, RADHP and YPI (Miguel et al., 2006). Once the stability of peptides to digestion is ensured, bioavailability studies need to be conducted, in order to determine the accessibility of potential biologically active peptides in the organism (Sánchez-Rivera et al., 2014). In this regard, mass spectrometry (MS)-based techniques have permitted the development of sensitive methods to detect and quantify small changes of peptides in plasma samples, reaching detection limits in the picogram range (van Platerink et al., 2006). These sensitive MS-based methods have been employed to detect bioactive peptides, such as casein-derived peptide IPP, in plasma of humans who ingested a peptide-enriched beverage containing this peptide (Foltz, et al., 2007). Likewise, Matsui et al. (2002) reported the presence of the bioactive dipeptide VY, from sardine muscle, in blood circulation

of humans after ingestion of a drink that contained VY. Van der Pijl et al. (2008) conducted a pharmacokinetic study to determine the peptides IPP, LPP and VPP in plasma of pigs after intravenous or intragastric administration thereof.

Moreover, having evaluated the changes through digestion and the absorption of peptides, the assessment of the peptide stability to plasma peptidases plays an important role on the exhibition of its antihypertensive effect. In this regard, some peptides, such as IVY, have been incubated in plasma with the aim of studying their metabolism, being hydrolysed by plasma peptidases to give rise to the active di-peptide VY (Matsui et al., 2000). Other authors studied the metabolism and distribution of tri-peptides (VPP and IPP) in aorta, lung, kidney, heart and brain of rats fed sour milk containing these peptides. They reported the presence of the intact tri-peptides in abdominal aorta (Masuda et al., 1996).

Previous studies of our group demonstrated that certain strains of *Enterococcus faecalis* were able to produce fermented milk that caused a decrease in systolic blood pressure (SBP) after its single oral administration to SHR (Muguerza et al., 2006), and after long-term intake (Miguel et al., 2005). The peptide LHLPLP, f(133-138) from β-casein, was one of the sequences that most contributed to the biological effect caused by the fermented milk, and its isolated form exerted ACE-inhibitory and antihypertensive activity (Quirós et al., 2007). This sequence was reported to be resistant to gastrointestinal digestion (Quirós et al., 2009). However, it was partly degraded by brush border enzymes when incubated with Caco-2 cells, releasing the peptide HLPLP, f(134-138), which underwent epithelial transport across the Caco-2 cell monolayer (Quirós et al., 2008). Recently, the intact absorption of β-casein f(133-138), HLPLP, into rat

blood circulation after oral administration thereof at a single dose of 40 mg/kg body weight has been demonstrated (Sánchez-Rivera et al., 2015). In this study, several derived fragments were detected after both, intravenous and oral administration of the penta-peptide HLPLP. However, whether the derived fragments released in plasma could retain any biological activity remains to be demonstrated.

The aim of this study is to identify if the peptide fragments derived from the action of plasmatic peptidases on HLPLP can exert in vivo antihypertensive activity. For this purpose, the peptide fragments generated during peptide incubation of the peptide in rat plasma were identified by ultra-high performance liquid chromatography (UPLC) coupled *on line* to a quadrupole-time-of-flight (Q-TOF) analyser. Identified fragments were chemically synthesized and their antihypertensive effect was evaluated on spontaneously hypertensive rats (SHR) after single oral administration. The ability to inhibit ECA, as a possible mechanism of action, was also assessed in isolated aortic rings.

2. Materials and methods

2.1 *Peptide synthesis and standard solution preparation*

Peptides were synthesized in-house by conventional fluorenyl-methoxy-carbonyl chloride (fmoc) solid-phase method, using a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany). The synthesized peptides corresponded to β-casein sequences: HLPLP f(134-138), HLPL, f(134-137), HLP, f(134-136), LPLP, f(135-138), and LPL, f(135-137). Peptide PLP, β-casein f(136-138), was purchased from SynPeptide CO, LDT (Shanghai, China). Their purities were above 93%, calculated by RP-HPLC-UV-MS. All other chemicals

were of the highest quality grade. To prepare the standard curve of HLPLP, HLPL, LPLP and HLP in plasma, the synthetic peptide diluted in Milli-Q water was added to plasma to reach peptide concentration from 2.5 ng mL^{-1} to 20 µg mL^{-1} and treated as described by Sánchez-Rivera et al. (2015).

2.2 Incubation of peptide β-casein f(134-138) in rat plasma

The peptide HLPLP, *β-casein* f(134-138) was incubated in rat control plasma obtained from female Wistar rats (250-300 g). The rats were anaesthetized using pentobarbital at intraperitoneal dose of 60 mg kg^{-1} body weight and the blood was extracted by cardiac puncture (5 mL per rat), with 5 mL 23G syringe. The blood was introduced in a lithium-heparin 9 mL tube, and centrifuged at 3600 rpm for 20 min at 4 ºC. The supernatant was collected and freezed at -20 ºC until use. Plasma was mixed 5:1 with the peptide aqueous solution to reach a final concentration of 0.06 mg mL^{-1}. This mixture was kept at 37 ºC during the experiment in a thermally controlled water bath (AQUAline AL25, LAUDA WOBSER, GmbH & Co., Postfach, Germany). Sampling was done at 5, 30, 60, 120 and 240 min after peptide addition to the plasma, and also an aliquot was taken just after the peptide was spiked in plasma and vortexed for 10 seconds (T0). The incubation was stopped using TFA (10%, v/v) and the plasma samples were treated as described by Sánchez-Rivera et al. (2015).

2.3 UPLC-Q-TOF analyses

The analyses of the plasma samples were performed on an UPLC from Waters (Milford, MA, USA), coupled to a MicroTOF-QII instrument (Bruker Daltonik, Germany). These analyses were carried out as described by Sánchez-Rivera et al. (2015) with some modifications. The pseudo-selected ion monitoring (SRM) method was set up to fragment the following parent ions, with *mass-to-charge ratio (m/z*) of 576.343, 439.284, 479.290, 366.206, 342.231, and 326.200, which corresponded to the sequences HLPLP, LPLP, HLPL, HLP, LPL and PLP, respectively. The amount of peptide fragments at different incubation times was estimated by the extraction of the corresponding molecular ions (*m/z*). Blank plasma was previously analyzed to confirm that there were no such fragments naturally present in plasma before these experiments.

2.4 Pharmacological activity assays.

All the animal trials were carried out according to the European Union guidelines for the ethical care and use of laboratory animals (European Directive 2010/63/EU). 12 to15- week old SHR were purchased from Elevage Janvier (Le Genest, Saint Isle, France). The animals were housed in groups of three rats and kept at 23 ºC with 12 h light/dark cycles in the Animal Experimentation Service of Salamanca University (NPAE SA001). Standard food (Global Diet 2014, Harlan, France) and water were available *ad libitum*. The synthesized peptides dissolved in ultrapure water were orally administered using an esophageal cannula, at a single dose of 7 mg kg^{-1} body weight and the control group received the same volume of water. The animals were deprived of solid food diet 12 h before experiments, only having access to sucrose (80 g L^{-1}) and NaCl (2 g L^{-1}) solution. The systolic blood pressure (SBP) were measured as previously described (Sánchez et al., 2011), using the CODA tail-cuff blood pressure system (Kent

Scientific, Torrington, CT, USA). The measurement of SBP was performed before peptide administration to estimate the basal blood pressure, and after administration thereof at 2, 4, 6, 8 and 24 h.

On the other hand, isolated thoracic aorta rings were mounted in organ bath with Krebs solution at 37 ºC aerated with carbogen as described elsewhere (Sánchez et al. 2011). After an equilibration period for 1 h at a resting tension of 2 g, the functionality of the rings was tested with a 120 mM solution of KCl. After that, rings were incubated with different peptides (HLPLP, LPLP, HLPL or HLP) at a dose of 10^{-4} M, for 30 min, and the contractile response to Ang I (10^{-7} M) was evaluated. Captopril (10^{-6} M) was used as positive control.

2.5 Statistical analysis

The mean values are expressed with the standard error of mean (SEM). The effect of each administered peptide over time and versus the control (water) was recorded. Two-way analysis of variance (ANOVA), using GraphPad Prism 5.0 (GraphPad software Inc., San Diego, USA) was performed. Bonferroni post-test was applied to establish the significant differences between the peptides and the control. Responses to Ang I were expressed as mg of contraction and statistical analysis was made using one way ANOVA comparing responses to Ang I in absence and presence of the peptides during the incubation period. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1 Hydrolysis of β-casein f(133-138), HLPLP, *by plasma peptidases*

To evaluate the resistance of β-casein f(133-138) HLPLP to plasma peptidases over time and to identify the formation of derived fragments, the penta-peptide was incubated in plasma for 4 h. A slight disappearance was observed from time zero to 5 min (Figure 1a); although from this point to 30 min, 6-fold decrease was observed. Then, plasma peptidases caused a pronounced depletion on its concentration from 30 to 60 min of incubation (10 fold decrease, from 0.9 to 0.09 μM). Nevertheless, the peptide was detected until 240 min. The formation of all possible tetra- and tri-peptides was monitored by pseudo-SRM and three derived fragments could be identified and quantified during incubation in plasma, i.e., LPLP, HLPL and HLP. The release of the peptide fragments occurred rapidly and the two tetra-peptides, HLPL (Figure 1b) and LPLP (Figure 1c) were detected at the first sample withdrawn from the incubation (time zero). These tetra-peptides suffered a decrease from 5 to 30 min. The first one, only resisted during 60 min of incubation, however the latter one, could be identified up to 120 min. The tri-peptide HLP (figure 1 d) was also detected at time zero, and increased significantly during the first 5 min of incubation in plasma, keeping a rising trend until 60 min; to finally suffer a pronounced decrease, but this fragment was detected until 240 min.

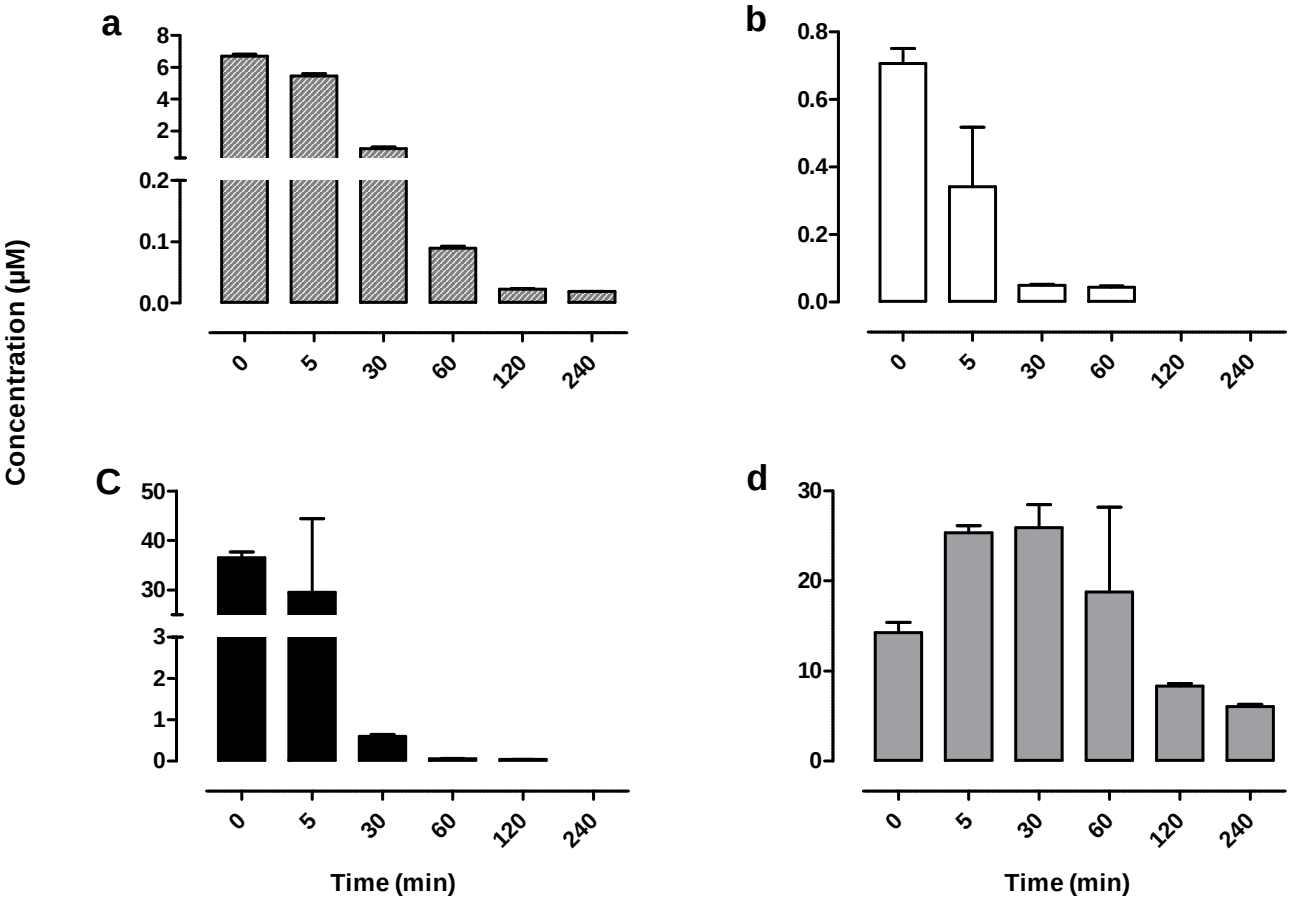


Figure 1. Concentrations (µM) of the peptides HLPLP (a), HLPL (b), LPLP (c) and HLP (d) identified during the incubation of the penta-peptide in plasma. Mean values ± SEM (n=3).

3.2 Evaluation of the antihypertensive activity

In order to assess the antihypertensive activity of the fragments released during plasma incubation of HLPLP, these chemically synthesized peptides were orally administered to SHR. Despite the tri-peptides LPL and PLP were not found during the in vitro incubation in plasma, their antihypertensive effect was also tested in SHR because their in vivo release cannot be excluded. Figure 2 shows the antihypertensive effect of the different β-casein peptides, orally administered to SHR at a single dose of 7 mg Kg^{-1} body weight. The administration of HLPLP and its derived peptide fragments led to different behaviours in regard to the

trend of SBP decrease. The parent peptide, HLPLP, (Figure 2a), showed a maximum decrease on SPB at 2 h post-administration (21.1 ± 3.4 mmHg). Subsequent recovery occurred from this point. However, the two tetra–peptides, HLPL and LPLP (Figures 2b and 2c) caused reduction on SBP that reached its maximum 4 h after administration (19.4 ± 3.8, and 16.2 ± 3.8 mmHg, respectively). The administration of the tri-peptides HLP, LPL and PLP to SHR led to a delay on the decrease of SBP compared to the previous ones. In the case of the tri-peptides HLP and LPL, 6 h were needed to reach a maximum peak of SBP reduction (15.2 ± 1.4 and 22.0 ± 2.5 mmHg, respectively). Finally, the administration of PLP induced the most effective SBP decrease in terms of endurance. This peptide caused a gradual drop of SBP values that reached its maximum at 8 h post-administration (21.2 ± 3.4 mmHg). The long-lasting effect of this peptide, led to a slow rate of recovery. At 24 h, significant differences on the reduction of SBP could be still observed.

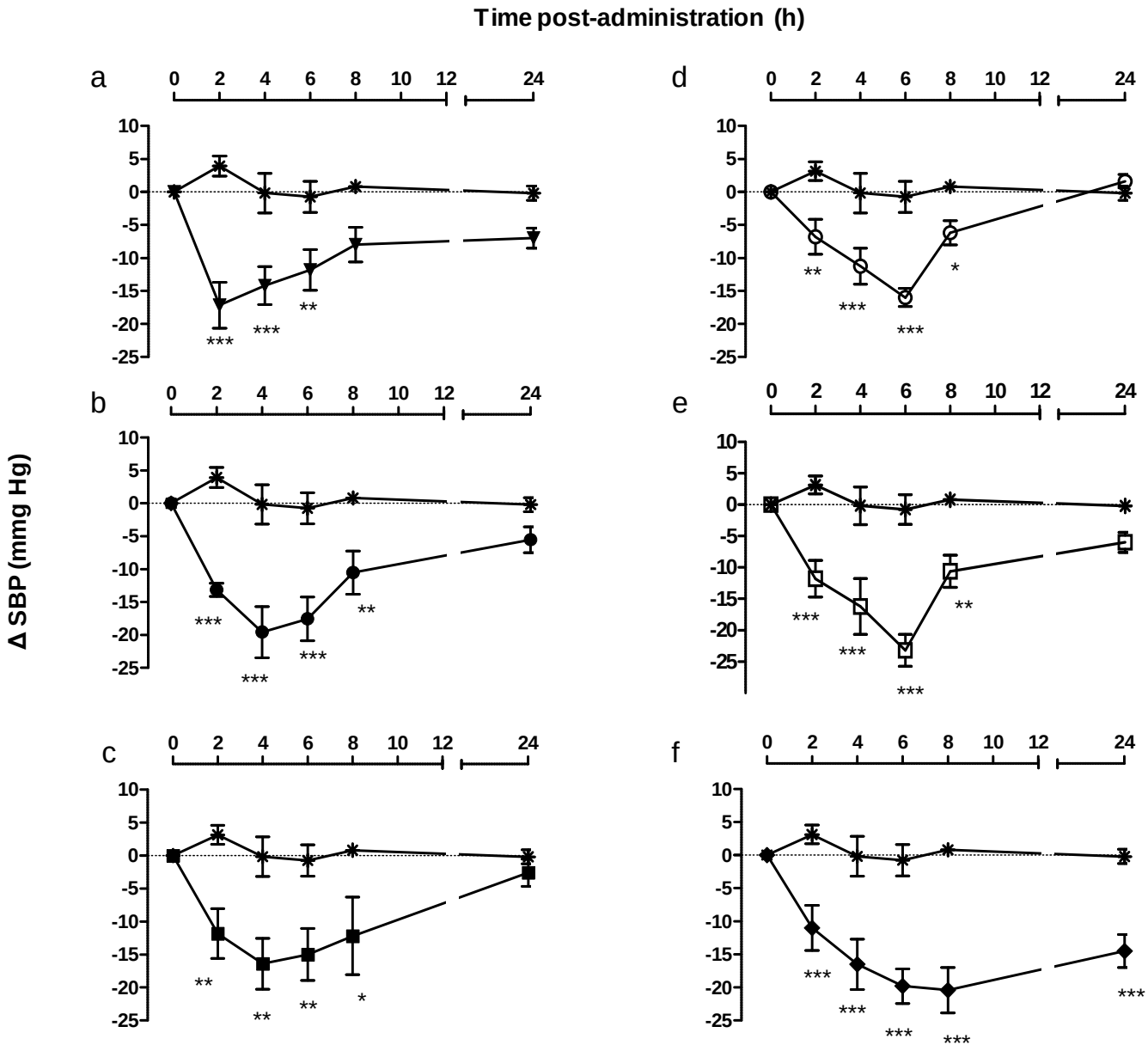
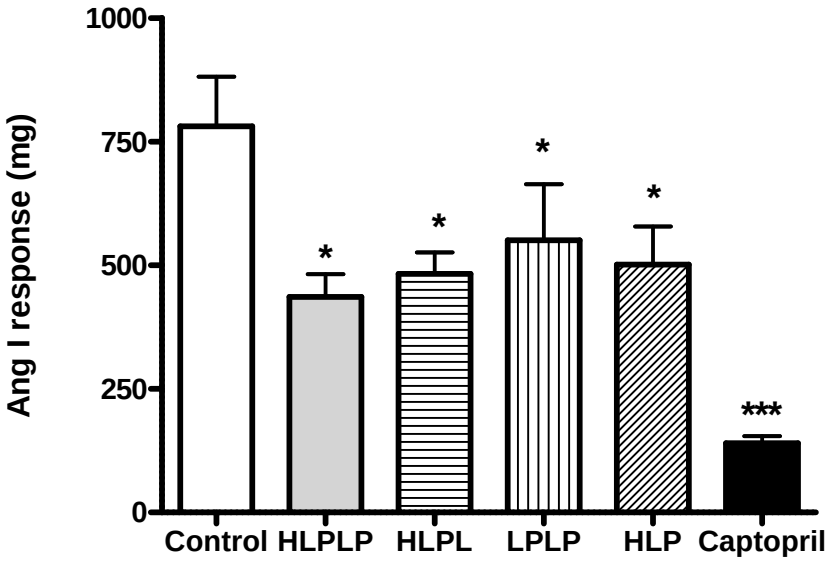


Figure 2. SBP decrease (Δ) caused in SHR after administration of water (*****), HLPLP (a), HLPL (b), LPLP (c), HLP (d), LPL (e) and PLP (f). Mean values ± SEM (n=5-6) are represented. Statistical differences were found at p≤0.05 (*), p≤0.01 (**) and p≤ 0.001(***).

To investigate whether the antihypertensive activity of these peptide could be due to ACE inhibition, the response of angiotensin I in isolated aortic rings was assessed. Angiotensin-I is converted to Angiotensin-II by tissue ACE activity, resulting in a contractile response. Figure 3 shows how the contractile response

to Angiotensin-I (780 +/- 100 mg) was significantly reduced in the presence of the peptides tested at 10^{-4} M dose. The highest reduction (44%) was achieved with HLPLP, but no significant differences were observed among all tested peptides. This reduction was much more efficient with the ACE inhibitor captopril (82%, at 10^{-6} M dose).



4. Discussion

Most studies on food-derived antihypertensive peptides associate the orally administered sequence with a given effect, although it is known that peptides are going to suffer further hydrolysis during digestion, absorption and, if they reach circulation, they could be further degraded by plasmatic peptidases. The region from β-casein f(133-138) has been shown to be especially resistant to digestion and related fragments have been found by us and others after simulated gastrointestinal digestion of dairy products (Dupont et al., 2010; Gómez-Ruiz et al., 2004; Hernández-Ledesma et al., 2004; Sánchez-Rivera et al., 2014b) or

even in human jejunal aspirates (Boutrou et al., 2013). This hexa-peptide was hydrolyzed, by the action of the brush-border peptidases, into the penta-peptide HLPLP, and in this form was rapidly transported across a Caco-2 cell monolayer (Quirós et al., 2008). Recently, the absorption of this penta-peptide in rats and the kinetic parameters of absorption and elimination have revealed that HLPLP is rapidly absorbed although the oral bioavailability (5.18%), calculated on the basis of HLPLP concentration in plasma, was found to be low. Interestingly, it was observed that this penta-peptide was further hydrolyzed into smaller fragments in vivo but it remained to ascertain if these derived fragments could retain antihypertensive activity.

In this work, the in vitro incubation of the penta-peptide in rat plasma resulted in the identification of two tetra-peptides and one tri-peptide, LPLP, HLPL and HLP. The increase in concentration undergone by the latter peptide from 5 to 60 min coincides with the decrease monitored for HLPL and HLPLP. These results could indicate the formation of the tri-peptide from both the penta- and tetra-peptide, by the action of plasma peptidases. In addition, the concentration calculated for HLPL is much lower than that of LPLP and HLP, suggesting that HLPL could be extensively hydrolyzed into HLP. The initial concentration of HLPLP detected in plasma was under the expected value, probably due to its fast degradation in favour of its derived fragments. A similar case was the rapid degradation by plasma peptidases undergone by Lactokinin [β-lactoglobulin f(142-148)] during its in vitro incubation in human serum (Walsh et al., 2004). The concentrations of LPLP and HLP were higher than that of the precursor peptide (6 and 5-fold, respectively), showing the rapid and extensive hydrolysis of the penta-peptide into smaller peptide fragments even a few

seconds after incubation (time zero). This is consistent with the degradation of Angiotensin II within seconds in plasma (Moskowitz, 2003). Thus, the formation of other peptide fragments cannot be excluded during the incubation of HLPLP in plasma. Undoubtedly, there is an inherent limitation of mass spectrometry techniques to identify short peptides, which could have impaired the identification of other derived fragments. Two of the peptide fragments (LPLP and HLPL) identified in this study during *in vitro* incubation of the penta-peptide are coincident with those recently reported after the oral and intravenous administration of HLPLP in rats (Sánchez-Rivera et al., 2015). Although these results are obtained in rat plasma, similar degradation could be expected in humans. In this regard, Matsui et al. (2000) reported the same degradation products of the peptide IVY, the smaller peptide fragments VY and Y, during its incubation in rat and human plasma although the parent peptide disappeared faster in human plasma.

Despite the tri-peptides LPL and PLP were not found during HLPLP incubation in plasma, the antihypertensive activity was also tested in SHR since their presence cannot be excluded. Probably, as demonstrated for the peptide LHLPLP (Quirós et al., 2009), the tested peptides survive gastrointestinal digestion due to the presence of Pro residues, which confer resistance to digestive enzymes (Haush et al., 2002; Vanhoof et al., 1995). This resistance could guarantee the arrival of the undegraded peptide to small intestine, concretely duodenum and upper jejunum, where the absorption mainly takes place (Langerholc et al., 2011); or where they might interact with receptors at the gut. From our results, it was surprising that, after oral administration, the shorter the peptide, the longer it took to reach the maximum SBP decrease. Likewise,

other tri-peptides, such as, IPP showed its maximum decrease on SBP at 8 h post-administration to SHR (Nakamura et al., 1995). The delay found on the antihypertensive effect after the administration of the tri-peptides (LPL, PLP and HLP) was also similar to that observed for other short peptide fragments from α_{s1}-casein, RYLG f(90-93) and RY f(90-91) with regard to the parent peptide (Contreras et al., 2013). In our previous report, it was found that HLPLP is rapidly absorbed, with absorption half-time ($T_{1/2a}$) of 2.79 min and time to reach the maximum concentration (T_{max}) of 11.8 min (Sánchez-Rivera et al., 2015). In the present work the time of maximum SBP decrease was found at 2 h. Likely, these parameters have been described for the tri-peptides VPP and IPP in a pig model ($T_{1/2a}$ 4.6 min; T_{max} 8.9 min and $T_{1/2a}$ 3.3 min; T_{max} 8.6 min, respectively), and the absorption time was similar to that of HLPLP, although the T_{max} was shorter for these two tri-peptides (van der Pijl et al., 2008). In spite of achieving the maximum concentration in plasma faster than HLPLP, these tri-peptides caused a maximum decrease on SBP in SHR at 4 and 8 h post-administration, respectively (Nakamura et al., 1995). These results suggest that there is a discrepancy between the parameters calculated for the absorption of peptides and the subsequent biological effect observed after administration. Activity of peptides may be affected by their further metabolism, once they reach circulation, as it could be the case of HLPLP. Hence, these results point out the importance of the pharmacokinetic studies focused on absorption and further metabolism of peptides.

In view of the results obtained on aortic rings, we can say that the antihypertensive effect of peptide HLPLP and those produced by the action of plasma peptidases is due, at least in part, to its ability to inhibit ACE. These

results are consistent with previous works published by our group where the precursor peptide of HLPLP i.e LHLPLP, β-casein f(133-138), showed antihypertensive activity and inhibited ACE activity *in vitro* (IC_{50} 5.4 uM) (Quirós et al., 2007). Similarly, IPP and VPP have been reported as ACE inhibitors *in vitro* with an IC_{50} value at micromolar level (Nakamura et al., 1995). In addition, these tri-peptides have also shown ACE-inhibitoty activity in mesenteric rings after 24h of incubation with the tri-peptides (Jäkala et al., 2009). Furthermore, other peptide fragments (LPLP) identified after incubation of the penta-peptide possess favourable structural features for ACE binding, such as the proline residue at C-terminal end and at the antepenultimate position of the sequence (Cheung et al., 1980; Rohrbach et al., 1981).

In addition, our results after oral administration, and especially the finding of the potent tri-peptide PLP, have allowed the identification of novel active β-casein tri-peptides, which could be produced by fermentation or hydrolysis with food-grade enzymes. The structure of PLP is favourable to bind and inhibit ACE, due to the presence of two proline residues, although further assays are needed to confirm this fact (Cheung et al., 1980; Rohrbach et al., 1981).

4. Conclusions

The incubation of the antihypertensive peptide β-casein f(134-138) HLPLP in rat plasma permitted the identification of several derived fragments (HLPL, LPLP and HLP), which are physiologically relevant in regard to the antihypertensive effect. Some of them, such f(134-136) HLP could be detected until 240 min of incubation time. All possible peptide-fragments derived from

HLPLP retained antihypertensive activity when orally administered to SHR. From all the peptide fragments, the tri-peptide PLP showed the longest lasting antihypertensive effect which was significant up to 24 h after administration. The incubation in plasma of HLPLP, which was known to be absorbed intact into circulation, was a good in vitro approach of its further degradation by plasmatic peptidases. Indeed, the in vitro incubation was in accordance to the peptide fragments found after intravenous and oral administration of HLPLP to rats. These results could play an important role in the overall physiological effect of the penta-peptide HLPLP. Inhibition of ACE is involved in the mechanism of antihypertensive action of these peptides, although further studies should be conducted to explore other possible mechanisms involved.

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2.7 Chapter VII

(Manuscript)

Implication of opioid receptors in the antihypertensive effect of a casein hydrolysate and α_{s1}-casein derived peptides

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ABSTRACT

The antihypertensive activity of three α_{s1}-casein-derived peptides and a casein hydrolysate containing these sequences was evaluated in the presence of naloxone. The activity of the casein hydrolysate was reverted by this opioid antagonist at 2, 4 and 6 h post-administration. Similarly, the antihypertensive effect of peptides RYLGY and AYFYPEL, [α_{s1}-casein f(90-94) and f(143-149), respectively] was antagonized by the co-administration of naloxone. However, the decrease of the systolic blood pressure caused by YFYPEL α_{s1}-casein f(91-94) was not significantly modified by naloxone. Peptidomic analysis of the active hydrolysate and a simulated gastrointestinal digest of the precursor casein revealed some differences that may explain the different in vivo effect on systolic blood pressure. Altogether, these results revealed that the activity of peptides from α_{s1}-casein, f(90-94) and f(143-149), and the effect of the casein hydrolysate is mediated by interaction with opioid receptors.

Keywords: naloxone, antihypertensive peptides, opioid receptors, casein hydrolysate, SHR

1. Introduction

Among bioactive peptides derived from food, antihypertensive peptides are undoubtedly the group from which more information is available (Jäkala & Vapaatalo, 2010; Hernández-Ledesma, Contreras, & Recio, 2011). Much work has been done to evaluate the *in vitro* activity of peptides on the angiotensin-I-converting enzyme (ACE), an enzyme which plays an important role in the regulation of blood pressure. However, for many peptides, an important lack of correlation has been found between the in vitro ACE-inhibitory activity and the in vivo antihypertensive activity. It has been postulated that this discrepancy can be due to the further degradation of peptides during gastrointestinal digestion, or because other mechanisms different than ACE inhibition may be involved (Martínez-Maqueda, Miralles, Recio, & Hernánadez-Ledesma, 2012). There are several examples where gastrointestinal digestion leads to an inactive form of the peptide (Walsh et al., 2004). On the contrary, several active sequences have been found to be resistant to digestion, especially those rich in proline residues (Quirós, Contreras, Ramos, Amigo, & Recio, 2009), and in other cases, the active form of the peptide is released during digestion (Maeno, Yamamoto, & Takano, 1996).

Regarding the mechanism of action, ACE-inhibition is the mechanism most widely studied for food derived peptides. For instance, it has been proved that spontaneously hypertensive rats (SHRs) fed a fermented milk containing the tri-peptides IPP and VPP showed a notable decrease of serum ACE activity (Jäkälä, Jauhiainen, Korpela, & Vapaatalo, 2009a; Jäkälä et al., 2009b). Microarray analysis showed a significant increase in the endothelial nitric oxide synthase (eNOS) gene expression in addition to other genes involved in blood pressure

regulation after the administration of these two tri-peptides to SHR for five days (Yamaguchi, Kawaguchi, & Yamamoto, 2009). The involvement of opioid receptors has been also demonstrated for some antihypertensive food-derived peptides, such as, α-lactorphin, [α-La f(50-53)], with sequence YGLF (Nurminen et al., 2000). This peptide has been found to lower blood pressure in SHR and produce and endothelium-dependent relaxation in mesenteric arteries which was reverted by an eNOS inhibitor (Sipola et al., 2002).

Our group has reported the antihypertensive activity of a peptic casein hydrolysate in SHR. Two α_{s1}-casein-derived peptides, f(90-94), RYLGY and f(143-149), AYFYPEL, with antihypertensive activity in addition to *in vitro* antioxidant and ACE-inhibitory activity, were identified as the main sequences responsible for the antihypertensive effect (Contreras, Carrón, Montero, Ramos, & Recio, 2009). These two peptides were partly resistant to gastrointestinal enzymes (Contreras et al., 2012) and were transported through intestinal cell monolayers, such as, a co-culture of 75/25 Caco-2 and HT29-MTX cells (Contreras, Sancho, Recio, & Mills, 2012). The administration of this casein hydrolysate for 6 weeks to SHR revealed that not only the development of hypertension was attenuated in the treated group of animals, but they showed improved aorta and mesenteric acetylcholine relaxations and increased eNOS expression in aorta. Treated animals also displayed a decreased left ventricular hypertrophy, compared with the control group, which was accompanied by a significant decrease in interstitial fibrosis (Sánchez et al., 2011). One of the proposed active peptides found in this casein hydrolysate is similar to a previously reported opioid peptide, α_{s1}-casein f(90-95), RYLGYL (Loukas, Varoucha, Zioudrou, Streaty, & Klee, 1983), and others, such as, AYFYPEL or

YFYPEL with two Tyr residues separated by Phe, resemble the proposed structural characteristics proposed for exogenous opioid peptides (Meisel, 1997).

The aim of this work was to evaluate, by the use of an opioid antagonist, if the activity of these α_{s1}-casein peptides, and the casein hydrolysate containing these, was mediated through the interaction with opioid receptors. In addition, the antihypertensive activity of the casein used as substrate for the peptic hydrolysis process was evaluated. A peptidomic study was performed to identify main differences in the profile of the active hydrolysate and the peptides released during casein simulated gastrointestinal digestion.

2. Materials and methods

2.1 *Peptide synthesis and casein hydrolysate preparation*

Peptides from α_{s1}-casein, fragment (f) (90-94), RYLGY, f(143-149), AYFYPEL and YFYPEL f(144-419) were synthesized in-house by using the method fluorenyl-methoxy-carbonyl chloride (Fmoc) solid-phase. A 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany) was employed. Their purities were calculated by reversed phase liquid chromatography-UV and mass spectrometry. The casein used was a commercial casein product (Promilk-85B Casein, Arras Cedex, France). The preparation of the casein hydrolysate (Lowpept®) was carried out with food-grade pepsin as previously described by Contreras et al. (2009). All other chemicals were obtained from commercial sources and of high quality grade.

2.2. Simulated gastrointestinal digestion

The simulation of gastrointestinal digestion was based on the method reported by Martos, Contreras, Molina, and López-Fandiño (2010), with some modifications. The gastric step in this work included 2.5 mM of $CaCl_2$, and to stop the reaction at the end of the duodenal step, Pefabloc SC (Fluka 76307) was used at a final concentration of 1 mM. The casein powder was dissolved in milliQ water at 5% (w/v) to start the digestion process at gastric phase.

2.3 Analysis by RP-HPLC-MS/MS

The casein hydrolysate and the casein subjected to simulated gastrointestinal digestion were analyzed by HPLC (Agilent Technologies, Waldbronn, Germany) coupled to an ion trap instrument (Esquire 3000, Bruker Daltonik GmbH, Bremen, Germany). The analyses and data processing were performed as previously described by Sánchez-Rivera et al. (2014). The column used was a Mediterranea Sea $_{18}$ 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). Solvent A (water/trifluoroacetic acid, 1000:0.37 v/v), and solvent B (acetonitrile/trifluoroacetic acid, 1000: 0.27 v/v). A linear gradient was used from 0 to 55% of solvent A and 45% of solvent B in 120 min. Spectra were acquired at mass/charge (*m/z*) range of 100-3000. The samples were run at two different target mass: *m/z* of 750 and 1500.

2.3 Animal assays and experimental design

All the animal trials were carried out in agreement with the European Union guidelines for the ethical care and use of laboratory animals (European Directive 86/609/CE). SHR aged from the 14th to the 16th-week were purchased from Elevage Janvier (Le Genest, Saint Isle, France). The animals were housed in

groups of three rats with 12h light/dark cycles and kept at a controlled temperature of 23℃. Standard food (Global Diet 201 4, Harlan, France) and water were available *ad libitum*. The synthetic peptides and the casein hydrolysate dissolved in pure water, were orally administered to rats by a cannula from mouth to stomach, at a single dose of 5 and 300 mg kg^{-1} body weight, respectively. The opioid antagonist, naloxone, was administered by subcutaneous injection, just after the administration of the peptide or the hydrolysate, at a dose of 10 mg kg^{-1} body weight. Control trials were performed by oral administration of water or casein (same dose as the hydrolysate, on protein basis); and by subcutaneous injection of naloxone (10 mg kg^{-1} body weight). The animals were deprived of solid food diet 12 h before experiments. During this period they only had access to NaCl (2 g L^{-1}) and sucrose (80 g L^{-1}) solution. The systolic blood pressure (SBP) were measured using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA), as previously described by Sánchez et al. (2011). The SBP was measured before each experiment to estimate the basal blood pressure. After administration of the peptides, hydrolysate, casein or water, the SBP was measured at 2, 4, 6, 8 and 24 h. However, after the administration of the peptide with naloxone or only naloxone (control), the first 6 h post-administration were recorded.

2.4 Statistical analysis

The effects of casein hydrolysate, peptides, casein, water or naloxone were calculated as changes in SBP (increase or decrease) from the baseline values. Data were expressed as the mean values ± standard error of mean (SEM). Two-way analysis of variance (ANOVA), using GraphPad Prism 5.0 (GraphPad software Inc., San Diego, USA) was carried out. A post-test (Bonferroni) was

applied to establish the significant differences between the effect of the peptides or the hydrolysate vs the controls. Significant differences were considered at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

3. Results

3.1. Antihypertensive activity of the peptic casein hydrolysate, its precursor casein and the synthetic peptides. Reversion by naloxone.

The effect on blood pressure of the casein hydrolysate and the casein used as substrate in its production was followed during 24 h after oral administration at 300 mg kg^{-1} body weight (Figure 1). Non-hydrolysed casein did not produce a significant decrease of the SBP in the animals ($p<0.05$). The peptic casein hydrolysate produced significant SBP decreases vs water at 2, 4, 6 and 8 h post-administration, with maximum decrease post-administration at 6 h (-14.3 ± 4.6 mm Hg). The subsequent recovery occurred from 6 to 8 h.

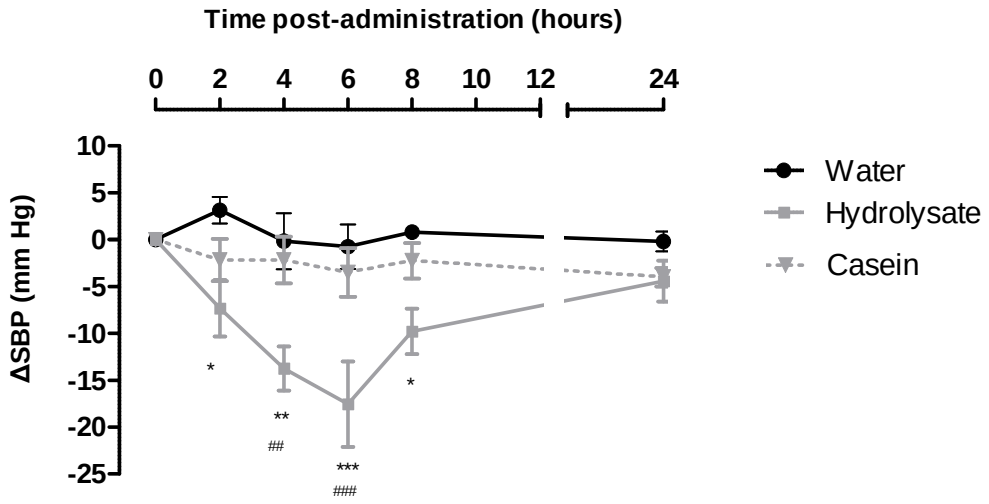

Figure 1. Changes in SBP after oral administration of the casein hydrolysate (300mg kg^{-1} body weight) vs administration of water or casein (300mg kg^{-1} body weight). Data are expressed as mean values ± SEM (n=4-6). Significant differences after administration of the casein hydrolysate vs water are indicated at P≤ 0.05(*) and P≤ 0.01 (**). Significant differences after administration of casein hydrolysate vs the control of casein were found at P≤ 0.01 (##).

The casein hydrolysate was also administered together with naloxone, a competitive antagonist of opioid receptors, and the antihypertensive effect was reverted at 2, 4 and 6 h post-administration (Figure 2A).

Three peptides, RYLGY, AYFYPEL and YFYPEL, contained in the active casein hydrolysate with sequences compatible with that described for casomorphins, were synthetized and orally administered to SHR. The three synthetic peptides produced a significant decrease in the SBP of the animals at all times recorded, i.e., 2, 4, 6 and 8 h (Figure 2 B, C and D). The decrease on SBP recorded after the administration of RYLGY and AYFYPEL was similar to that previously obtained by Contreras et al. (2009) for these peptides at same oral dose. The maximum decrease after the administration of RYLGY (Figure 2A)

was at 6 h (-23.8 ± 2.5 mm Hg), for AYFYPEL the maximum was reached at 4 h (-21.1 ± 3.2 mm Hg) (Figure 2B), and for YFYPEL the maximum was recorded at 6 h (-18.8 ± 3.5 mm Hg) (Figure 2D). When naloxone was subcutaneously co-administered, the antihypertensive effect of RYLGY and AYFYPEL was completely reverted at 2, 4 and 6 h post-administration. In the case of RYLGY, at 8 h, significant differences were still found when co-administered with naloxone. However, in the case of YFYPEL, the opioid antagonist did not significantly affect the blood depressor effect caused by this peptide at 4 and 6 h. Naloxone alone did not exert any effect of the blood pressure of SHRs (Figure 2).

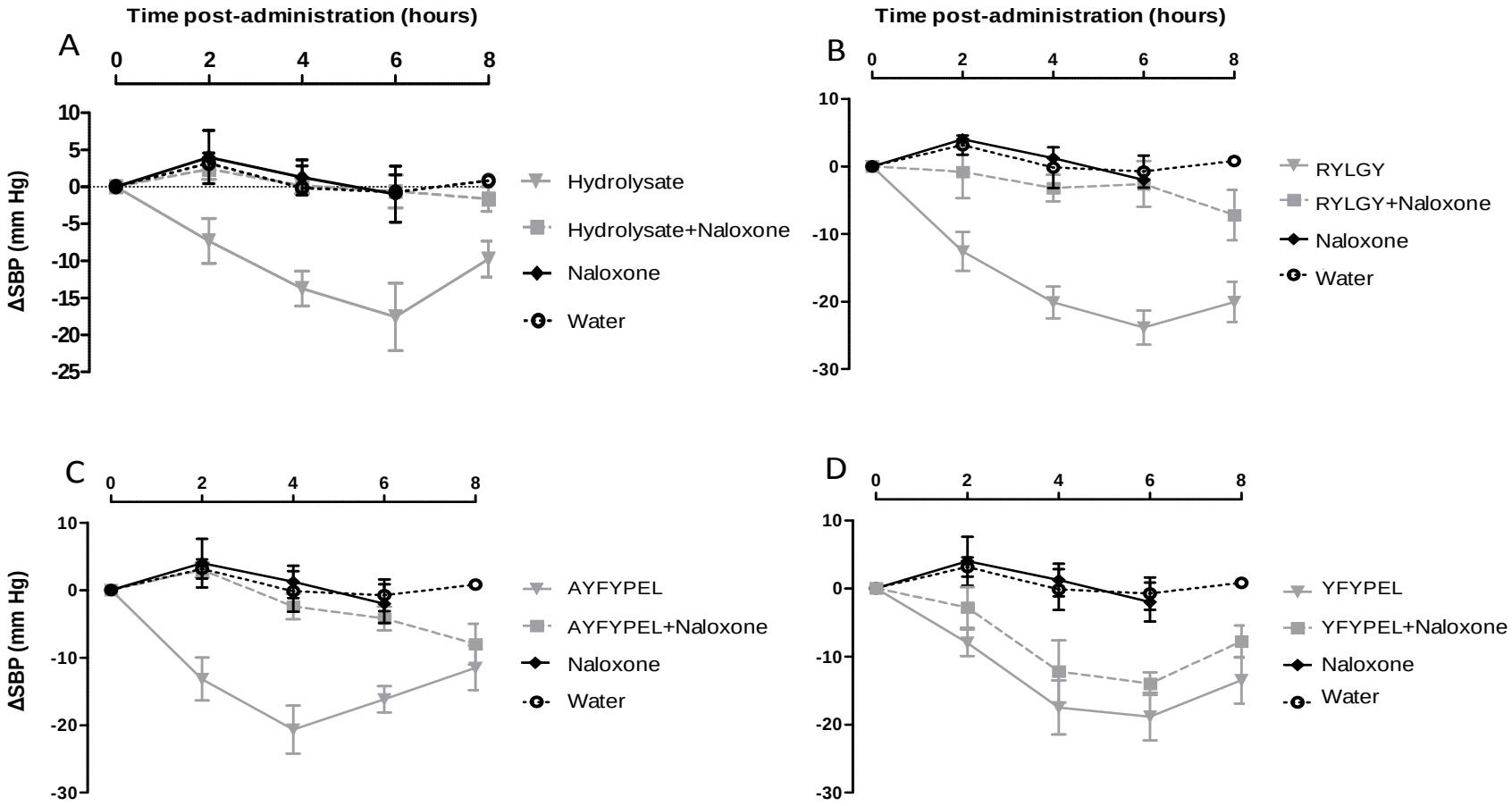

Figure 2. Changes in SBP after oral administration of (A) casein hydrolysate (300mg kg^{-1} body weight), and (B) peptide RYLGY, (C) peptide AYFYPEL and (D) peptide YFYPEL (5mg kg^{-1} body weight), vs co-administration thereof with naloxone or control of naloxone (10mg kg^{-1} body weight, subcutaneous). Data are expressed as mean values ± SEM (n=4-6). Significant differences after administration of the peptide vs the peptide with naloxone at P≤ 0.05(*), P≤ 0.01 (**) and P≤ 0.001(***). Significant differences after administration of the peptide with naloxone vs the control of naloxone at P≤ 0.05(#), P≤ 0.01 (##) and P≤ 0.001(###). Significant differences between the administration of the synthetic peptides and water control were at P≤ 0.05(a), P≤ 0.01 (b) and P≤0.001(c).

3.2. Peptidome of the casein hydrolysate and the casein gastrointestinal digest.

The analysis of the peptidomic profile derived from the peptic hydrolysate is shown in Table 1. The comparison of the hydrolysate peptide profile with the precursor-casein gastrointestinal digest is shown for α_{s1}-casein (Figure 3), since the three active peptides belong to this protein. As it can be observed, most sequences are not coincident. Among others, the active peptide α_{s1}-casein f(90-94), RYLGY, could not be found after simulated digestion of casein. On the contrary, some common peptides could be identified in both samples i.e. f(25-31), VAPFPEV, f(143-149), AYFYPEL, f(144-149), YFYPEL f(165-172), YYVPLGTQ, and f(173-179), YTDAPSF (Figure 3).

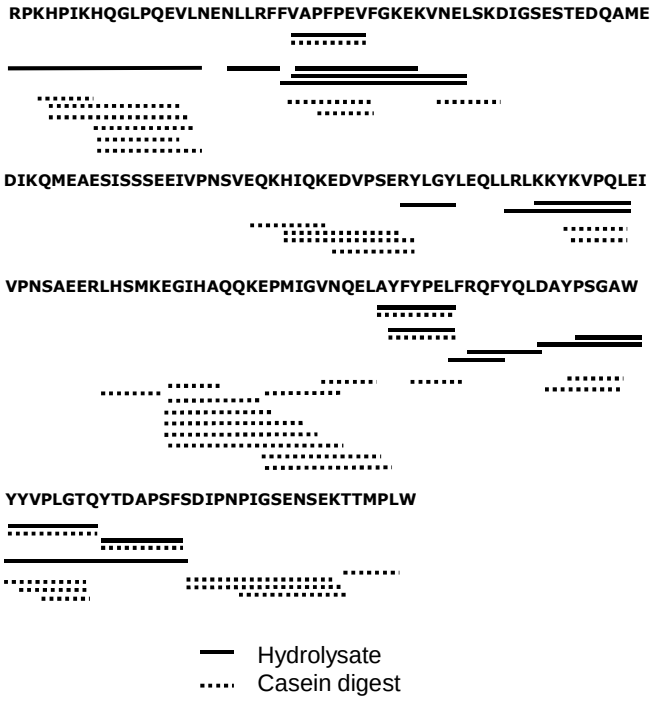

Figure 3. Identified peptides from α_{s1}-casein present in the casein hydrolysate and in the precursor-casein digest.

Table 1. Identified peptides from casein (CN) hydrolysate. Peptides with favorable structural features for opioid receptor binding are highlighted in bold.

Observed Mass	Calculated Mass	Range	Sequence
β-CN			
675.300	674.293	1-5	RELEE
1284.700	1283.693	1-11	RELEELNVPGE
1300.700	1299.693	59 - 70	VYPFPGPIPNSL
1320.800	1319.793	81 - 92	PVVVPPFLQPEV
1451.900	1450.893	81 - 93	PVVVPPFLQPEVM
1057.600	1056.593	85 - 93	PPFLQPEVM
746.500	745.493	94 - 100	GVSKVKE
1244.700	1243.693	94 - 105	GVSKVKEAMAPK
1473.900	1472.893	128 - 140	TDVENLHLPLPLL
1587.000	1585.993	127 - 140	LTDVENLHLPLPLL
1689.000	1687.993	128 - 142	TDVENLHLPLPLLQS
1472.900	1471.893	130 - 142	VENLHLPLPLLQS
1372.800	1371.793	129 - 140	DVENLHLPLPLL
1257.800	1256.793	130 - 140	VENLHLPLPLL
1698.900	1697.893	143 - 156	WMHQPHQPLPPTVM
787.400	786.393	157 - 163	FPPQSVL
748.400	747.393	193 - 198	YQEPVL
1782.200	1781.193	193 - 208	YQEPVLGPVRGPFPII
α_{s1}-CN			
939.000	1875.985	1-16	RPKHPIKHQGLPQEVL
662.400	661.393	19 - 23	NLLRF
1837.100	1836.093	24 - 39	FVAPFPEVFGKEKVNE
758.400	757.393	25 - 31	VAPFPEV
1219.700	1218.693	25 - 35	VAPFPEVFGKE
1690.000	1688.993	25 - 39	VAPFPEVFGKEKVNE
671.400	670.393	90 - 94	**RYLGY**
693.500	1384.985	99 - 109	LRLKKYKVPQL
1003.700	1002.693	102 - 109	KKYKVPQL
902.400	901.393	143 - 149	**AYFYPEL**
831.400	830.393	144 - 149	**YFYPEL**
710.500	709493,0	149 - 153	LFRQF
854.500	853.493	151 - 156	RQFYQL
866.400	865.393	157 - 164	DAYPSGAW
517.300	516.293	160 - 164	PSGAW
940.500	939.493	165 - 172	YYVPLGTQ
1721.900	1720.893	165 - 179	YYVPLGTQYTDAPSF
800.300	799.293	173 - 179	YTDAPSF
κ-CN			
1422.900	1421.893	18 - 29	FSDKIAKYIPIQ
1585.900	1584.893	18 - 30	FSDKIAKYIPIQY
833.400	832.393	43 - 49	YQQKPVA
946.600	945.593	43 - 50	YQQKPVAL
1253.700	1252.693	56 - 66	LPYPYYAKPAA
804.400	803.393	162 - 169	VQVTSTAV

Table 1 continue

α_{s2}-CN			
973.500	972.493	89 - 95	**YQKFPQY**
888.400	887.393	187 - 193	QKAMKPW

4. Discussion

The three α_{s1}-casein-derived peptides studied caused a significant decrease in SBP of SHR at 2, 4 and 6 h post-administration. To the best of our knowledge, this is the first report on the antihypertensive activity of YFYPEL. The blood pressure reduction caused by RYLGY, AYFYPEL and the casein hydrolysate was antagonized by the co-administration of the opioid antagonist, naloxone at all the times studied (Figure 2). The opioid activity of homologous, but slightly longer peptides than RYLGY, had been previously reported. Concretely, α_{s1}-casein f(90-96), RYLGYLE, showed the most potent opioid activity in different bioassays, followed by α_{s1}-casein f(90-95), RYLGYL (Loukas et al., 1983). These α_{s1}-casein-derived peptides have been described as, moderate in strength, δ-opioid agonists. Some food-derived peptides, for instance, α-lactorphin, which is described as a μ-opioid receptor ligand with low potency, could produce a decrease in SBP after administration to SHR by interaction with opioid receptors (Nurminen et al., 2000). The implication of central opioid receptors would be undesirable in these types of food products, and in fact, the effect produced by α-lactorphin was not related to central opioid receptors (Ijäs et al., 2004). This opioid peptide was also demonstrated to improve endothelial function associated to an increase of nitric oxide (Sipola et al., 2002). Opioid receptors have been reported to play a role in the heart and

cardiovascular system and it has been proposed that a deficiency of the κ-opioid receptor may cause hypertension, since this receptor may be a component in the central nervous system involved in the regulation of blood pressure (for a review, see Pugsley, 2002). Several studies have also indicated the contribution of endogenous opioid peptides in the regulation of blood pressure. After i.v. administration of μ- and δ- and κ-opioid agonists to stressed rats, the involvement of μ- and δ-opioid receptors was demonstrated and they were able to reduce blood pressure and heart rate (Sun, Liu, Li, & Ingenito, 1996). Endomorphin 2 and its homologous (D[Ala^2]-endomorphin 2) endogenous ligands for the μ-opioid receptor, decrease heart rate, cardiac output, and total peripheral resistance when administered i.v., and these responses were reverted by naloxone and were mediated by the release of nitric oxide from the vascular endothelium (Champion & Kadowitz, 1998). Similarly, the active casein hydrolysate tested in this study had previously demonstrated an improvement of endothelial function in aortic and mesenteric rings accompanied by an increase of eNOS expression in aorta (Sánchez et al., 2011). In the same study, the animals fed with the hydrolysate had a significant reduction of left ventricle weight (compared with the control group) and a significant decrease in collagen deposition measured in left ventricle. Receptor binding studies in the heart, have confirmed the existence of the δ-opioid receptor (Krumins, Faden, & Feuerstein, 1985; Ventura, Bastagli, Bernardi, Caldarera, & Guarnieri, 1989), although the distribution of the δ-opioid receptor in the rat heart favors atrial tissue and the right side of the heart more than the left side of the heart (Barron, 1999).

In the case of peptide AYFYPEL, naloxone antagonized the effect of this peptide on SBP. Naloxone is a non-selective opioid antagonist with slightly higher

affinity for μ-opioid receptors and with lower affinity for κ- and δ-opioid receptors. The common structural features between endogenous and exogenous opioid peptides are the presence of Tyr at N-terminal end and Phe or Tyr at third or fourth position, except for α-casein opioids, which contain an Arg as N-terminal residue (Meisel, 1997). In spite of the structural similarities between AYFYPEL and its related peptide, YFYPEL, which possesses the favorable features for opioid receptor binding, naloxone did not significantly affect the antihypertensive effect of the latter one. Although, to the best of our knowledge, the presence of Ala at N-terminal has not been reported as a favorable feature for opioid receptor binding, in this case it seemed to be crucial for the activity of AYFYPEL, and indeed, the absence of antagonism for YFYPEL.

In addition, RYLGY and AYFYPEL were also found to have potent in vitro ACE-inhibitory activity (IC_{50} values of 0.71 μM ± 0.08 and 6.58 μM ± 0.50, respectively) and antioxidant activity (2.829 and 3.216 μmol equiv Trolox μmol^{-1} peptide, respectively, determined by the method of oxygen radical absorbance capacity) (Contreras et al., 2009). The contribution of the ACE-inhibitory activity of food derived peptides has been extensively studied (Ricci-Cabello, Herrera, & Artacho, 2012; Korhonen, 2009). Moreover, it has been proposed the beneficial effects of food antioxidants in hypertension and cardiovascular disease (Schiffrin, 2010). For instance, some studies have revealed that antioxidant vitamins reduce blood pressure and arterial stiffness in patients with diabetes (Mullan, Young, Fee, & McCance, 2002). In any case, although further studies focused on the contribution of other potential mechanisms are already in progress, our results demonstrated that the two previously identified active peptides (RYLGY and AYFYPEL) in the hydrolysate exert their effect mediated by the opioid system.

According to our results, the antihypertensive activity of YFYPEL did not seem to involve opioid receptors. The mechanism of action of this new antihypertensive peptide deserves further investigations. Under our trial conditions the activity of the hydrolysate is mediated by interaction with opioid receptors in a naloxone-antagonizable manner. This would suggest that the contribution of the opioid mechanism in its activity is predominant over other pathways.

Since the precursor casein of the hydrolysate showed no antihypertensive activity compared with the hydrolysate, a peptidomic analysis thereof and the precursor-casein digest was performed in order to understand the different behaviour. Some differences were revealed. Interestingly, α_{s1}-casein f(90-94), RYLGY, present in the peptic casein hydrolysate, was not found after gastrointestinal digestion of the precursor casein, as revealed by the peptidomic analysis. On the contrary, peptides α_{s1}-casein f(143-149), AYFYPEL, and α_{s1}-casein f(144-149), YFYPEL, were found in both samples among others [f(25-31), f(173-179) and f(165-172)]. Moreover, AYFYPEL had been previously found in calves' stomach content after ingestion of whole casein (Yvon & Pelissier, 1987), and in human gastric effluents after ingestion of milk, although not in duodenum (Chabance et al., 1998). In this latter study, YFYPEL was found in duodenal effluents. In any case, this study confirms the antihypertensive activity of AYFYPEL, and reports for the first time that of YFYPEL after their individual oral administration to SHR, although the mechanism involved in the effect produced by the latter one deserves future investigations. Thus, the possible matrix effect and the synergism between peptides in the overall activity of the hydrolysate should not be discarded.

The peptidomic study of the casein-simulated gastrointestinal digest and the active hydrolysate showed that although the casein hydrolysate is produced by pepsin, a gastric enzyme, the nature of the peptides or their concentration within the hydrolysate is different than that generated during gastrointestinal digestion. These differences, between casein and the hydrolysate, are sufficient to elicit or not an antihypertensive effect when orally administered to SHR at the same dose.

4. Conclusions

These results provided evidence of involvement of the opioid receptors on the antihypertensive activity of a peptic casein hydrolysate. This suggested that several peptides present in this hydrolysate could exert antihypertensive activity related to the opioid system. The results obtained for peptide RYLGY and AYFYPEL showed that the opioid system is involved on their antihypertensive effect. In the case of YFYPEL, although its effect is mediated by other mechanism of action since there was no evidence of such implication under our trial conditions, the present work represents the first report on the antihypertensive activity of this peptide. In this respect, the involvement of other mechanisms of action deserves future investigations. In addition, the casein hydrolysate produced a significant decrease in SBP, while the precursor casein administered at the same dose did not. The peptidomic study showed that although there are similarities between the casein digest and the casein hydrolysate, the appearance of certain peptides under these conditions of hydrolysis with food-grade pepsin would explain the difference in activity.

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3. DISCUSSION

In the last years, the effect of the in vivo processes undergone by the food components in the organism once ingested has been increasingly pointed out, since the resulting products may have subsequent health implications. In addition, the production of active sequences within food matrices has been widely studied by using enzymatic hydrolysis or fermentation with acid lactic bacteria. Part of the work included in this thesis is related to the analysis of food matrices by the application of peptidomic techniques targeted at active peptides or untargeted. In this regard, peptidomics was applied to the characterisation of casein hydrolysates produced by different strains of yeasts, focused on certain antihypertensive sequences (chapter I). Furthermore, the whole peptide profile of cheeses where different packaging technologies have been used, and its evolution during storage time, was studied in chapter II. Subsequently, the changes of milk proteins during gastrointestinal digestion and the generation of peptides through this process, focusing on bioactive sequences were evaluated in this thesis (chapter III and IV). In chapter III, the influence of the proteolytic state of two dairy matrices on the release of peptides during digestion was assessed. Likely, food processing, such as heat treatment, can affect the behavior of proteins and the formation of peptides through digestion, as shown in chapter IV. Moreover, in publication V the oral bioavailability and the pharmacokinetic parameters of an antihypertensive peptide derived from β-casein, f(134-138), have been estimated in a rat model. In this study, several fragments derived from metabolism of this peptide were detected in plasma. In order to confirm the possible derived fragments generated from this β-casein sequence, in vitro incubation of the synthetic peptide in plasma was performed, and the antihypertensive activity of the identified derived fragments was assayed (chapter VI). Finally, the involvement of the opioid system in the antihypertensive activity of a peptic casein hydrolysate produced by food-grade pepsin and three of its main peptides was evaluated in chapter VII.

3.1 Application of a peptidomic tools to the identification of peptides during production, shelf-life of the product and changes during digestion.

Fermentation and enzymatic hydrolysis with food-grade enzymes has been widely used to produce bioactive peptides in dairy matrices. For instance, the antihypertensive sequences RYLGY and AYFYPEL from α_{s1}-casein f(90-94) and f(143-419), respectively, were previously identified by our group as two of the main peptides present in a casein hydrolysate produced with food-grade pepsin (Contreras et al., 2009). Similarly, the group also identified the antihypertensive peptide f(133-138), LHLPLP, as one of the major peptides in fermented milk produced by *Enterococcus faecalis* (Quirós et al., 2007). However, the pathogenic risks of *Enterococcus* genus strains may limit their use in the food industry (Franz et al., 1999; Ogier & Serror, 2008). Thus, an optimisation of enzymatic hydrolysis for the production of HLPLP was carried out (Quirós et al., 2012), since this penta-peptide was also shown to exert an antihypertensive effect, and it was released by the action of brush border enzymes (Quirós et al., 2008; Miguel et al., 2010). Recently, casein was demonstrated to be a suitable substrate for yeast strains such as *Debariomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* to produce antihypertensive hydrolysates (García-Tejedor et al., 2013). This could represent a good alternative to fermentation with lactic acid bacteria as they are considered GRAS (Generally Recognised As Safe) microorganisms. These fermentation processes usually lead to complex peptide profiles. In this regard, in chapter I, the casein hydrolysates produced by different yeasts strains of *D. hansenii*, *K. lactis* and *K. marxianus* where ACE-inhibitory activity was observed, were analysed by MS-based techniques. This analysis was targeted at previously reported active peptides present in an enzymatic hydrolysate i.e RYLGY, AYFYPEL (Contreras et al., 2009), in fermented milk i.e. LHLPLP, and its derived peptide (HLPLP); and in sour milk i.e. VPP and IPP (Nakamura et al., 1995a). The peptides RYLGY, AYFYPEL, VPP and IPP could not be identified in any of the

hydrolysates produced by the yeasts strains used in chapter I. However, LHLPLP and HLPLP were present in two of the casein hydrolysates produced by strains of *D.hansenii* (Dh1 and Dh14) at concentrations of 15.6 and 52.7 µg/mg in Dh1 and Dh14, respectively in the case of the first sequence, and 43.5 and 46.4 µg/mg in Dh1 and Dh14, respectively, for the latter peptide. In spite of these differences in the concentration of LHLPLP between the two hydrolysates, no significant differences in ACE-inhibitory values were found among the two hydrolysates. These results suggest the implication of other sequences in the potency of ACE-inhibitory activity of the hydrolysates. Indeed, the zymography assay carried out on the hydrolysates to evaluate the extracellular proteolytic activity, indicated a different proteolytic profile for both hydrolysates, which was reflected on the peptidic profile. The β-casein region containing residues 128-140 gave rise to numerous peptides beside the active sequences LHLPLP and HLPLP, i.e. LHLPLPL and HLPLPL, among others. The two latter peptides have been also identified in Valdeón cheese digest (chapter III), and in jejunal content of humans after ingestion of casein (Boutrou et al., 2013). In spite of the absence of IPP in the hydrolysates produced by *D. hansenii*, several sequences identified shared some residues with this active peptide i.e IPPLT, IPPLTQT or IPPLTQTP. In this regard, gastrointestinal digestion could play an important role in the release of active fragments from some of these sequences, which deserves further investigations.

In any case, the generation of the two sequences LHLPLP (f(133-138) and HLPLP (134-138) by yeasts within a casein matrix provides an alternative to the use of lactic acid bacteria for the production of active hydrolysates. The results provided by this work are relevant since this region of the β-casein (residues 128-140) is a well conserved domain between different species, and has been underlined as a resistant core of the protein to *in vitro,* static and dynamic, gastrointestinal digestion (chapter III

and IV), and the shorter fragment, HLPLP, was absorbed into blood circulation in rats (chapter V).

On the other hand, technological processing leads to modifications in food components, which can ultimately affect the protein behavior and the nutritional value of food, as pointed out in previous sections. Once the production has been studied and optimised, as it could be the case of a fermented product or an active hydrolysate (chapter I), the packaging and the storage time of the final product is an important issue to take into account, especially for cheese (Pantaleão et al., 2007). Cheese undergoes proteolysis during ripening. In this regard, the application of MS-based techniques to study the proteolysis phenomena in cheese has been reported as a useful tool (Piraino et al., 2007). In addition, when cheese is packed in order to extend its life-time the impact of packaging on the ripening process should be assessed. However, to our knowledge, few comparative studies on the peptide profile of cheese in regard of the packaging technologies have been carried out. Thus, in chapter II, peptidomic analysis of semi-hard cheese packed with two different systems, vacuum packaging (VP), and modified atmosphere packaging (MAP) was studied. The evolution of the peptide profile along the storage time and their differences depending on the packaging systems were evaluated. The UV chromatograms were not different between VP and MAP cheeses. However, the MS profile revealed some differences. Numerous peptides from the N-terminal region of α_{s1}-casein (i.e. residues 1-23) were found in both cheeses (VP and MAP). The peptide bond Phe^{23}-Phe^{24} is susceptible of cleavage by chymosin, giving rise to two major fragments: α_{s1}-casein f(1-23) and f(24-199) (McSweeney & Fox, 1993). In this regard, the results obtained are in accordance to what it was reported for Emmental cheese (Gagnaire et al., 2001). However, in Valdeón cheese (chapter III) this trend at N-terminal was not observed before digestion; in contrast, more homogeneous distribution of the peptide profile throughout the protein was observed. This could be due to the different proteolysis phenomena

undergone by semi-hard and blue cheeses. Conversely to α_{s1}-casein, the peptides derived from β-casein built up a profile homogenously distributed throughout the protein sequence in agreement to the results reported during Emmental cheese ripening (Gagnaire et al., 2001). Overall, although slight qualitative differences in the peptide profile from α_{s1}-casein with respect of the packaging technology, no differences were found for β-casein. However, the relative abundance of some sequences was different in VP and MAP at 90 days of storage time. For instance α_{s1}-casein f(24-32) and (25-32), and β-casein f(44-52) achieved greater intensities in VP. On the contrary, the relative abundance of α_{s1}-casein f(1-16) and f(46-52) and f(74-82) was higher in MAP. While the behavior of β-casein peptides during the storage time was similar regardless of the packaging technology, differences could be detected for α_{s1}-casein peptides among VP and MAP.

In terms of bioactivity, some of the identified peptides in both semi-hard cheeses have been previously reported as active sequences. Among these sequences (nine), only three of them were common for both semi-hard and Valdeón blue cheese before digestion (chapter III). Nonetheless, out of those three sequences, only the ACE-inhibitor β-casein f(47-52), DKIHPF (Gómez-Ruiz et al., 2004; 2006), survived gastrointestinal digestion of the blue cheese (chapter III), which is consistent with the identification of this sequence *in vivo* (Boutrou et al., 2013). However, the other two β-casein sequences i.e. the opioid peptide f(60-68), YPFPGPIPN (β-CM 9) (Jinsmaa & Yoshikawa, 1999) and the antihypertensive sequence f(169-175), KVLPVPQ (Maeno et al., 1996), did not resist in vitro digestion of cheese (chapter III). Nevertheless, other active peptides, not identified in the peptidomic profiles of semi-hard or Valdeón cheese, were generated through gastrointestinal digestion, as in the case of the antihypertensive f(133-138), LHLPLP from β-casein (chapter III). In any case, great number of biologically active peptides could be found after gastrointestinal digestion

although it remains to be clarified if these are produced in vivo in a significant amount to exert a physiological effect.

The important role of gastrointestinal digestion on the formation and bioavailability of peptides derived from milk proteins has been highlighted. The physiological changes that food undergoes upon ingestion determine their bioaccesibility, and ultimately their bioactivity (Fernández-García et al., 2009). Once the food matrices have been characterised, in terms of production of active sequences i.e. HLPLP and LHLPLP in yeasts hydrolysates, or the influence of packaging technologies and the storage time in the peptide profile of proteolised matrices, the next step was to study the impact of digestion on food proteins and on the stability or formation of peptides. In this respect, it is known that certain sequences that showed *in vitro* activity, did not exert any *in vivo* biological effect, and vice versa. In some cases, the explanation lies on the effect of digestion on the stability of these peptides. For instance the case of Lactokinin, which was not stable enough to digestion to maintain its activity (Walsh et al., 2004). In addition, some conditions may affect proteins and the subsequent release of peptides upon digestion, such as the free ends and cleavage sites which are determined by the proteolytic state of the matrix. Thus, in chapter III, Spanish blue cheese (Valdeón) and skimmed milk powder (SMP), two dairy matrices containing the same proteins but in different degree of proteolysis were digested using an *in vitro* static digestion model (Martos et al., 2010). In this work, the exhaustive peptidomic analysis of undigested cheese revealed the presence of several previously reported active sequences in cheese. Nevertheless, the simulated digestion process of both food matrices led to the release of a high number of peptide sequences, among them, previously reported active peptides, such as antihypertensive, antibacterial or antioxidant sequences. The total peptide homology estimated for the cheese before and after digestion (12%) and between cheese and SMP digests (19%) suggested that digestion process could bring closer the resulting products from certain regions of the

protein, even if the matrices and the proteolysis degree were initially different. For instance, the β-casein domains 60-93, 128-140 and 193-209 were found to be resistant in both, cheese and SMP digests, probably due to the presence of hydrophobic residues such as Pro (Vanhoof et al., 1995). The region comprised between residues 128-140 was found to be especially resistant to hydrolysis during cheese ripening and digestion thereof, and also in SMP digest. This is consistent with results from in vitro dynamic gastric digestion of SMP (chapter IV), *in vitro* static digestion of β-casein (Dupont et al., 2010a) and also from humans studies after ingestion of casein (Boutrou et al., 2013). In this sense, these results are relevant since they highlight the resistance of this β-casein domain to in vitro (dynamic and static models) and in vivo digestion that hosts the active sequences LHLPLP and HLPLP.

A higher number of peptides was found in cheese digest compared to SMP. This can be attributed to the extensive proteolysis phenomena undergone by blue cheese (Cantor et al., 2004;, Sousa et al., 2001; Diezhandino et al., 2013; Fresno et al., 2013), which exposes numerous cleavage sites to digestion enzymes. It has to be underlined that the α_{s1}-casein sequence YFYPEL f(144-149), described as antioxidant (Suetsuna et al., 2000), mucin production stimulant (Martínez-Maqueda et al., 2013) and antihypertensive (chapter VII), was found in cheese and SMP digests. However, its related peptide, the sequence AYFYPEL f(143-149), was not detected after digestion of SMP, but only in digested cheese. These results are interesting because both sequences (YFYPEL and AYFYPEL) are present in the active casein hydrolysate produced with food-grade pepsin used in chapter VII, and were both identified in SMP after gastric dynamic digestion (chapter IV). Likely, AYFYPEL was found in stomach effluents from calves after ingestion of milk, nevertheless YFYPEL was identified in duodenal contents in the same study (Chabance et al., 1998). These two sequences are formed in gastric phase by the action of pepsin, but it seems that YFYPEL can survive intestinal digestion. Since AYFYPEL could be found in blue cheese, but not in SMP after gastrointestinal simulation, this could suggest the influence of the food

matrix on the release of peptides during digestion. Likewise, the antihypertensive peptide LHLPLP f(133-138) from β-casein, was present in cheese digests, but not detected in digested SMP. This fact could be related to the need of a precursor sequence in the matrix in order to release this active peptide through digestion. Moreover, the presence of this peptide has been recently reported in jejunal effluents from humans after ingestion of casein (Boutrou et al., 2013). In this sense, fermented milk containing LHLPLP, the major responsible for its activity, caused blood depressor effect on SHR after long term intake compared to the fermented milk lacking LHLPLP, with no ACE activity, used as negative control (Miguel et al., 2005). Thus, this fact brings up the question whether the amount of this sequence released *in vivo* from casein could elicit appreciable biological effect.

Dynamic digestion models are more realistic in terms of physiological parameters compared to the static ones (Guerra et al., 2012). Therefore, in this thesis, chapter IV represents a forward step in the study of digestion of milk proteins after the static *in vitro* model. Some technological processes such as heat treatment may influence the behavior of milk proteins through digestion. Therefore, the impact of heat treatment on protein and the subsequent release of peptides during gastric dynamic digestion were evaluated in chapter IV.Heat treatment affected the behavior of proteins in different manner, as shown in the electrophoretic runs. The increase of the resistance to digestion observed for caseins was in accordance to previously reported results in caseins (Picariello et al., 2010), β-casein or in raw milk (Dupont et al., 2010a; 2010c). However, the β-Lg became more susceptible to hydrolysis by pepsin, even though it is known to be resistant to gastric digestion. Probably the heat-induced structural changes that led to the exposure of the cleavage sites made the protein more susceptible to the action of pepsin (Peram et al., 2013). The different behavior of these proteins through digestion of heated and unheated milk could also be explained by the heat-induced aggregates that may be formed between β-Lg and caseins (Guyomarc'h et al., 2003).

Although the impact of heat treatment on the behavior of milk proteins during digestion has been widely evaluated, the application of MS-based techniques to study peptide release has been less studied (Kopf-Bolanz et al., 2014; Dupont et al., 2010b; 2010c). Heat treatments irreversibly affect conformational status and aggregation of the protein matrix. Therefore, peptidomes arising from digestion are expectedly divergent, at least to some extent (Nyemb et al., 2014). The peptidomic analysis in this work has permitted to evaluate the influence of heating on the final products resulting from milk protein digestion, focusing on the active sequences. The peptide homology calculated in this work allowed to highlight the difference in the pattern of peptide release at short times of digestion and the importance of the exposure time to enzymes on the release of peptides. Indeed, it is not after 50 min that digestion starts to bring closer the peptidome of certain regions of proteins from heated and unheated milk. Nevertheless, at the end of digestion only 48% of peptide homology was achieved, which points out the impact of the heat treatment on the identity of the peptides generated through digestion. The resistance of certain regions to gastrointestinal enzymes has been observed, and some of them were common for heated and unheated milk. This is the case of the β-casein domains comprised between residues 76-93, 128-140 or the C-terminal part of the protein, as also reported in chapter III, and coincident with highly hydrophobic areas at low pH (Dupont et al., 2010c). The MS-analysis also revealed some differences between heated and unheated milk, not only in the identity of peptides but also in the pattern of release thereof. These differences could have health implications from the bioactivity point of view and also regarding the generation or resistance of epitopes. The region 76-93 of the protein hosts various binding epitopes, for instance f(82-93), still identified at 405 min of digestion in unheated milk, and previously described as an IgE binding fragment (Benedé et al., 2014). With respect to the time of peptide release some sequences had different hydrolysis rate in heated and non-heated milk. This fact could represent an important aspect to consider in terms of nutritional and physiological efficiency, since the absorption rate and the postprandrial

N utilization is related to this issue (Lacroix et al., 2006b, c; Deglaire et al., 2009a). The gastric step plays a key role in digestion since the peptides generated during this phase will be delivered to duodenum, where they can be further hydrolysed, be absorbed or interact with intestinal receptors in situ (Langerholc et al., 2011; Shimizu et al., 2010). Both, the absorption rate of these products as well as the interaction with receptors at intestinal epithelium may be affected by the hydrolysis rate of peptides during digestion. Some examples of the identified active sequences with different rate of hydrolysis in heated and unheated milk are α_{s1}-casein f(25-32) (VAPFPEVF), α_{s1}-casein f(90-94) (RYLGY) or β-casein f(94-105) (GVSKVKEAMAPK), β-casein f(132-140) (NLHLPLPLL), α_{s1}-casein f(144-149) (YFYPEL), among others. The two latter peptides were also found in chapter III, in cheese and SMP digests. In addition, these sequences could be detected in intestinal effluents of humans after ingestion of casein (Boutrou et al., 2013) and milk (Chabance et al., 1998), respectively. These results indicate that they may be formed at gastric phase (chapter IV), and they can resist intestinal digestion in vivo, regardless of the matrix ingested, a protein fraction (casein) or milk. Likely, bioactive peptides from other proteins could be found in heated or unheated milk, for instance, α_{s2}-casein f(183-206) and f(183-207), reported as antibacterial (Recio et al., 1999), and also identified in effluents gathered from mini-pigs after ingestion of milk (Barbé et al., 2014). These results imply the identification of several peptide sequences during dynamic gastric digestion that are also generated *in vivo*, which in sufficient amount may elicit a biological effect, and in some cases also correlate with their formation *in vitro.* Altogether, these results suggest the importance of digestion in the formation or resistance of bioactive peptides.

3.2 Bioavailability of food-derived peptides. ADME studies

Once the resistance of some peptides to digestion has been assessed by the application of gastrointestinal digestion models, it is important to establish their bioavailability. Little information is available on the absorption of hypotensive food-derived peptides, which is essential to determine their mechanism of action and a cause-effect relationship. Previous works of our group had identified the β-casein peptide LHLPLP in fermented milk produced by different strains of *E. faecalis* (Quirós et al., 2007). chapter I, shows that this peptide can be also produced with similar efficiency by food-grade yeasts. In fact, LHLPLP was specifically proven to be resistant to gastrointestinal digestion (Quirós et al., 2009), although it was further hydrolysed by brush border enzymes, releasing the penta-peptide, HLPLP, which was transported through Caco-2 monolayer (Quirós et al., 2008). The penta-peptide retained hypertensive activity (Miguel et al., 2010). In addition, the β-casein domain 128-140, where these sequences are included, was found to be resistant to digestion as previously discussed (chapter s III and IV). This background brought up the question about the ability of the penta-peptide HLPLP to reach blood circulation, since generally only amino acids, di- or tri-peptides derived from food had been previously demonstrated to be absorbed in plasma. For instance IPP, LPP and VPP from casein reported in pigs (van der Pijl et al., 2008) and in humans (Foltz et al., 2007). In chapter V, the peptide f(134-138), HLPLP, from β-casein was identified in plasma samples collected from rats fed the synthetic peptide, using an UPLC coupled to a Q-TOF instrument. This is the first report of the absorption and of the pharmacokinetic parameters for a food-derived penta-peptide (HLPLP) in rats fed the synthetic peptide. In addition, several derived fragments from HLPLP could be detected in blood stream as a result of the action of plasmatic peptidases on this peptide. The MS-based analytical method developed after the exhaustive optimisation of the parameters was shown to be sensitive enough to detect and quantify the penta-peptide in plasma with a

detection limit of 0.03 nmol/L (0.02 ng/mL). It has to be underlined that the oral bioavailability obtained for HLPLP (5.18%), was greater than that observed for the tri-peptides above mentioned, estimated at 0.059-0.077% in a pig model (van der Pijl et al., 2008).

Two derived fragments from β-casein i.e f(135-138), LPLP and f(134-137), HLPL, were detected in plasma after intravenous administration of HLPLP. After oral administration they could also be detected, although not quantified, because they were under the LOQ of the analytical method. After intravenous administration, the peptides LPLP and HLPL represented 206.7% and 21.8%, respectively, of the precursor peptide, which suggest a wide biotransformation of HLPLP by plasma peptidases. The formation of LPLP could be predominant compared to HLPL, or the latter might undergo faster degradation, which would lead to a lower concentration of HLPL in either case. Probably, the cleavage at His-Leu from HLPLP is favored compared to Leu-Pro, since the Pro confers more resistance to peptidases (Vanhoof et al., 1995). Moreover, the two Pro residues present in the sequence LPLP may have impaired its further degradation. Keeping in mind the metabolism of HLPLP after oral and intravenous administration, the oral bioavailability calculated for this sequence could have been underestimated, since this parameter was established only considering the intact amount of the penta-peptide, and not any of its derived fragments. These results represent relevant and valuable information since these derived fragments generated in vivo by plasmatic peptidases have influence on the pharmacokinetic behavior of HLPLP and might affect the *in vivo* physiological effect produced by this peptide.

In chapter VI, the in vivo metabolism of HLPLP was confirmed by in vitro incubation thereof in rat plasma. The analytical method used in chapter V was adapted to achieve the best conditions focused on the possible fragments derived from HLPLP metabolism. After *in vitro* incubation of the peptide HLPLP in plasma the fragments LPLP, HLPL and HLP were identified. The tri-peptide HLP could not be detected in

vivo, nor after intravenous neither after oral administration of the penta-peptide (chapter V). Probably, this fact was a matter of concentration that precludes its detection in vivo. Matsui et al. (2000) conducted an *in vitro* incubation of the peptide IVY in rat and human plasma that resulted in the identification of VY and Y. Although the hydrolysis of the parent sequence occurred faster in human plasma compared to rat, both in vitro incubations resulted in the formation of the same derived fragments. Therefore, as the in vitro incubation in rat plasma was similar to that in human in qualitative terms, it could be considered a suitable approach to confirm the generation of the derived fragments, as in the case of our work.

The sequences derived from HLPLP metabolism were orally administered to SHR to evaluate their antihypertensive activity. All the peptide fragments identified after in vitro incubation of HLPLP in plasma (LPLP, HLPL and HLP) retained antihypertensive activity after oral administration to SHR. These results are important because the effective absorption of HLPLP was estimated only considering the parent peptide, and this work demonstrates the hypotensive effect of its derived fragments when orally administered. Thus, the in vivo effect of HLPLP is influenced by the generation of these peptide fragments. Furthermore, two additional peptide fragments that could possibly be generated from HLPLP in plasma were also tested in SHR i.e LPL and PLP, and showed antihypertensive activity. Indeed, PLP, which has Pro residues at ultimate and penultimate position, meets structural features that favour the binding to ACE (Cheung et al., 1980; Rohrbach et al., 1981), and also confer resistance to peptidases. In this regard, this work provides a novel tri-peptide from β-casein (PLP) that could derive from metabolism of the penta-peptide in plasma, which showed long-lasting hypotensive effect even at 24h post-administration.

Considering the $T_{1/2a}$ (2.79 min) and the time to reach maximum concentration ($T_{max,}$ 11.88 min) estimated for HLPLP in chapter V, the peptide was rapidly absorbed, and the maximum SBP decrease was observed at 2h post-administration (chapter VI).

This value of $T_{1/2a}$ was similar to that found for IPP (3.3 min) and for VPP (4.6 min); although their T_{max} 8.6 and 8.9 min, respectively, (van der Pijl et al., 2008), were lower than that observed for HLPLP. Interestingly, the maximum decrease on SBP produced by IPP and VPP was recorded at 8 and 4h after their respective administration (Nakamura et al., 1995). In the case of HLPLP, the further degradation in plasma into smaller fragments seems to influence its in vivo effect that reaches its maximum decrease at 2h post-administration, and afterwards starts to recover. This could suggest the importance of establishing the suitable pharmacokinetic criteria, not only in regard of absorption, distribution and elimination of peptides, but also of their further metabolism, that correlate to the subsequent effect.

3.3 Evaluation of alternative mechanisms of action of food-derived peptides

The blood depressor effect of food-derived peptides has been widely studied, however, their mechanism of action is still a controversial issue. Some of the known antihypertensive peptides have shown ACE-inhibitory activity, nevertheless, the correspondence between the effect in vivo and in vitro has only been demonstrated in few cases, in rats after single oral administration (Nakamura et al., 1995b), or after long-term intake (Sipola et al., 2001), and in humans (Seppo et al., 2003). This discrepancy could be due to further degradation of the compound during gastrointestinal digestion or because other mechanisms of action different from ACE are implicated (Martinez-Maqueda et al., 2012). The involvement of endogenous opioid peptides in the regulation of blood pressure has been demonstrated (Siren et al., 1992; Czapla et al., 1998). Indeed, the decrease on mean arterial pressure produced by a quimeric opioid peptide (YPFPFRTic-NH2) based on morphiceptin and the neuropeptide FF, were antagonised by the co-administration of naloxone, and it was suggested the implication of endothelial NO pathway in the response (Li et al., 2013).

However, the implication of opioid receptors in the antihypertensive effect of food-derived peptides has only been demonstrated in few cases, for instance α-Lactorphin (Nurminen et al., 2000). Moreover, this peptide was shown to improve endothelial function associated to NO (Sipola et al., 2002b).

One of the objectives of this thesis was to evaluate the implication of opioid receptors on the antihypertensive activity of peptides RYLGY, AYFYPEL and YFYPEL, and the casein hydrolysate containing the same, by the co-administration of naloxone. In previous works of our group, the production of a hypotensive casein hydrolysate using food-grade pepsin was developed, where two of the main peptides from α_{s1}-casein showed antihypertensive effect i.e. RYLGY [f(90-95)] and AYFYPEL [f(143-149)] (Contreras et al., 2009). They were also demonstrated to inhibit ACE in vitro and to exert antioxidant activity. Later on, Sanchez et al. (2011) reported an improvement of aorta and mesenteric acetylcholine relaxation after the administration of this hydrolysate to SHR during 6 weeks, in addition to an increase on eNOS gene expression in aorta. Interestingly, in chapter VII, the results provided evidence of the involvement of opioid receptors in the casein hydrolysate effect and for two of the main peptides (RYLGY and AYFYPEL), although not for YFYPEL. Two sequences similar to RYLGY, i.e. RYLGYL and RYLGYLE, had been previously reported as opioid peptides (Loukas et al., 1983). In addition, the Tyr at N-terminal end of the sequence and the presence of Phe and Tyr at third or fourth position have been described as favourable structural features for opioid receptor binding, except for α_{s1}-casein opioids that could present Arg at N-terminal (Meisel et al., 1997). The peptide AYFYPEL, whose structure does not contain Tyr at N-terminal, showed antagonism with naloxone. Conversely, YFYPEL, which displayed the structural traits favourable for the interaction with opioid receptors, did not show any evidence of such antagonism. Although the presence of Ala in first position at N-terminal end has not been described as an

important structural trait for the interaction with opioid receptors, it seemed to be crucial for the antihypertensive activity of AYFYPEL that involved opioid receptors.

The antagonism of the hydrolysate when co-administered with naloxone indicates the implication of opioid receptors in its effect. These results suggest the contribution of both sequences (RYLGY and AYFYPEL) to the overall effect of the hydrolysate. Moreover, the antihypertensive effect of the peptide YFYPEL, present in the hydrolysate is reported for the first time. However, the possibility of synergic effect with other active peptides present in the hydrolysate should not be discarded.

Furthermore, the differences on the peptide profile between the casein hydrolysate and the precursor casein, suggested that even though the hydrolysate was produced with a gastric enzyme, food-grade pepsin, the pattern of peptide release and probably the amount thereof differs from that generated during gastrointestinal digestion. These differences could explain the absence of biological effect in the casein preparation and the significant antihypertensive effect of the peptic casein hydrolysate after their oral administration to SHR, as shown in results. Thus, the importance of gastrointestinal digestion on the biological effect of food matrices was pointed out.

The results derived from this work provide important information since they demonstrate the implication of opioid receptors in the antihypertensive effect of two milk-derived peptides present in a casein hydrolysate, for which such implication was also evidenced. Furthermore, the antihypertensive activity of YFYPEL was demonstrated for the first time, although its mechanism of action deserves future investigations. Therefore, this work highlights the contribution of other mechanisms of action different to ACE-inhibition in the in vivo antihypertensive effect.

4. CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

1. Two strains of *Debaryomyces hansenii,* isolated from ewe's and goat's milk cheese, are able to produce the β-casein-derived antihypertensive sequences f(133-138), LHLPLP, and f(134-138), HLPLP, by fermentation of commercial casein.

2. The packaging technology, vacuum or modified atmosphere, does not lead to differences in the qualitative peptide profile of semi-hard cheese in the course of storage time, although it affects the relative abundance of the peptides.

3. β-casein domains comprised between residues 60-93, 126-140 and 190-209, are resistant to simulated gastrointestinal digestion regardless of the dairy matrix: blue cheese, skim milk powder or heated and unheated milk, and of the in vitro digestion model used (static or dynamic). These regions are well conserved between species and host several bioactive sequences. The proteolytic state of food-stuffs is a determinant factor in the presence of these sequences' precursors.

4. Heat treatment of milk induces an increase in the resistance of caseins to hydrolysis by pepsin, in contrast to β-Lg, which becomes more susceptible to dynamic simulated gastric digestion. The pattern of peptides released is also affected by the heat treatment applied, being only 48% of peptide homology among heated and non-heated milk samples.

5. The absorption of the penta-peptide from β-casein f(134-138), HLPLP, into blood circulation in rats was demonstrated for the first time. The total effective absorption reached was 5.18%. The peptide was further hydrolysed by the action of plasma peptidases *in vivo* giving rise to the fragments HLPL, f(134-137) and LPLP, f(135-138).

6. The *in vivo* metabolism of the penta-peptide in plasma was confirmed *in vitro* by incubation, giving rise to the two fragments generated *in vivo*, and in addition to the tri-peptide HLP, f(134-136). These three peptide fragments, as well as f(135-137), LPL and f(136-138), PLP, show a potent antihypertensive activity in spontaneously hypertensive rats.

7. Opioid receptors could be involved in the mechanism of action of the antihypertensive α_{s1}-casein peptides f(90-94), RYLGY and f(143-149), AYFYPEL and the casein hydrolysate comprising both, since their activity is antagonised by naloxone.

CONCLUSIONES

1. Dos cepas de *Debaryomyces hansenii,* aisladas de quesos de leche de cabra y oveja, son capaces de liberar las secuencias antihipertensivas derivadas de la β-caseína, LHLPLP, f(133-138) y HLPLP, f(134-138), mediante la fermentación de caseína comercial.

2. En queso semicurado, el tipo de envasado, al vacío o en atmósfera modificada, no da lugar a diferencias cualitativas en el perfil peptídico durante el tiempo de almacenamiento, aunque sí en la abundancia relativa de los péptidos.

3. Los dominios de la β-caseína comprendidos entre los residuos 60-93, 126-140 y 190-209 son resistentes al proceso de simulación de la digestión gastrointestinal independientemente del producto lácteo digerido: queso azul, leche en polvo, leche tratada y no tratada térmicamente y del modelo de digestión utilizado, bien estático o dinámico. Estas regiones están bien conservadas entre especies y albergan numerosas secuencias bioactivas. El estado proteolítico del alimento es un factor determinante en la presencia de los precursores de estas secuencias.

4. El tratamiento térmico de la leche provoca un aumento de la resistencia de las caseínas a la digestión por pepsina mientras que la β-Lg resulta más susceptible a la digestión gástrica dinámica. El calentamiento al que se somete la leche afecta al patrón de liberación de péptidos de los digeridos, alcanzándose sólo un 48% de homología entre los péptidos presentes en las muestras de leche con diferente tratamiento térmico.

5. Se demuestra por primera vez la absorción de un penta-péptido derivado de la β-caseína, f(134-138), HLPLP, llegando intacto al torrente sanguíneo de rata tras su administración oral. Su valor de absorción efectiva fue de 5.18%. El péptido se hidroliza *in vivo* en el plasma dando lugar a los fragmentos HLPL, f(134-137) y LPLP, f(135-138).

6. El metabolismo *in vivo* del penta-péptido fue confirmado *in vitro* en plasma dando lugar a los dos fragmentos identificados *in vivo*, y además encontrándose el tri-peptido HLP, f(134-136). Estos tres fragmentos, así como f(135-137), LPL y f(136-138), PLP, muestran una potente actividad antihipertensiva en ratas espontáneamente hipertensas.

7. Los receptores opioides podrían estar involucrados en el mecanismo de acción de los péptidos antihipertensivos α_{s1}-caseína f(90-94), RYLGY, y α_{s1}-caseína f(143-149), AYFYPEL, así como del hidrolizado que los contiene, dado que su actividad antihipertensiva se antagoniza con la coadministración de naloxona.

5. REFERENCES

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6. ANNEXES