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Chapter 4

Whey Proteins as Source of Bioactive Peptides Against Hypertension

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Additional information is available at the end of the chapter

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1. Introduction

A food can be considered as functional if, beyond its nutritional outcomes, it provides benefits upon one or more physiological functions, thus improving health while reducing the risk of illness [1]. This definition – originally proposed by the European Commission Concerted Action on Functional Food Science in Europe (FuFoSE), should be refined in that: (i) the functional effect is different from the nutritional one; and (ii) the benefit provided requires scientific consubstantiation in terms of improvement of physiological functions, or reduction of occurrence of pathological conditions. The concept of functional food emerged in Japan during the 80's, chiefly because of the need to improve the quality of life of a growing elderly population – who typically incurs in much higher health costs [2]. Nowadays, a growing consumer awareness of the relationship between nutrition and health has made the market of functional foods to boom.

Bioactive peptides can be commercially sold as nutraceuticals; a nutraceutical is an edible substance possessing health benefits that may accordingly be used to prevent or treat a disease. However, a distinction should be made between nutraceuticals taken to prevent diseases – and which are present as natural ingredients of functional foods consumed as part of the daily diet, and nutraceuticals used as adjuvants for treatment of diseases – which require pharmacologically active compounds.

Milk and dairy products have been concluded to be functional foods; a number of studies have indeed shown that many peptides from milk proteins play a role in several metabolic processes, so a considerable interest has arisen from the part of the dairy industry towards large-scale production of dairy proteins in general, and bioactive peptides in particular. Manufacture of bioactive peptides is usually carried out through hydrolysis using digestive, microbial, plant or animal enzymes, or by fermentation with lactic starter cultures. In some cases, a combination of these processes has proven crucial to obtain functional peptides of

small size [3,4]. Proteins recovered from whey released by cheese manufacture already found a role as current ingredients on industrial scale. Use of these proteins (concentrated or isolated), and mainly of biologically active peptides derived therefrom as dietary supplements, pharmaceutical preparations or functional ingredients is of the utmost interest for the pharmaceutical and food industries – while helping circumventing the pollution problems associated with plain whey disposal.

2. Cheese whey

Despite having been labeled over the years as polluting waste owing to its high lactose and protein contents [5], whey is a popular protein supplement in various functional foods and the like [6]. In fact, whey compounds exhibit a number of functional, physiological and nutritional features that make them potentially useful for a wide range of applications (Table 1).

Advantageous features	Disadvantageous features
High nutritional value of protein fraction in terms of amino acid residues (e.g. Lys, Thr, Leu, Ser)	High dilution requiring costly dehydration
Possibility of lactose production in parallel	High salt content (ca. 10 % of dry matter)
Reduction in pollution owing to biochemical oxygen demand of proteins	High sugar content requiring delactosation
	Highly perishable raw material
	Widely dispersed cheese production facilities
	Technical innovation needed in separation (e.g. ultrafiltration and diafiltration)

Table 1. Major features associated with use of whey (adapted from Alais [62])

Whey can be converted into lactose-free whey powder, condensed whey, whey protein concentrates and whey protein isolates [7] – all of which are commercially available at present. In the case of bovine milk, ca. 9 L of whey is produced from 10 L of milk during cheesemaking; estimates of worldwide production of cheese in 2011 point at ca. 15 million tonnes (United States Department of Agriculture – Foreign Agricultural Service). For environmental reasons, whey cannot be dumped as such into rivers due to its high chemical and biological oxygen demands. On the other hand, whey can be hardly used as animal feed or fertilizer due to economic unfeasibility.

2.1. Physicochemical composition

There are two types of whey, depending on how it is obtained; when removal of casein is via acid precipitation at its isoelectric point (pH 4.6 at room temperature) [8], it is called acid whey; however, the most common procedure is coagulation via enzymatic action, so the product obtained is called sweet whey [9-10].

Despite containing ca. 93 % water, whey is a reservoir of milk components of a high value: it indeed contains ca. half of the nutrients found in whole milk. Said composition depends obviously on how the cheese is produced and the milk source; the compound found to higher level is lactose (4.5-5 %, w/v), followed by soluble proteins (0.6-0.8 %, w/v), lipids (0.4-0.5 %, w/v) and minerals (8-10 %, $w/w_{dry\ extract}$) – particularly calcium, and vitamins such as thiamine, riboflavin and pyridoxin [11-13]. In fact, whey is now considered as a co-product rather than a by-product of cheese production, in view of its wide range of potential applications [13-15].

2.2. Protein composition

Milk has been recognized as one of the main sources of protein [16] in feed for young animals and food for humans of all ages [17]. Bovine milk contains ca. 3 % protein [9] – of which 80 % is caseins and 20 % is whey proteins [18]. Whey comprises a heterogeneous group of proteins that remain in the supernatant after precipitation of caseins; they are characterized by genetic polymorphisms that usually translate into replacement of one or more amino acid residues in their original peptide sequence.

Two major types of proteinaceous material can be found in whey: β-lactoglobulin (β-Lg) and α-lactalbumin (α-La); and proteose-peptone (derived from hydrolysis of β-casein, β-CN), small amounts of blood-borne proteins (including bovine serum albumin, BSA, and immunoglobulins, Igs), and low molecular weight (MW) peptides derived from casein hydrolysis (soluble at pH 4.6 and 20 °C) [16, 19]. Whey proteins have a compact globular structure that accounts for their solubility (unlike caseins that exist as a micellar suspension, with a relatively uniform distribution of non-polar, polar and charged groups). These proteins have amino acid profiles quite different from caseins: they have a smaller fraction of Glu and Pro, but a greater fraction of sulfur-containing amino acid residues (i.e. Cys and Met). These proteins are dephosphorylated, easily denatured by heat, insensitive to Ca^{2+}, and susceptible to intramolecular bond formation via disulfide bridges between Cys sulfhydryl groups. Selected physicochemical parameters typical of whey proteins are tabulated in Table 2.

Proteins	Concentration (gL^{-1})	MW (kDa)	Isoelectric point (pI)
β-Lg	3 – 4	18.4	5.2
α-La	1.5	14.2	4.7 – 5.1
BSA	0.3 – 0.6	69	4.7 – 4.9
IgG, IgA, IgM	0.6 – 0.9	150 – 1000	5.5 – 8.3
Lactoperoxidase	0.006	89	9.6
Lactoferrin	0.05	78	8.0
Protease-peptone	0.5	4 – 20	
Caseinomacropeptide		7	

Table 2. Characteristics of major whey proteins (adapted from Zydney [186])

2.2.1. β-Lactoglobulin (β-Lg)

The major protein in ruminant whey is β-Lg, which represents ca. 50 % of the total whey protein inventory in cow's milk and 12 % of the total milk proteins [9, 20-21]. Although it can be found in the milk of many other mammals, it is essentially absent in human milk [22]. This is a globular protein, with 162 amino acid residues in its primary structure and a MW of 18.4 kDa. There are at least twelve genetic variants of β-Lg (A, B, C, D, D_R, D_{YAK}/E, F, G, H, I, W and X) – of which A is the most common.

The monomer of β-Lg has a free thiol group and two disulfide bridges – which makes β-Lg exhibit a rigid spacial structure [8]; however, its conformation is pH-dependent [23] – and at temperatures above 65 °C (at pH 6.5), β-Lg denatures, thus giving rise to aggregate formation [24]. Between pH values 5.2 and 7.2, that protein appears as a dimmer – with a MW of 36.0 kDa [8]. At low pH, association of monomers leads to octamer formation; and, at high temperatures, the dimer dissociates into its monomers. The solubility of β-Lg depends on pH and ion strength – but it does not precipitate during milk acidification [25].

A number of useful nutritional and functional features have made β-Lg become an ingredient of choice for food and beverage formulation: in fact, it holds excellent heat-gelling [26] and foaming features – which can be used as structuring and stabilizer agents in such dairy products as yogurts and cheese spreads. This protein is resistant to gastric digestion, as is stable in the presence of acids and proteolytic enzymes [22, 27-30]; hence, it tends to remain intact during passage through the stomach. It is also a rich source of Cys, an amino acid bearing a key role in stimulating synthesis of glutathione (GSH) – composed by three amino acids, Glu, Cys and Gly [31].

Many techniques have been developed for purification of β-Lg – which normally rely on its precipitation [32-35]; when large scale purification is intended, precipitation is usually complemented by ion exchange [35-36].

2.2.2. α-Lactalbumin (α-La)

α-La appears quantitatively second in whey; it comprises ca. 20 % of all proteins in bovine whey, and 3.5 % of the total protein content of whole milk [9]. It is a calcium metalloprotein composed of 123 amino acids, with a MW of 14.4 kDa [37]; and appears as three genetic variants (A, B and C), with B being the most common [38]. Chromatographic and electrophoretic analysis within stability studies carried out at various times and temperatures (pH 6.7) indicated that α-La is more heat resistant than β-Lg – in part due to its denaturation being reversible below 75 °C [39]. Owing to such a relatively high thermal stability, it holds a poor capacity to gel; however, it can be easily incorporated in fluid or viscous products to increase their nutritional value. This protein is commercially used in supplements for infant formulae, because of its similarity in structure and composition to human milk proteins – coupled with its higher content of Cys, Trp, Ile, Leu and Val residues, which make it also the ingredient of choice in sport supplements [13, 40-41]. Regarding tertiary structure, α-La is a compact globular protein consisting of 26 % α-helix,

14 % β-sheet and 60 % other motifs; it is also very similar to lysozyme [9]. This protein is one of the most studied proteins with regard to understanding the mechanism of protein stability and folding/unfolding [42]; at low pH [43], high temperature [44] or moderate concentrations of denaturants – e.g. guanidine hydrochloride [45], α-La adopts a conformational structure called molten globule. A partially unfolded state, the apo-state, is formed at neutral pH upon removal of Ca^{2+} by ethylenediamine tetracetic acid (EDTA) [46-47]; this state preserves its secondary, but not its tertiary structure [48].

The molten globule state of α-La retains a high fraction of its native secondary structure, as well as a flexible tertiary structure [45, 48-49]; it accordingly appears as an intermediate in the balance between native and unfolded states [50-51]. This structure of α-La is highly heterogeneous, with proeminence of α-helix driven mainly by weak hydrophobic interactions – while the β-sheet domain is significantly unfolded.

2.2.3. Caseinomacropeptide (CMP)

CMP is a heterogeneous polypeptide fraction derived from cleavage of Phe_{105}-Met_{106} in κ-casein (κ-CN). When milk is hydrolyzed with chymosin during cheesemaking, κ-CN is hydrolyzed into two portions: one remains in the cheese (*para*-κ-CN) and the other (CMP) is lost in whey; the latter is relatively small, with 63 residues and a MW of ca. 8 kDa [52]. Further to its polymorphisms, CMP may exist in various forms depending on the extent of post-transcriptional changes: it glycosylates through an *O*-glycoside bridge, and phosphorylates via a Ser residue. Note that post-transcriptional modifications of κ-CN occur exclusively in the CMP portion of the molecule.

The amino acid sequence of CMP is well-known; it lacks aromatic amino acid residues (Phe, Trp and Tyr) and Arg, but several acidic and hydroxyl amino acids are present [53]. CMP from cow is soluble at pH in the range 1-10, with a minimum solubility (88 %) between pH 1 and 5 [54-55]. CMP appears to remain essentially soluble following heat treatment at 80-95 °C for 15 min at pH 4 and 7 [55]. Its emulsifying activity exhibits a minimum near the isoelectric point [54]. Dziuba and Minkiewicz [56] showed that a decrease in pH leads to a decrease in CMP volume, owing to reduction of internal electrostatic forces and steric repulsion; this apparently has a significant influence upon its emulsifying capacity.

2.2.4. Bovine serum albumin (BSA)

BSA is derived directly from the blood, and represents 0.7-1.3 % of all whey proteins [8]. Its molecule has 582 amino acid residues and a MW of 69 kDa – and contains 17 disulfide bonds and one free sulphydryl group [9]. Because of its size and higher levels of structure, BSA can bind free fatty acids and other lipids, as well as flavor compounds [57] – but this feature is severely hampered upon denaturation. Its heat-induced gelation at pH 6.5 is initiated by intermolecular thiol-disulphide interchange – similar to what happens with β-Lg [58].

2.2.5. Immunoglobulins (IGs)

IGs represent 1.9-3.3 % of the total milk proteins, and are derived from blood serum [8]; they constitute a complex group, the elements of which are produced by β-lymphocytes. Igs encompass three distinct classes: IgM, IgA and IgG (IgG_1 and IgG_2) – with IgG_1 being the major Ig present in bovine milk and colostrum [8], whereas IgA is predominant in human milk. The physiological function of Igs is to provide various types of immunity to the body; they consist of two heavy (53 kDa) and two light (23 kDa) polypeptide chains, linked by disulfide bridges [9]. The complete Ig, or antibody molecule has a MW of about 180 kDa [59]. Igs are partially resistant to proteolytic enzymes, and are in particular not inactivated by gastric acids [59].

2.2.6. Lactoferrin (LSs)

LFs are single-chain polypeptides of ca. 80 kDa, containing 1-4 glycans depending on the species. Bovine and human LFs consist of 989 and 691 amino acids, respectively [60]: the former is present to a concentration of ca. 0.1 mg mL^{-1} [25, 61], and is an iron-binding glycoprotein - so it is thought to play a role in iron transport and absorption in the gut of young people.

2.2.7. Proteose-peptones (PPs)

The total PP fraction (TPP) of bovine milk represents ca. 10 % of the whole whey protein content; it is accounted for by the whey protein fraction soluble after heating at 95 °C for 30 min, followed by acidification to pH 4.6 [62]. The TPP fraction is often divided in two main groups: the first one includes PPs originated from casein hydrolysis; its principal components have been labeled as 5 (PP5), 8 fast (PP8 fast) and 8 slow (PP8 slow), according to their electrophoretic mobility [62, 63]. PP3 constitutes the second group, and it is not derived from casein (it is actually found only in whey); it is known for its extreme hydrophobicity.

2.3. Functional ingredients from whey proteins

Whey proteins have unique characteristics [64] beyond their great importance in nutrition; they exhibit chemical, physical, physiological, functional and technological features also useful for food processing [14]. Based on these properties, more and more individual proteins and protein concentrates of whey have been incorporated in food at industrial scale. Therefore, whey proteins address two major issues in practice: nutritionally, they supply energy and essential amino acids, besides being important for growth and cellular repair; in terms of functionality, they play important roles upon texture, structure and overall appearance of food – e.g. gel formation, foam stability and water retention.

A few physiological properties useful in therapies have been found [65]: a number of reviews have accordingly examined to some length the bioactive properties of whey proteins in general [66-67], or of β-Lg and α-La in particular [26]; other authors have covered

mainly such biological activities as anticarcinogenic [68] and immunomodulatory [69]. It was observed that whey proteins trigger immune responses that are significantly higher than those by diets containing casein or soy protein. Antimicrobial and antiviral actions, immune system stimulation and anticarcinogenic activity (among other metabolic features) have indeed been associated with ingestion of β-Lg and α-La, as well as LF, LP, BSA and CMP; the main biological activities of whey proteins are listed in Table 3.

With regard to bioactive peptides, research has undergone a notable intensification during the past decade [4, 70]. Advances in nutritional biochemistry and biomedical research have in fact helped unravel the complex relationships between nutrition and disease, thus suggesting that food proteins and peptides originated during digestion (or from *in vitro* proteolysis) may play important roles in preventing or treating diseases associated with malnutrition, pathogens and injuries [71-72].

Protein/Peptide	Treatment	Biological function	Reference
Whole whey protein		Prevention of cancer	[74]
		Breast and intestinal cancer;	[14, 75]
		Chemically-induced cancer	[76-77]
		Increment of gluthatione levels	[64]
		Increase of tumour cell vulnerability	[78-79]
		Antimicrobial activities	[80]
		Increment of satiety response	
		Increment in plasma amino acids, cholecystokinin and glucagon-like peptide	[81]
	Enzyme hydrolysis	**ACE[a]-inhibitor**	[82]
		Antiulcerative	
		Prostaglandin production	[83-85]
	Enzyme hydrolysis	Antiulcerative	[83, 86]
β-Lactoglobulin		Transporter	
		Retinol	[9, 41, 87, 88]
		Palmitate	[89]
		Fatty acids	[90]
		Cellular defence against oxidative stress and detoxification	[31, 65, 91-93]
		Enhancement of pregastic esterase activity	[94]
		Transfer of passive immunity	[95]
		Regulation of mammary gland phosphorus metabolism	[96]
	Enzyme hydrolysis; Fermentation	**ACE[a]-inhibitor**	[97-107]
	Enzyme hydrolysis	Antimicrobial against several gram-positive bacteria	[108-111]
	Enzyme hydrolysis	Antimicrobial (bactericidal)	[112-113]
	Enzyme hydrolysis	Hypocholesterolemic	[113-114]

Protein/Peptide	Treatment	Biological function	Reference
	Enzyme hydrolysis	Opioid agonist	[73, 97, 115]
	Enzyme hydrolysis	**Antihypertensive**	[99, 116-117]
	Enzyme hydrolysis	Ileum contracting	[97, 99]
	Enzyme hydrolysis	Antinociceptive	[118]
		Prevention of cancer	
	Enzyme hydrolysis	Intestinal cancer	[14]
α-Lactalbumin		Prevention of cancer	[119]
		Apoptosis of tumoral cells	[120-122]
		Lactose synthesis	[25, 123]
		Treatment of chronic stress-induced disease	[124]
		Antimicrobial (bactericidal)	
		Streptococcus pneumonia	[125]
		Stress reduction	[123, 126]
		Immunomodulation	[127]
	Enzyme hydrolysis	Antimicrobial against several gram-positive bacteria	[108-110]
	Enzyme hydrolysis	Opioid agonist	[97, 115, 128]
	Enzyme hydrolysis	**ACE[a]-inhibitor**	[26, 97-98, 101, 107]
	Enzyme hydrolysis	**Antihypertensive**	[117, 129]
	Enzyme hydrolysis	Ileum contracting	[97]
		Antiulcerative	
		Prostaglandin production	[130-132]
Bovine serum albumin		Fatty acid binding	[13]
		Antioxidant	[133-134]
		Prevention of cancer	[135]
	Enzyme hydrolysis	**ACE[a]-inhibitor**	[136-137]
	Enzyme hydrolysis	Ileum contracting	[138]
	Enzyme hydrolysis	Opioid agonist	[97, 128, 139]
Immunoglobulins		Immunomodulation	[140]
		Disease protection through passive immunity	[141-142]
		Antibacterial	[143-145]
		Antifungal	[146]
		Opioid agonist	[147]
Caseinomacropeptide		Antithrombotic	[148-153]
		ACE[a]-inhibitor	[154-156]
		Antimicrobial	[56, 111, 157-160]
	Enzyme hydrolysis	Prebiotic	[161]
		Increment in plasma amino acids and cholecystokinin peptide	[162-165]

ACE[a]- angiotensin-converting enzyme

Table 3. Biological functions of whey proteins/peptides (adapted from Madureira *et al.* [87])

Although inactive within the primary structure of their source proteins, hydrolysis (e.g. mediated by a protease) may release peptides with specific amino acid sequences possessing biological activity. A number of chemical and biological methods of screening have accordingly been developed to aid in search for specific health effects; however, only some of those found *in vitro* have eventually been confirmed in studies encompassing human volunteers [73].

Scientific evidence has shown that whey proteins contain a wide range of peptides that can play crucial physiological functions and modulate some regulatory processes (see Table 3). Due to its high biological value, coupled with excellent functional properties and clean flavor, whey has earned the status of a recommended source of functional ingredients [71] – designed to reduce or control chronic diseases and promote health, thus eventually reducing the costs of health care [3, 166].

Favorable health effects have indeed been claimed for some peptides derived from food proteins – being able to affect the cardiovascular, nervous, digestive or immune systems; these encompass antimicrobial properties, blood pressure-lowering (or angiotensin-converting enzyme (ACE)-inhibitory) effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of mineral absorption and/or bioavailability thereof, cyto- or immunomodulatory effects, and opioid features. With regard to the mechanisms underlying the physiological roles of bioactive peptides, a few involve action only upon certain receptors, whereas others are enzyme inhibitors; they may also regulate intestinal absorption, and exhibit antimicrobial or antioxidant activities. Recall that oxidative metabolism is essential for survival of cells, but it generates free radicals (and other reactive oxygen species) as side effect – which may cause oxidative damage. Antioxidant activity has been found specifically in whey proteins, probably via scavenging of such radicals via Tyr and Cys amino acid residues – which is predominantly based on proton-coupled single electron or hydrogen atom transfer mechanisms; or else chelation of transition metals [167-168].

On the other hand, bioactive peptides derived from food proteins differ in general from endogenous bioactive peptides in that they can entail multifunctional features [98]. Furthermore, bioactive peptides that cannot be absorbed though the gastrointestinal tract may exert a direct role upon the intestinal lumen, or through interaction with receptors in the intestinal wall itself; some of these receptors have been implicated in such diseases as cancer, diabetes, osteoporosis, stress, obesity and cardiovascular complications.

3. Production of bioactive peptides in whey

Bioactive peptides derived from whey proteins constitute a new concept, and have open up a wide range of possibilities within the market for functional foods [4, 169]; of special interest are those released via enzymatic action – as happens during clotting in cheesemaking.

The enzymes used to bring about milk coagulation are selected protein preparations that provide in general a high clotting activity – i.e. a considerable, but selective proteolytic

activity. Animal rennet obtained from the calf stomach, composed by 88-94 % and 6-12 % chymosin and pepsin, respectively, has been the coagulant of choice for cheesemaking. However, due to increased world production of cheese, the supply of animal rennet has lied below its demand; the increased prices have driven a search for alternative coagulants (including plant and microbial sources). With regard to animal rennet substitutes, pig pepsin has enjoyed a remarkable commercial success; with regard to rennet from microbial origin, the proteases from *Mucor miehei*, *Mucor pusillus* and *Endothia parasitica* are the most successful [170]. Recombinant bovine chymosin is, nowadays, one of the proteinases with greater commercial expression – even though its use is still prohibited in certain countries [171].

Chymosin and the other rennet substitutes are aspartic proteases, with optimal activity at acidic pH, and possessing high degree of homology in primary and 3-dimensional structures, 3-dimensional structure and catalytic mechanism. The specificity towards the substrate is, however, rather variable; although they have a greater tendency to break peptide bonds between hydrophobic amino acids having bulky side residues, they hydrolyze a large number of bond types [172]. Of particular interest is vegetable rennet, which – with few exceptions, enjoys a still limited use worldwide. Many plant enzyme preparations proved indeed to be excessively proteolytic for manufacture of cheese, causing defects in terms of flavor and texture of the final product. These difficulties arise from the presence of non-specific enzymes that belong to complex enzyme systems (which, as such, are difficult to control). An exception to the poor suitability of vegetable coagulants is the proteinases in aqueous extracts of plants of the *Cynara* genus – which have been employed for traditional cheesemaking in Portugal and Spain since the Roman period.

Bioactive peptides derived from whey proteins can be released at industrial scale via enzyme-mediated hydrolysis with digestive enzymes – and pepsin, trypsin and chymotrypsin have been the most frequent vectors therefor [4, 169, 173]. However, whey proteins are not easily broken down by proteases in general – a realization that also explains their tendency to cause allergies upon ingestion [174]. Hence, less conventional sources of proteolytic enzymes have been sought that can cleave the whey protein backbone at specific and usual sites. This is the case of aspartic proteinases present in the flowers of *Cynara cardunculus* – a plant related to the (common) globe artichoke. They can cleave the whey protein backbone next to hydrophobic amino acid residues, especially Phe, Leu, Thr and Tyr [82, 175], and act mainly on α-La, either in whole whey or following concentration to whey protein concentrate (WPC) [176-178]; conversely β-Lg appears not to be hydrolyzed thereby to a significant extent [82, 175].

4. Recovery of proteins/peptides from whey

The relatively low concentration of proteins in whey requires concentration processes to assure high hydrolysis productivity. Development of membrane separation techniques has been essential toward this endeavor – and food industry has taken advantage of its relatively easy scale-up, as well as its being inexpensive when compared with preparative

chromatographic techniques [41]. Furthermore, the absence of heat treatment allows the bioactive components to remain intact (or become only slightly affected) during processing. Recall that membrane separation allows differential concentration of a liquid, provided that the solute of interest is larger in molecular diameter than the membrane pores – so the liquid that percolates the membrane (filtrate) contains only components smaller than that size threshold [179].

The dairy industry has pioneered development of equipment and techniques for membrane filtration, which recovers whey proteins in a non-denatured state. Typical procedures include: (i) basic membrane separation, e.g. reverse osmosis, ultrafiltration and diafiltration [180-186], that permits fractionation of proteins, as well as concentration and purification thereof; (ii) nanofiltration (or ultraosmosis) that allows removal of salts or low MW contaminants; and (iii) microfiltration to remove suspended solid particles or microorganisms [179, 187]. Note that isolation of individual whey proteins on laboratory scale has resorted chiefly to salting out, ion exchange chromatography and/or crystallization [188]; such a fractionation allows fundamental studies of their immunological properties to be carried out, which are necessary to establish and support industrial interest [189-190].

5. Activity of peptides from whey upon hypertension

Hypertension is a major public health issue worldwide that affects nearly one fourth of the population; and it is usually associated with such other disorders as obesity, pre-diabetes, renal disease, atherosclerosis and heart stroke [191-194]. Its specific treatment will likely reduce the risk of incidence of cardiovascular diseases, which currently account for 30 % of all causes of death [195].

Blood pressure can be regulated through diet changes and physical exercise, as well as administration of calcium T channel antagonists, angiotensin II receptor antagonists, diuretics and ACE inhibitors [104]. A few mechanisms have been described that rationalize how peptides lower blood pressure. Traditionally, control of hypertension has focused on the renin-angiotensin system, via inhibition of ACE [173]. Captopril, enalapril and lisinopril have accordingly been used as antihypertensive drugs that act essentially as ACE inhibitors; they found a widespread application in treatment of patients with hypertension, heart failure or diabetic nephropathies [193, 196-197]. However, they bring about undesirable side effects, so safer (and, hopefully, less expensive) alternatives are urged [198-199].

In fact, increasing evidence has been provided that mechanisms other than ACE inhibition may be involved in blood pressure decrease arising from consumption of many food-derived peptides [200]; although there are few studies to date with antihypertensive peptides obtained from whey. One of them corresponds to interaction with opioid receptors that are present in the central nervous system and in peripheral tissues, while another is based on release of nitric oxide (NO) that causes vasodilatation and thus affects blood pressure. Those peptides hold the advantage of no side effects, unlike happens with such other opiates as morphine [102]. One example is α-lactorfin, a tetrapeptide derived from α-La [129, 201], for which studies showed that antihypertensive effects are mediated through

the vasodilatory action of binding to opioid receptors. Furthermore, endothelium-dependent relaxation of mesenteric arteries in spontaneously hypertensive rats (SHR, which is the animal model normally accepted to study human hypertension) that was inhibited by an endothelial nitric oxide synthase (eNOS) inhibitor was also observed [202]. That peptide may even chelate minerals, and thus facilitates calcium absorption [200].

Alternative mechanisms are other routes of vasoregulator substance synthesis – e.g. kallikrein-kinin, neutral endopeptidase and endothelin-converting enzyme systems. The release of vasodilator substances like prostaglandin I_2 or carbon monoxide could be implied in dependent and independent mechanisms of ACE inhibition responsible for antihypertensive effects [203-205]; an example is the peptide ALPMHIR, which inhibits release of an endothelial factor (ET-1) that causes contractions in smooth muscle cells [206].

In the last decade, production of antioxidant peptides from whey has been reported [207]. Experimental evidence – including SHR and human studies, claimed that oxidative stress is one of the causes of hypertension and several vascular diseases, via increase production of reactive oxygen species and reduction of NO synthesis and bioavailability of antioxidants [208].

Nevertheless, the most intensively studied peptides – i.e. VPP and IPP derived from caseins, showed possible mechanisms of action that could be found also in other peptides. In studies performed with rats, VPP and IPP increased plasma renin levels and activity [202]; and decreased ACE activity in the serum; they also showed endothelial function protection in mesenteric arteries [208]. The influence of VPP and IPP on gene expression of SHR abdominal aorta unfolded a significant increase of genes related with blood pressure regulation – the eNOS and connexin 40 genes [208]. Other studies have highlighted the peptide effects on the vasculature itself, showing that the antihypertensive activity of the peptide rapakinin is induced mainly by CCK_1 and IP-receptor-dependent vasorelaxation; this peptide relaxes the mesenteric artery of SHR via prostaglandin I_2-IP receptor, followed by CCK-CCK_1 receptor pathway; other peptides improve aorta and mesenteric acetylcholine relaxation, and decrease left ventricular hypertrophy, accompanied by significant decrease in interstitial fibrosis [209]. In order to prevent hypertension, two alternative enzyme inhibitors were suggested: renin (a protease recognized as the initial compound of the renin–angiotensin system) and platelet-activating factor acetylhydrolase (PAF-AH) (a circulating enzyme secreted by inflammatory cells and involved in atherosclerosis) [208].

5.1. Inhibition of angiotensin-converting enzyme (ACE)

Since diet has a direct relationship to hypertension, the food industry (in association with research and public health institutions) has promoted development of novel functional ingredients that can contribute to keep a normal blood pressure – thus avoiding the need to take antihypertensive drugs [73, 173, 209-212]. Various investigators have accordingly hypothesized that certain peptides formed through hydrolysis of food proteins have the ability to inhibit ACE; López-Fandiño [173], FitzGerald [104, 137], Gobetti [213], Meisel

[214], Korhonen and Pihlanto [4], Silva and Malcata [215], Vermeirssen [216], and Martínez-Maqueda [208] have comprehensively reviewed this subject. In general, it has been claimed that a diet rich in foods containing antihypertensive peptides is effective toward prevention and treatment of hypertension [173, 201].

ACE-inhibitory peptides may be obtained from precursor food proteins via enzymatic hydrolysis, using viable or lysed microorganisms or specific proteases [3, 73, 137, 169]. Although *in vitro* studies are useful at screening stages, the efficacy and safety of such peptides requires *in vivo* testing – first in animals, and then in human volunteers [217]. This issue is particularly relevant because *in vitro* ACE inhibition does not necessarily correlate with *in vivo* antihypertensive features, as peptides often undergo breakdown during gastrointestinal digestion that hampers manifestation of their potential physiological function. Conversely, antihypertensive activity may be promoted after long-chain peptide precursors release bioactive fragments by gastrointestinal enzymes [73].

In the latest two decades, various active peptides have been identified from animal proteins, including some with antihypertensive effects in animals (e.g. SHR) and even in humans [3, 73, 137, 169, 173, 201, 208, 212, 217, 299]: bovine plasma proteins [218], egg proteins [203, 219] and tuna proteins [220]; but also plant proteins, e.g. from soy [221], wine [222] and maize [223]. Nevertheless, milk proteins still appear to be the best source of ACE-inhibitory peptides.

Recall that caseins are the most abundant proteins in milk, and have an open and flexible structure that makes them susceptible to attack by proteases; hence, many ACE-inhibitors have been obtained via enzyme-mediated approaches [224-225] – e.g. casokinins. Studies on peptides with ACE-inhibitory activity obtained from whey proteins (called lactokinins) are more limited – which may be due to the rigid structure of β-Lg (the major whey protein) that makes it particularly resistant to digestive enzymes. However, bioactive protein fragments with ACE-inhibitory activity have been found in whey protein hydrolyzates [107, 217, 226-228]; and Manso and López-Fandiño [155] also identified this activity in CMP hydrolyzates. Characterization of hydrolyzates of the main whey proteins – including the amino acid sequences of peptides therein that exhibit *in vitro* ACE-inhibiting activity, is provided in Table 4.

The ACE-inhibitory activity depends on the protein substrate and the proteolytic enzymes used to break it down. ACE (i.e. a dipeptidyl carboxypeptidase) is an enzyme ubiquitous in tissues and biological fluids – where it plays an important physiological role upon regulation of the cardiovascular function, including a basic role in regulation of peripheral blood pressure via the renin-angiotensin system [229-230]. ACE inhibitors and angiotensin II receptor blockers [231-232] have been therapeutically important, since they act as efficient drugs and bring about very few collateral effects.

ACE-inhibitor peptide can reduce blood pressure in a process regulated (in part) by the renin-angiotensin system: renin — a protease secreted in response to various physiological stimuli, cleaves the protein angiotensinogen to produce the inactive decapeptide

Source protein	Enzyme	Peptide fragment	Amino acid sequence	IC_{50} (μM)[a]	Reduction in SBP[b] (mm Hg) (Dose (mg kg^{-1}_{bw}))	Refe-rences
Whole whey protein	Fermentation + trypsin + chymotrypsin	β-Lg f9-14	GLDIQK	580		[104, 233]
	Yogurt starter + trypsin + pepsin	β-Lg f15-20	VAGTWY	1682		[100]
	Fermentation with lactic acid bacteria + prozyme 6	β-Lg f17-19	GTW	464.4		[105]
	Cardosins	β-Lg f33-42	DAQSAPLRVY[c]	12.2	10 (5)	[107, 117]
	Proteinase K	β-Lg f78-80	IPA[c]	141	31 (8)	[136]
	Cardosins	α-La f16-26	KGYGGVSLPEW[c]	0.7	20 (5)	[107, 117]
	Cardosins	α-La f97-103	DKVGINY[c]	99.9		[107]
	Cardosins	α-La f97-104	DKVGINYW[c]	25.4	15 (5)	[107, 117]
	Fermentation + trypsin + chymotrypsin	α-La f105-110	LAHKAL	621		[100]
	Fermentation by cheese microflora	α-La f104-108	WLAHK	77		[233]
	Neutrase	α-La f105-110	INYWL	11		[234]
β-Lactoglobulin	Trypsin	f7-9	MKG	71.8		[103]
	Trypsin	f10-14	LDIQK	27.6		[103]
	Pepsin + trypsin + chymotrypsin	f15-19	VAGTW	1054		[233]
	Trypsin	f22-25	LAMA	556		[233]
	Trypsin	f32-40	LDAQSAPLR	635		[233]
	Protease N Amano	f36-42	SAPLRVY	8		[235]
	Thermolysin	f58-61	LQKW[c]	34.7	18.1 (10)	[103, 236]
	Trypsin	f81-83	VKF	1029		[233]
	Pepsin + trypsin + chymotrypsin	f94-100	VLDTDYK	946		[106, 233]
	Pepsin + trypsin + chymotrypsin	f102-103	YL[c]	122		[214]
	Pepsin + trypsin + chymotrypsin	f102-105	YLLF[c]	172		[113]
	Thermolysin	f103-105	LLF[c]	79.8	29 (10)	[113, 236]
	Pepsin + trypsin + chymotrypsin	f106-111	CMENSA	788		[233]
	Pepsin + trypsin + chymotrypsin	f142-145	ALPM[c]	928	21.4 (8)	[116]
	Pepsin + trypsin + chymotrypsin	f142-146	ALPMH[c]	521		[233]
	Trypsin	f142-148	ALPMHIR[c]	43		[226]
α-Lactalbumin	Thermolysin	f15-26	LKGYGGVSLPEW	83		[237]
	Thermolysin	f18-26	YGGVSLPEW	16		[237]
	Thermolysin	f20-26	GVSLPEW	30		[237]
	Thermolysin	f21-26	VSLPEW	57		[237]
	Synthetic	f50-51 or f18-	YG[c]	1522		[98]

Source protein	Enzyme	Peptide fragment	Amino acid sequence	IC_{50} (μM)[a]	Reduction in SBP[b] (mm Hg) (Dose (mg kg^{-1}_{bw}))	References
		19				
	Pepsin + trypsin + chymotrypsin	f50-52	YGL	409		[233]
	Pepsin	f50-53	YGLF[c]	733	23.4 (0.1)	[129, 233]
	Synthetic	f52-53	LF[c]	349		[26]
	Trypsin	f99-108	VGINYWLAHK	327		[233]
	Trypsin	f104-108	WLAHK	77		[233]
Bovine serum albumin	Proteinase K	f208-216	ALKAWSVAR[c]	3		[238]
	Proteinase K	f221-222	FP	315	27 (8)	[136]
Caseinomacropeptide	Trypsin	f106-112	MAIPPKK		28 (10)	[239]
Lactoferrin	Pepsin	f20-25	RRWQWR		16.7 (10)	[240]
	Pepsin	f22-23	WQ		11.4 (10)	[240]

[a] Concentration of peptide needed to inhibit 50 % of original ACE activity.
[b] Systolic blood pressure.
[c] Synhetic peptides used.

Table 4. Primary structural characteristics of whey peptides with ACE-inhibitory activity and antihypertensive activity in spontaneously hypertensive rats, and vectors of generation thereof.

angiotensin I. Cleavage of angiotensin I – via removal of two amino acid residues from the C-terminal end by ACE, produces the active octapeptide angiotensin II that is a potent vasoconstrictor; however, there are alternative routes to generate angiotensin II [198, 241-242]. Angiotensin II activates angiotensin II type 1 (AT_1) receptor — a member of the G-protein-coupled-receptor superfamily, which plays various roles, e.g. vasoconstriction, as well as stimulation of aldosterone synthesis and release (which leads to sodium retention, and thus increases blood pressure) [198, 217, 242]. In addition, ACE acts on the kallikrein-kinin system, catalyzing degradation of the nonapeptide bradykinin – which is a vasodilator [241]. ACE-inhibitor peptides exert a hypotensive effect by preventing angiotensin II formation and degradation of bradykinin, thus reducing blood pressure in hypertensive patients [217].

Several tests on SHRs – probably the best experimental model for antihypertensive studies because they exhibit vascular reactivity and renal function similar to those in human beings [243], have been described that prove control of arterial blood pressure following a single oral administration of known ACE-inhibitory hydrolyzates or/and peptides derived from whey proteins. The antihypertensive effect associated with some of those peptides is comparable to that exhibited by VPP – an antihypertensive peptide included in functional foods that is already available in the market [117, 129, 137, 154, 201, 210-212, 242, 244-247]. To measure ACE-inhibitory activity, distinct biological, radiochromatographic, colorimetric and radioimmunologic methods have been employed – using angiotensin I as substrate. Chemical methods are sensitive, and resort to a tripeptide with a substituted amino-terminus, Z-Phe-His-Leu, as ACE-substrate – from which the dipeptide His-Leu is released

and quantified by specific fluorometric procedures. A similar tripeptide used as substrate of ACE is Bz-Gly-His-Leu, or Hippuryl-His-Leu (HHL); upon incubation with the enzyme, hippuric acid is formed and the dipeptide His-Leu is released, which is subsequently measured by one of several colorimetric [248] or fluorometric methods [249], or even by capillary electrophoresis [250].

One of the most performing methods to measure ACE-inhibitory activity was developed by Cushman and Cheung [251], and is based on spectrophotometric measurement at 228 nm of hippuric acid formed by incubating the substrate HHL with ACE – in the presence of selected inhibitory substances. More recently, a modified tripeptide, furanacriloil Gly-Phe-Gly, has been chosen as substrate for a spectrophotometric method [252]. The ACE-inhibitory activity is usually measured in terms of IC_{50} (i.e. the concentration of inhibitory substance required to inhibit 50 % of ACE activity); a low IC_{50} value means that a small concentration of inhibitory substance is required to produce enzyme inhibition, so that substance displays a potent inhibitory activity.

As shown in Table 4, ACE-inhibitor peptides are produced mainly by enzymatic hydrolysis, but active sequences have also been obtained via chemical synthesis [253]. Starter and non-starter bacteria are commonly used in cheese manufacture – taking advantage namely of their proteolytic system, which contains at least 16 different peptidases that have already been characterized. Some of these bacteria were found to have ACE-inhibitory activity, or release peptides with this activity. For instance, *Lactobacillus helveticus* is able to release ACE-inhibitory peptides; the best-known ACE-inhibitory peptides – *viz.* VPP and IPP, have indeed been identified in milk fermented with *L. helveticus* strains [154, 244, 254]. More recently, an ACE-inhibitory peptide derived from β-CN – FFVAPFPEVFGK, was successfully expressed by genetic engineering in *Escherichia coli* [255].

5.1.1. Structure/activity relationships

ACE-inhibitor peptides contain usually between 2 and 12 amino acid residues – even though larger peptides may also exhibit such an activity [173]. Ondetti [229] rationalized the interaction of competitive inhibitors for the ACE active site based on enzyme homology with carboxypeptidase A; the first ACE-inhibitor (i.e. captopril), which is one of the oral drugs widely used to treat hypertension, was designed based on this model. Recently, this model was reviewed and used to design even more potent ACE inhibitors [229, 256-257]. The base model proposes that residues of the carboxy-terminal (C-terminal) tripeptide interact with the S_1, S'_1 and S'_2 subunits of the enzyme active site. One of the subunits has a positively charged group that forms an ionic bond with the C-terminal peptide group. The following subunit contains a group capable of interacting with the peptidic bond of the C-terminal amino acid – probably through hydrogen bonding. The third subunit has a Zn^{2+} atom able to carry the carbonyl group of the peptidic bond between the one before the last and the last amino acid residue of the substrate – thus making it more susceptible to hydrolysis [256].

Although the relationships between structure and activity have not been fully elucidated, ACE-inhibitory peptides possess a number of analogies with each other. The tripeptide at the C-terminus is crucial – because this is where the peptide binds to the active site of the enzyme [256]. ACE prefers substrates (or competitive inhibitors) with hydrophobic residues (e.g. Trp, Tyr, Phe and Pro) at the C-terminus, and shows poorer affinity to substrates containing dicarboxylic amino acids in the final position, or those that have a Pro residue in the one before the last position. However, presence of Pro as the last residue [258], or in the third position from the terminus [259] favors binding of peptide to enzyme, in much the same way as when Leu appears in the last position [260,261].

Bioinformatics has been used more recently to find the structural requirements of ACE-inhibitor peptides; these are termed quantitative structure/activity relationship (QSAR) models. Through a QSAR model, Pripp [262] concluded – for milk-derived peptides up to six amino acids in length, that there is a relationship between ACE-inhibitory activity and presence of a hydrophobic (or positively charged) amino acid residue in the last position of the sequence; however, no special relation was found with the structure of the N-terminus. Based on the QSAR model for peptides containing between 4 and 10 amino acid residues, Wu [263] claimed that the residue of the C-terminal tetrapeptide may determine the potency of ACE inhibition – with preference for Tyr and Cys in the first C-terminal position; His, Trp and Met in the second; Ile, Leu, Val and Met in the third; and Trp in the fourth position. Results from other QSAR-based studies aimed at finding ACE-inhibitory activity of di- and tripeptides derived from food proteins have shown that dipeptides with hydrophobic chains, as well as tripeptides with an aromatic amino acid residue at the C-terminus, a positively charged residue at the intermediate position and a hydrophobic amino acid residue at the N-terminus are likely to exhibit ACE-inhibitory power [263].

On the other hand, a biopeptide may adopt a different configuration depending on the prevailing environmental conditions; but the final structural conformation may be crucial for its ACE-inhibitory activity. The fact that the catalytic center of ACE has different structural requirements may unfold the need to develop complex mixtures of peptides, with different structural conformations, so as to produce more complete inhibition than a single peptide [264]. Meisel [265] postulated that the mechanism of ACE inhibition may involve interaction of inhibitor with the subunits that are not normally occupied by substrate, or with the anionic bond site that is different from the enzyme catalytic center. Moreover, somatic ACE has two homologous domains – each of which has an active site with distinct biochemical characteristics. *In vitro* ACE-inhibition studies showed that it is necessary to block the two active centers for complete inhibition of its action upon angiotensin I and bradykinin. Nevertheless, *in vivo* studies in rats showed that the selective inhibition of the N- or C-terminal domains of ACE prevents conversion of angiotensin I to II, but not of bradykinin [266].

Despite the importance arising from the three amino acids in their C-terminus, it was shown that peptides with identical sequences at the C-terminus may exhibit quite different ACE-inhibitory activities from each other. One example is VRYL and VPSERYL, both identified in

Manchego cheese; despite having the same C-terminal tripeptide sequence, they exhibit IC_{50} values of 24.1 μM and 249.5 μM, respectively – i.e. the latter is 10-fold less active than the former. If Val were replaced by a dicarboxylic amino acid at the fourth position of the C-terminus, e.g. via synthesis of ERVL, the IC_{50} measured would be 200.3 μM, which corresponds to an ACE-inhibitory activity 8-fold lower than VRYL – hence demonstrating the crucial role of Val in that position for the intended bioactivity [261].

5.1.2. Bioavailability

Among the several bioactive peptides studied to date, ACE-inhibitory peptides have received particular attention because of their beneficial effects upon hypertension [226, 233, 267]. Note that such effects depend on their ability to reach the target organs without having undergone decay or transformation. Tests encompassing hypertensive animals and human clinical trials have shown that certain sequences can lower blood pressure; however, it is difficult to establish a direct link between the ability to inhibit ACE *in vitro* and the actual antihypertensive activity *in vivo*. Knowledge of the mechanism of action of such bioactive peptides is obviously crucial before manufacture of functional foods with physiological properties is in order [268].

Some peptides with ACE-inhibitory and antihypertensive activities can be transported through the intestinal mucosa via the $PepT_1$ transporter [269]; likewise, there is evidence that other peptides may exert a direct role upon the intestinal lumen [151, 270-271]. Digestive enzymes, absorption through the intestinal tract and blood proteases can bring about hydrolysis of ACE-inhibitor peptides, thus producing fragments with lower or greater activity than their precursor sequences [216]. Hence, for ACE-inhibitor peptides exert an *in vivo* effect, they should not act as substrates of the enzyme. Peptides may accordingly be classified into three groups based on their behavior regarding ACE: (1) true inhibitors, for which IC_{50} is not modified when incubated with the enzyme; (2) ACE-substrates, which are hydrolyzed during incubation, thus giving rise to fragments with a lower ACE-inhibition activity; and (3) peptides that are converted to real inhibitors by ACE and gastrointestinal protease action. Note that only sequences belonging to groups 1 and 3 may show an antihypertensive effect [245].

Effective inclusion of ACE-inhibitory peptides in the diet consequently requires them to somehow resist the strong stomach hydrolysis that may cause loss of bioactivity [104], and afterwards be able to pass into the blood stream – where they should be resistant to peptidases therein, so as to eventually reach the target sites where they are supposed to exert their physiological effects *in vivo*. The structure and bioactivity of short-chain peptides are more easily preserved through gastrointestinal passage than those of their long-chain counterparts [272] – whereas sequences containing Pro residue(s) are generally more resistant to degradation by digestive enzymes [273]. Furthermore, peptides absorbed following digestion may accumulate in specific organs, and then exert their action in a systematic and gradual manner [274, 275]. However, antihypertensive peptides that cannot

be absorbed from the digestive tract may still exert their function directly in the intestinal lumen – e.g. via interaction with receptors on the intestinal wall [97, 265, 276].

Besides carrying out protein degradation to varying extents, gastrointestinal digestion plays a key role in formation of ACE-inhibitory peptides [216, 277]; hence, it is relevant to assess the gastrointestinal bioavailability of any potentially interesting peptides. Several studies have accordingly provided evidence for this realization – as happened with Manchego cheese, as well as with other fermented solutions and infant formulae [100, 261, 278-281]; for instance, a potent antihypertensive peptide was released via gastrointestinal digestion from a precursor with poor ACE-inhibitory activity *in vitro* [282] – and some peptides possess a remarkable intrinsic stability, whereas others are susceptible to unwanted degradation [136, 261, 281]; however, whether of any of those options will apply cannot be known in advance.

Animal and human trials are therefore nuclear when assessing bioactivity of peptides; peptides that do not show *in vitro* activity may exhibit *in vivo* antihypertensive activity, and vice versa. For instance, YKVPQL identified in a casein hydrolyzate and released by a proteinase from *L. helveticus* CP790, had a high *in vivo* ACE-inhibitory activity (IC_{50} 22 μM) but did not show any antihypertensive one [282] – probably as a consequence of degradation during the digestion process [137]. When the hydrolyzate was purified, another peptide sequence (KVLPVPQ) was found. Unlike the previous case – with a low *in vitro* ACE-inhibitory activity (IC_{50} > 1000 μM), the latter showed a potent *in vivo* antihypertensive activity. It was claimed that this was due to pancreatic digestion that releases Gln, thus forming KVLPVP; furthermore, this fragment showed ACE-inhibitory activity *in vitro*, characterized by an IC_{50} of only 5 μM. Finally, there are reports on peptides with a low ACE-inhibitory activity *in vitro* that possess antihypertensive activity *in vivo* – owing to a hypotensive mechanism of action distinct from that of ACE inhibition. One example is YP, the IC_{50} of which is 720 μM; however, it significantly decreases blood pressure between 2 and 8 h after oral administration to SHR [283]. It should be emphasized that *in vivo* tests of (putatively) promising bioactive peptides should not come into play before careful *in vitro* models have been checked – as they can provide useful preliminary information on the stability of such peptides upon exposure to the various peptidases and proteinases that they will likely find in the gastrointestinal tract, prior to eventual transport across the intestinal barrier [278-279].

Simulated (physiological) digestion is a useful tool to assess the stability of peptides with ACE-inhibitory activity against digestive enzymes. However, the degree of hydrolysis of a given peptide depends not only on its size and nature, but also on the presence of other peptides in its vicinity [272] – which would make it difficult to test the required number of possibilities in a rather limited experimental program. Several *in vitro* studies were carried out that show the importance of digestion upon formation and degradation of ACE-inhibitor peptides [107, 272, 278-280, 284]. In these studies, peptides were subjected to two stages of hydrolysis that mimic digestion in the body. First, hydrolysis with pepsin, at acidic pH, intended to simulate the digestion process prevailing in the stomach; and second, digestion with a pancreatic extract, at basic pH as prevailing during intestinal digestion.

Results encompassing prior or subsequent hydrolysis of peptides showed that *in vitro* digestion controls bioavailability of ACE-inhibitor peptides [162, 278]

Some authors used whey proteins, fermented (or not at all) with *L. helveticus* and *Saccharomyces cerevisiae*, and then subjected them to gastrointestinal digestion; they reached a maximum ACE-inhibitory activity, and unfermented samples were the most active. However, some peptides with *in vivo* antihypertensive activity – as is the sequence KVLPVPQ, and which did not show *in vitro* ACE-inhibitory activity, could be transformed to active forms via gastrointestinal digestion [282]. Simulation of digestion is also useful in studies of the mechanism of action of antihypertensive peptides with demonstrated *in vivo* activity. For example, Miguel [277] found that YAEERYPIL derived from ovalbumin – which is a powerful ACE-inhibitor (IC_{50} = 4.7 μM) and exhibits antihypertensive activity, was susceptible to degradation by digestive enzymes; that peptide was indeed fully hydrolyzed during simulated gastrointestinal digestion, thus giving rise to fragments YAEER and YPI. Tests on mice showed that YAEER could not significantly lower blood pressure, but the peptide YPI exhibited a significant antihypertensive effect. This fragment may possibly be the active form hidden in the sequence YAEERYPIL, and may exert its action via a different mechanism of ACE-inhibition [285].

In vitro models provide useful information to assess the stability of bioactive peptides to different peptidases and proteinases of the body, yet transport across the intestinal barrier raises an extra resistance – so they have limitations. *In vitro* simulated digestion is in fact not entirely reliable; the degree of hydrolysis depends on the size, nature and neighborhood of the peptide [272], so *in vivo* studies (with laboratory animals and human volunteers) are eventually necessary to ascertain in full the behavior of the peptide. Another example is the release of *potent* ACE-inhibitory peptides from WPC brought about by aqueous extracts from the plant *C. cardunculus.* A peptide mixture – in which 3 peptides were pinpointed: α-La f(16-26), with the sequence KGYGGVSLPEW; α-La f(97-104), with the sequence DKVGINYW; and β-Lg f(33-42), with the sequence DAQSAPLRVY, produced ACE-inhibition (see Table 4). Such peptides were then exposed to simulated gastrointestinal digestion: no peptide was able to keep its integrity, but even total hydrolysis to smaller peptides did not significantly compromise the overall ACE-inhibitory activity observed. In view of their ACE-inhibitory activities, both in the absence or following gastrointestinal digestion, peptides KGYGGVSLPEW and DAQSAPLRVY are expected to eventually exhibit notable antihypertensive activities *in vivo* [107].

6. Concluding remarks

Processing of whey proteins yields several bioactive peptides able to trigger physiological effects in the human body. Such peptides, in concentrated form, can be commercially appealing because their claimed health-promoting features are nowadays an important driver for consumers' food choices. Hence, they may constitute an excellent alternative for whey upgrade. Use of selective membranes to isolate, and eventually purify whey proteins and peptides has substantially increased the number and depth of studies

encompassing those molecules and their hydrolysates. The technology developed is not excessively expensive, and can easily be implemented in dairy plants – of either small or large dimension. Most whey peptides bearing biological activity are released by enzymatic hydrolysis, so new alternatives to enzymes of animal origin have been under scrutiny.

This chapter focused on studies of whey peptides with antihypertensive activity – including their mechanisms of action (especially ACE inhibition), as well as the bioavailability of these peptides, and highlighting the main *in vitro* and *in vivo* results, as well as clinical trials in humans.

Although a good deal of data have been generated encompassing food bioactive peptides, much is still left to do with whey peptides. Hence, several opportunities for further research exist, on incorporation of said ingredients in food products for human consumption. However, several scientific, technological and regulatory issues should be addressed before such peptide concentrates (and pure peptides) will have a chance to be marketed at large, aiming at both human nutrition and health.

More detailed studies are indeed welcome for a better understanding of antihypertensive mechanisms. In particular, the antihypertensive activity should be checked with extra detail – including deep studies on the blood pressure-reducing mechanisms, such as the effects of peptides on neutral endopeptidases and their putative beneficial activity upon cardiovascular diseases. The pharmacological effect of said peptides should be determined both on post- and prejunctional receptors. More extensive clinical trials should also be performed – after thorough bioavailability studies *in vitro*, such as stability to gastrointestinal digestion and passage through the blood barrier, have taken place.

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7. References

[1] Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. Scientific concepts of functional foods in Europe consensus document. British Journal of Nutrition 1999; 81, 1-27.

[2] Arai S. Studies on functional foods in Japan–state of the art. Bioscience, Biotechnology and Biochemistry 1996; 60, 9-15.

[3] Korhonen H, Pihlanto A. Food-derived bioactive peptides – opportunities for designing future foods. Current Pharmaceutical Design 2003; 9, 1297-1308.

[4] Korhonen H, Pihlanto A. Bioactive peptides: production and functionality. International Dairy Journal 2006; 16, 945-960.

[5] Pintado ME, Pintado AE, Malcata FX. Controlled whey protein hydrolysis using two alternative proteases. Journal of Food Engineering 1999; 42, 1-13.

[6] Recio I, López-Fandiño R. Ingredientes y productos lácteos funcionales, bases científicas de sus efectos en la salud. In: Alimentos funcionales (ed.) Fundación Espanõla para la Ciencia y la Tecnología; 2005.

[7] Mulvihill DM. Production, functional properties and utilization of milk protein products. In: Fox PF. (ed.) Advanced dairy chemistry – proteins, vol 1. London, UK: Elsevier; 1992. p369-404.

[8] Nakai S, Modler HW. Food proteins, properties and characterization. New York, USA: Wiley – VCH Publishers; 1996.

[9] Fox PF, McSweeney PLH. Milk Proteins. In: Dairy chemistry and biochemistry. London, UK: Blackie Academic and Professional; 1998.

[10] Pintado ME, Macedo AC, Malcata FX. Review: technology, chemistry and microbiology of whey cheeses. Food Science and Technology International 2001; 7, 105-116.

[11] Blenford DE. Whey: from waste to gold. International Food Ingredients 1996; 1, 27-29.

[12] Barth CA, Behnke U. Nutritional significance of whey and whey components. Nahrung-Food 1997; 41, 2-12.

[13] Walzem RL, Dilliard CJ, German JB. Whey components: millenia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. Critical Reviews in Food Science and Nutrition 2002; 42, 353-375.

[14] McIntosh GH, Royle PJ, le Leu RK, Regester GO, Johnson MA, Grinsted RL, Kenward RS, Smithers GW. Whey proteins as functional food ingredients? International Dairy Journal 1998; 8, 425-434.

[15] Balagtas JV, Hutchinson FM, Krochta JM, Sumner DA. Anticipating market effects of new uses for whey and evaluating returns to research and development. Journal of Dairy Science 2003; 86, 1662-1672.

[16] Miller GD, Jarvis JK, Mcbeon LD. Handbook of Dairy Foods and Nutrition. Boca Raton, USA: CRC, 2000.

[17] Sgarbieri VC. Proteinas em alimentos protéicos: propriedades, degradações, modificações. Varela, São Paulo 1996; 139-157.

[18] Pihlanto-Leppälä A, Korhonen H. Bioactive peptides and proteins. Advances in Food and Nutrition Research 2003; 47, 175-276.

[19] Whitney RM. Milk Proteins In: Fundamentals of dairy chemistry. van Nostrand Reinhold. New York, USA; 1988.
[20] Law AJR, Leaver J, Banks JM, Horne DS. Quantitative fractionation of whey proteins by gel permeation FPL. Milchwissenschaft 1993; 48, 663-666.
[21] Creamer L. K., Sawyer L. β-Lactoglobulin. In: Roginski H, Fuquay JW, Fox PF (ed) Encyclopedia of Dairy Sciences. New York: Academic Press; 2003.
[22] Sawyer L, Kontopidis G. The core lipocalin, bovine β-lactoglobulin. Biochimica et Biophysica Acta 2000; 1482, 136-148.
[23] Imafidon IG, Farkye YN, Spanier MA. Isolation, purification, and alteration of some functional groups of major milk proteins: a review. Food Science and Nutrition 1997; 37, 663-689.
[24] Gough P, Jenness R. Heat denaturation of β-lactoglobulins A and B. Journal of Dairy Science 1961; 44, 1163-1168.
[25] Walstra P, Geurts TJ, Noomen A, Tellema A, van Bockel. MAJS. Principles of milk properties and processes – Dairy Technology. New York, USA: Marcel Dekker; 1999.
[26] Chatterton DEW, Smithers G, Roupas P, Brodkorb A. Bioactivity of β-lactoglobulin and α-lactalbumin – technological implications for processing. International Dairy Journal 2006; 16, 1229-1240.
[27] Papiz MZ, Sawyer L, Eliopoulos EE, North AC, Findlay JB, Sivaprasadarao R, Jones TA, Newcomer ME, Kraulis PJ. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. Nature 1986; 324, 383-385.
[28] Mansouri A, Haertle T, Gerard A, Gerard H, Gueant JL. Retinol free and retinol complexed β-lactoglobulin binding sites in bovine germ cells. Biochimica et Biophysica Acta 1997; 1357, 107-114.
[29] Mansouri A, Gueant JL, Capiaumont J, Pelosi P, Nabet P, Haertle T. Plasma membrane receptor for β-lactoglobulin and retinol-binding protein in murine hybridomas. Biofactors 1998; 7, 287-298.
[30] Barros RM, Ferreira CA, Silva SV, Malcata FX. Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate. Enzyme and Microbial Technology 2001; 29, 541-547.
[31] Anderson ME. Glutathione: an overiew of biosynthesys and modulation. Chemico-Biological Interaction – Limerick 1998; 111, 1-14.
[32] Armstrong JM, McKenzie HA, Sawyer WH. On the fractionation of β-lactoglobulin and α-lactalbumin. Biochimica et Biophysica Acta 1967; 147, 60-72.
[33] Monaco HL, Zanotti G, Spadon P, Bolognesi M, Sawyer L, Eliopoulos EE. Crystal structure of the trigonal form of bovine β-lactoglobulin and of its complex with retinol at 2.5 Å resolution. Journal of Molecular Biology 1987; 197, 695-706.
[34] Felipe X, Law AJ. Preparative-scale fractionation of bovine, caprine and ovine whey proteins by gel permeation chromatography. Journal of Dairy Research 1997; 64, 459-464.
[35] de Jongh HH, Groneveld T, de Groot J. Mild isolation procedure discloses new protein structural properties of β-lactoglobulin. Journal of Dairy Science 2001; 84, 562-571.

[36] Outinen M, Tossavainen O, Tupasela T, Koskela P, Koskinen H, Rantamaki P, Syvaoja EL, Antila P, Kankare V. Fractionation of proteins from whey with different pilot scale processes. Food Science and Technology 1996; 29, 411-417.

[37] Hiraoka Y, Segawa T, Kuwajima K, Sugai S, Murai N. α-Lactalbumin: a calcium metalloprotein. Biochemical and Biophysical Research Communication 1980; 95, 1098-1104.

[38] Eigel WN, Butler JE, Ernstrom CA, Farrell H, Harwalkar VR, Jenness R, Whitney RM. Nomenclature of proteins of cows milk – 5th revision. Journal of Dairy Science, 1984; 67, 1599-1631.

[39] Law AJR, Horne DS, Banks JM, Leaver J. Heat-induced changes in the whey proteins and caseins. Milchwissenschaft 1994; 49, 125-129.

[40] Heine WE, Klein PD, Reeds PJ. The importance of α-lactalbumin in infantil nutrition. Journal of Nutrition 1991; 121, 277-283.

[41] Tolkach A, Kulozik, U. Fractionation of whey proteins and caseinomacropeptide by means of enzymatic crosslinking and membrane separation techniques. Journal of Food Engineering 2005; 67, 13-20.

[42] Chang J, Bulychev A, Li L. A stabilized molten globule protein. FEBS Letters 2000; 487, 298-300.

[43] Dolgikh DA, Abaturov IA, Bolotina, Brazhnikov EV, Bychkova VE, Gilmanshin RI, Lebedev YO, Semisotnov GV, Tiktopulo EI, Ptitsyn OB. Compact state of a protein molecule with pronounced small-scale mobility. European Biophysics Journal 1985; 13, 109-121.

[44] Vanderheeren G, Hanssens I. Thermal unfolding of bovine α-lactalbumin. Comparison of circular dichroism with hydrophobicity measurements. Journal of Biological Chemistry 1994; 269, 7090-7094.

[45] Kuwajima K. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein struture. Protein: Structure, Function and Genetics 1989; 6, 87-103.

[46] Kuwajima K, Hiraoka Y, Ikeguchi M, Sugai S. Comparison of the transient folding intermediates in lysozyme and α-lactalbumin. Biochemistry 1985; 24, 874-881.

[47] Kuwajima K. The molten globule state of α-lactalbumin. The FASEB Journal 1996; 1, 102-109.

[48] Dolgikh DA, Gilmanshin RI, Brazhnikov EV, Bychkova VE, Semisotnov GV, Venyaminov S, Ptitsyn OB. Alpha-lactalbumin: compact state with fluctuating terciary structure? FEBS Letters 1981; 136, 311-315.

[49] Ptitsyn OB. Molten globule and protein folding. Advances in Protein Chemistry 1995; 47, 83-229.

[50] Arai M, Kuwajima K. Role of the molten globule state in protein folding. Advances in Protein Chemistry 2000; 53, 209-282.

[51] Leandro P, Gomes CM. Protein misfolding in conformational disorders: rescue of folding defects and chemical chaperoning. Mini-Reviews in Medicinal Chemistry 2008; 8, 901-911.

[52] Delfour A, Jollès J, Alais C, Jollès P. Caseino-glycopeptides: characterization of a methionine residue and of the N-terminal sequence. Biochemical and Biophysical Research Communications 1965; 19, 452-455.
[53] Manso MA, López-Fandiño R. κ-Casein macropeptides from cheese whey, physicochemical, biological, nutritional, and technological features for possible uses. Food Reviews International 2004; 20, 329-355.
[54] Chobert JM, Touati A, Bertrand-Harb C, Dalgalarrondo M, Nicolas MG. Solubility and emulsifying properties of κ-casein and its caseinomacropeptide. Journal of Food Biochemistry 1989; 13, 457-473.
[55] Moreno FJ, López-Fandiño R, Olano A. Characterization and functional properties of lactosyl caseinomacropeptide conjugates. Journal of Agricultural and Food Chemistry, 2002; 50, 5179-5184.
[56] Dziuba J, Minkiewicz P. Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's κ-casein. International Dairy Journal 1996; 6, 1017-1044.
[57] Kinsella E, Whitehead DM. Proteins in whey: chemical, physical and functional properties. Advances in Food and Nutrition Research 1989: 33, 343-438.
[58] de Wit JN. The use of whey protein products. In: Fox PF. (ed.) Developments in dairy chemistry 4. Functional milk proteins. Barking, UK: Elsevier Science Publishers; 1989. p323-345.
[59] Korhonen H, Marnila P, Gill H. Milk immunoglobulins and complement factors, a review. British Journal of Nutrition 2000; 84, 75-80.
[60] Steijns JM. Milk ingredients as nutraceuticals. International Journal of Dairy Technology 2001; 54, 81-88.
[61] Fonseca ML, Fonseca CSP, Brandão SCC. Propriedades anticarcinogênicas de componentes do leite. Indústria de Laticínios 1999; 4, 5-55.
[62] Alais C (ed.). Science du lait: principes des techniques laitières 4. Paris: SEPAIC; 1984.
[63] Innocente N, Corradini C, Blecker C, Paquot M. Emulsifying properties of the total fraction and the hydrophobic fraction of bovine milk proteose-peptones. International Dairy Journal 1998; 8, 981-985.
[64] Parodi PW. A role for milk protein in cancer prevention. Australian Journal of Dairy Technology 1998; 53, 37-47.
[65] Regester GO, Mcintosh GH, Lee VWK, Smithers GW. Whey proteins as nutritional and functional food ingredients. Food Australia 1996; 48, 123-127.
[66] Ha E, Zemel MB. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). Journal of Nutritional Biochemistry 2003; 14, 251-258.
[67] Smithers GW. Whey and whey proteins – 'from gutter-to-gold'. International Dairy Journal 2008; 18, 695-704.
[68] Bouchard D, Morisset D, Bourbonnais Y, Tremblay GM. Proteins with whey acidic-protein motifs and cancer. Lancet Oncology 2006; 7, 167-174.
[69] Gauthier SF, Pouliot Y, Saint-Sauveur D. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. International Dairy Journal 2006; 16, 1315-1323.

[70] Xu RJ. Bioactive peptides in milk and their biological and health implications. Food Reviews International 1998; 14, 1-16.
[71] Prates JAM, Mateus CMRP. Functional foods from animal sources and their physiologically active components. Revue de Médicine Vétérinaire 2002; 153, 155-160.
[72] Amaya-Farfán J. Avanços no conhecimento sobre a função das proteínas nas dietas para desempenho físico. 5º Simpósio Latino Americano de Ciência de Alimentos – Desenvolvimento Científico e Tecnológico e Inovação na Indústria de Alimentos. Campinas-Unicamp, 2003.
[73] Hartmann R, Meisel H. Food-derived peptides with biological activity: from research to food applications. Current Opinion in Biotechnology 2007; 18, 163-169.
[74] Gill HS, Rutherford KJ, Cross ML. Bovine milk: a unique source of immunomodulatory ingredients for functional foods. In: Buttriss J, Saltmarsh M. (eds.) Functional foods II – claims and evidence. Cambridge, UK: Royal Society of Chemistry Press; 2000. p82-90.
[75] Badger TM, Ronis MJJ, Hakkak R. Developmental effects and health aspects of soy protein isolate, casein and whey in male and female rats. International Journal of Toxicology 2001; 20, 165-174.
[76] Hakkak R, Korourian S, Shelnutt SR, Lensing S, Ronis MJJ, Badger TM. Diets containing whey proteins or soy protein isolate protect against 2,12-dimethylbenzanthracene-induced mammary tumours in female rats. Cancer Epidemiology, Biomarkers and Prevention 2000; 9, 113-117.
[77] Rowlands JC, He L, Hakkak R, Ronis MJJ, Badger TM. Soy and whey proteins down regulate DMBA-induced liver and mammary gland CYP1 expression in female rats. Journal of Nutrition 2001; 131, 3281-3287.
[78] Micke P, Beeh KM, Schlaak JF, Buhl R. Oral supplementation with whey proteins increases plasma GSH levels of HIV-infected patients. European Journal of Clinical Investigation, 2001; 31, 171-178.
[79] Micke P, Beeh KM, Buhl R. Effects of long-term supplementation with whey proteins on plasma GSH levels of HIV-infected patients. European Journal of Nutrition 2002; 41, 12-18.
[80] Clare DA, Catignani GL, Swaisgood HE. Biodefense properties of milk, the role of antimicrobial proteins and peptides. Current Pharmaceutical Design 2003; 9, 1239-1255.
[81] Hall WL, Millward DJ, Long SJ, Morgan LM. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. British Journal of Nutrition 2003; 89, 339-348.
[82] Tavares TG, Contreras MM, Amorim M, Martín-Álvarez PJ, Pintado ME, Recio I, Malcata FX. Optimization, by response surface methodology, of degree of hydrolysis, antioxidant and ACE-inhibitory activities of whey protein hydrolyzates obtained with cardoon extract. International Dairy Journal 2011; 21, 926-933.
[83] Rosaneli CF, Bighetti AE, Antônio MA, Carvalho JE, Sgarbieri VC. Efficacy of a whey protein concentrate on the inhibition of stomach ulcerative lesions caused by ethanol ingestion. Journal of Medicinal Food 2002; 5, 221-228.

[84] Pacheco MTB, Bighetti EA, Antônio M, Carvalho JE, Possenti A, Sgarbieri VC. Antiulcerogenic activity of fraction and hydrolyzate obtained from whey protein concentrate. Brazilian Journal of Food Technology, III JIPCA 2006; 15-22.

[85] Mezzaroba LFH, Carvalho JE, Ponezi AN, Antônio MA, Monteiro KM, Possenti A, Sgarbieri, VC. Antiulcerative properties of bovine α-lactalbumin. International Dairy Journal 2006; 16, 1005-1012.

[86] Tavares TG, Monteiro KM, Possenti A, Pintado ME, Carvalho JE, Malcata FX. Antiulcerogenic activity of peptide concentrates obtained from hydrolysis of whey protein brought about by proteases from *Cynara cardunculus*. International Dairy Journal 2011; 21, 934-939.

[87] Madureira AR, Pereira CI, Gomes AMP, Pintado ME, Malcata FX. Bovine whey proteins – overview on their main biological properties. Food Research International 2007; 40, 1197-1211.

[88] Puyol P, Dolores-Perez M, Sanchez L, Ena JM, Calvo M. Uptake and passage of β-lactoglobulin, palmitic acid and retinol across the Caco-2 monolayer. Biochimica et Biophysica Acta – Biomembranes 1995; 1236, 149-154.

[89] Wu SY, Pérez MD, Puyol P, Sawyer L. β-Lactoglobulin binds palmitate within its central cavity. Journal of Biological Chemistry 1999; 274, 170-177.

[90] Puyol P, Pérez MD, Ena JM, Calvo M. Interaction of β-lactoglobulin and other bovine and human whey proteins with retinol and fatty acids. Agricultural and Biological Chemistry 1991; 10, 2515-2520.

[91] Bounous G, Batist G, Gold P. Whey proteins in cancer prevention. Cancer Letters 1991; 57, 91-94.

[92] Baruchel S, Wang T, Farah R, Jamali M, Batist G. *In vivo* modulation of tissue glutathione in rat mammary carcinoma model. Biochemical Pharmacology 1995; 50, 1505-1508.

[93] Bounous G. Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. Anticancer Research 2000; 20, 4785-4792.

[94] Perez MD, Sanchez L, Aranda P, Ena JM, Oria R, Calvo M. Effect of β-lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. Biochimica et Biophysica Acta 1992; 1123, 151-155.

[95] Warme PKF, Momany A, Rumball SV, Tuttle RW, Scherag HA. Computation of structures of homologous proteins. α-Lactalbumin from lysozyme. Biochemistry 1974; 13, 768-782.

[96] Farrel HM, Bede MJ, Enyeart JA. Binding of *p*-nitrophenyl phosphate and other aromatic compounds by β-Lg. Journal of Dairy Science 1987; 70, 252-258.

[97] Meisel H, Schlimme E. Bioactive peptides derived from milk proteins: ingredients for functional foods? Kieler Milchwirtschaftliche und Forschungsberichte 1996; 48, 343-357.

[98] Mullally MM, Meisel H, Fitzgerald RJ. Synthetic peptides corresponding to α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. Biological Chemistry Hoppe-Seyler 1996; 377, 259-260.

[99] Pihlanto-Leppälä A, Paakkari I, Rinta-Koski M, Antila P. Bioactive peptide derived from *in vitro* proteolysis of bovine β-lactoglobulin and its effect on smooth muscle. Journal of Dairy Research 1997; 64, 149-155.

[100] Pihlanto-Leppälä A, Rokka T, Korhonen H. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. International Dairy Journal 1998; 8, 325-331.

[101] Pihlanto L. Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. Trends in Food Science and Technology 2000; 11, 347-356.

[102] Ijäs H, Collin M, Finckenberg P, Pihlanto-Leppälä A, Korhonen H, Korpela R, Vapaatalo H, Nurminen ML. Antihypertensive opioid-like milk peptide α-lactorphin: lacks effect on behavioural tests in mice. International Dairy Journal 2004; 14, 201-205.

[103] Hernández-Ledesma B, López-Expósito I, Ramos M, Recio I. Bioactive peptides from milk proteins. In: Pizzano R (ed.) Immunochemistry in dairy research. Kerala, India: Trivandrum; 2006. p37-60.

[104] FitzGerald RJ, Murray BA. Bioactive peptides and lactic fermentations. International Journal of Dairy Technology 2006; 59, 118-125.

[105] Chen G-W, Tsai J-S, Pan BS. Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. International Dairy Journal 2007; 17, 641-647.

[106] Roufik S, Gauthier S, Turgeon S. Physicochemical characterization and *in vitro* digestibility of β-lactoglobulin/β-Lg f142-148 complexes. International Dairy Journal 2007; 17, 471-480.

[107] Tavares TG, Contreras MM, Amorim M, Pintado ME, Recio I, Malcata FX. Novel whey-derived peptides with inhibitory activity against angiotensin-converting enzyme: *in vitro* activity and stability to gastrointestinal enzymes. Peptides 2011; 32, 1013-1019.

[108] Expósito IL, Récio I. Antibacterial activity of peptides and holding variants from milk proteins. International Dairy Journal 2006; 16, 1294-1305.

[109] Pihlanto-Leppälä A, Marnila P, Hubert L, Rokka T, Korhonen HJT, Karp M. The effect of α-lactalbumin and β-lactoglobulin hydrolysates on the metabolic activity of *Escherichia coli* JM103. Journal of Applied Microbiology 1999; 87, 540-545.

[110] Pellegrini A, Thomas U, Bramaz N, Hunziker P, Fellenberg R. Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. Biochimica et Biophysica Acta 1999; 1426, 439-448.

[111] Bruck WM, Graverholt G, Gibson GR. A two-stage continuous culture system to study the effect of supplemental α-lactalbumin and glycomacropeptide on mixed populations of human gut bacteria challenged with enteropathogenic *Escherichia coli* and *Salmonella* serotype *Typhimurium*. Journal of Applied Microbiology 2003; 95, 44-53.

[112] Pellegrini A, Dettling C, Thomas U, Hunziker P. Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. Biochimica et Biophysica Acta 2001; 1526, 131-140.

[113] Groleau PE, Morin P, Gauthier SF, Pouliot Y. Effect of physicochemical conditions on peptide-peptide interactions in a tryptic hydrolysate of β-lactoglobulin and

identification of aggregating peptides. Journal of Agricultural and Food Chemistry 2003; 51, 4370-4375.

[114] Nagaoka S, Futamura Y, Miwa K, Awano T, Yamauchi K, Kanamaru Y, Kojima T, Kuwata T. Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. Biochemical and Biophysical Research Communications 2001; 281, 11-17.

[115] Antila P, Paakkari I, Järvinen A, Mattila MJ, Laukkanen M, Pihlanto-Leppälä A, Mäntsälä P, Hellman J. Opioid peptides derived from *in vitro* proteolysis of bovine whey proteins. International Dairy Journal 1991; 1, 215-229.

[116] Murakami M, Tonouchi H, Takahashi R, Kitazawa H, Kawai Y, Negishi H, Saito T. Structural analysis of a new anti-hypertensive peptide (β-lactosin B) isolated from a commercial whey product. Journal of Dairy Science 2004; 87, 1967-1974.

[117] Tavares TG, Sevilla MA, Montero MJ, Carrón R, Malcata FX. Acute effects of whey peptides upon blood pressure of hypertensive rats, and relationship with their angiotensin-converting enzyme inhibitory activity. Molecular Nutrition and Food Research 2011; doi: 10.1002/mnfr.201100381.

[118] Yamauchi R, Sonoda S, Jinsmaa Y, Yoshikawa M. Antinociception induced by β-lactotensin, a neurotensin agonist peptide derived from β-lactoglobulin, is mediated by NT2 and D1 receptors. Life Sciences 2003; 73, 1917-1923.

[119] de Wit JN. Nutritional and functional characteristics of whey proteins in food products. Journal of Dairy Science 1998; 81, 597-608.

[120] A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C. Apoptosis induced by a human milk protein. Proceedings of the National Academy of Sciences of USA 1995; 92, 8064-8068.

[121] Svensson M, Sabharwal H, Hakansson A, Mossberg AK, Lipniunas P, Leffler H, Svanborg, C, Linse S. Molecular characterization of α-lactalbumin folding variants that induce apoptosis in tumor cells. Journal of Biological Chemistry 1999; 274, 6388-6396.

[122] Svensson M, Hakansson A, Mossberg AK, Linse S, Svanborg C. Conversion of α-lactalbumin to a protein inducing apoptosis. Proceedings of the National Academy of Sciences of USA 2002; 97, 4221-4226.

[123] Markus CR, Olivier B, Haan E. Whey protein rich in α-lactalbumin increases the ratio of plasma tryptophan to the sum of the other large neutral amino acids and improves cognitive performance in stress-vulnerable subjects. American Journal of Clinical Nutrition 2002; 75, 1051-1056.

[124] Ganjam LS, Thornton WH, Marshall RT, MacDonald RS. Antiproliferative effects of yoghurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. Journal of Dairy Science 1997; 80, 2325-2329.

[125] Hakansson A, Svensson M, Mossberg AK, Sabharwal H, Linse S, Lazou I, Lönnerdal B, Svanborg C. A folding variant of α-lactalbumin with bactericidal activity against *Streptococcus pneumoniae*. Molecular Microbiology 2000; 35, 589-600.

[126] Markus CR, Olivier B, Panhuysen GEM, Gugten J, van Der, Alles MS, Tuiten A, Westenberg HGM, Fekkes D, Kopperschaar HF, Haan E. The bovine α-lactalbumin increases the plasma ratio of tryptophan to the other large neutral amino acids, and in

vulnerable subjects raises brain serotonin activity, reduces cortisol concentration, and improves mood under stress. American Journal of Clinical Nutrition 2000; 71, 1536-1544.

[127] Montagne PM, Cuiliere ML, Mole CM, Bene MC, Faure GC. Dynamics of the main immunologically and nutritionally available proteins of human milk during lactation. Journal of Food Composition and Analysis 2000; 13, 127-137.

[128] Meisel H, FitzGerald RJ. Opioid peptides encrypted in milk proteins. Journal of Nutrition 2000; 84, 27-31.

[129] Nurminen ML, Sipola M, Kaarto H, Pihlanto-Leppälä, Piilola K, Korpela R, Tossavainen O, Coronen H, Vapaatalo H. α-Lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. Life Science 2000; 66, 1535-1543.

[130] Matsumoto H, Shimokawa Y, Ushida Y, Toida T, Hayasawa H. New biological function of bovine α-lactalbumin: protective effect against ethanol- and stress-induced gastric mucosal injury in rats. Bioscience, Biotechnology and Biochemistry 2001; 65, 1104-1111.

[131] Uchida K, Tateda T, Takagi S. Hypothetical mechanism of prostaglandin E1-induced bronchoconstriction. Medical Hypotheses 2003; 61, 378-384.

[132] Rosaneli CF, Bighetti AE, Antônio MA, Carvalho JE, Sgarbieri VC. Protective effect of bovine milk whey protein concentrate on the ulcerative lesions caused by subcutaneous administration of indomethacin. Journal of Medicinal Food 2004; 7, 309-314.

[133] Tong LM, Sasaki S, McClements DJ, Decker EA. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. Journal of Agricultural and Food Chemistry 2000; 48, 1473-1478.

[134] Smith C, Halliwell B, Aruoma O I. Protection of albumin against the pro-oxidant actions of phenolic dietary components. Food Chemistry and Toxicology 1992; 30, 483-489.

[135] Laursen I, Briand P, Lykkesfeldt AE. Serum albumin as a modulator of the human breast cancer cell line MCF-7. Anticancer Research 1990; 10, 343-351.

[136] Abubakar A, Saito T, Kitazawa H, Kawai Y, Itoh T. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. Journal of Dairy Science 1998; 81, 3131-3138.

[137] FitzGerald RJ, Murray BA, Walsh DJ. Hypotensive peptides from milk proteins. Journal of Nutrition 2004; 134, 980-988.

[138] Yamauchi K. Biologically functional proteins of milk and peptides derived from milk proteins. IDF Bulletin 1992; 272, 51-57.

[139] Tani F, Shiota, Chiba H, Yosliikawa M. Serophin an opioid peptide derived from bovine serum albumin. In: Brantl V. (ed.) β-Casomorphins and relate peptides: recent developments. Weinheim, Germany: VCH-Verlag; 1993. p49-53.

[140] Ormrod DJ, Miller TE. The anti-inflammatory activity of a low molecular weight component derived from the milk of hyperimmunized cows. Agents and Actions 1991; 32, 160-166.

[141] Mitra AK, Mahalanabis D, Unicomb L, Eechels R, Tzipori S. Hyperimmune cow colostra reduces diarrhoea due to rotavirus: a double-blind, controlled clinical trial. Acta Paediatrica 1995; 84, 996-1001.

[142] Tomita M, Todhunter DA, Hogan JS, Smith KL. Immunisation of dairy cows with an *Escherichia coli* J5 lipopolysaccharide vaccine. Journal of Dairy Science 1995; 78, 2178-2185.

[143] Oona M, Rägö T, Maaroos HI, Mikelsaar M, Loivukene K, Salminen S, Korhonen H. *Helicobacter pylori* in children with abdominal complaints: has immune bovine colostrum some influence on gastritis? Alpe Adria Microbiology Journal 1997; 6, 49-57.

[144] Freedman DJ, Tacket CO, Delehanty A, Maneval DR, Nataro J, Crabb JH. Milk immunoglobulin with specific activity against purified colonization factor antigens can protect against oral challenge with enterotoxigenic *Escherichia coli*. Journal of Infectious Diseases 1998; 177, 662-667.

[145] Loimaranta V, Laine M, Söderling E, Vasara E, Rokka S, Marnila P, Korhonen H, Tossavainen O, Tenovuo J. Effects of bovine immune- and non-immune whey preparations on the composition and pH response of human dental plaque. European Journal of Oral Science 1999; 107, 244-250.

[146] Okhuysen PC, Chappell CL, Crabb J, Valdez LM, Douglass E, DuPont HL. Prophylactic effect of bovine anti-*Cryptosporidium hyperimmune* colostra immunoglobulin in healthy volunteers challenged with *Cryptosporidium parvum*. Clinical Infectious Diseases 1998; 26, 1324-1329.

[147] Sharpe SJ, Gamble GD, Sharpe DN. Cholesterol-lowering and blood pressure effects of immune milk. American Journal of Clinical Nutrition 1994; 59, 929-934.

[148] Jollès P, Fiat AM. The carbohydrate portions of milk glycoproteins. Journal of Dairy Research 1979; 46, 187-191.

[149] Jollès P, Levy-Toledano S, Fiat AM, Soria C, Cillessen D, Thomaidis A, Dunn FW, Caen JP. Analogy between fibrinogen and casein. Effect of an undecapeptide isolated from κ-casein on platelet function. European Journal of Biochemistry 1986; 158, 379-382.

[150] Shebuski RJ, Berry DE, Bennett DB, Romoff T, Storer BL, Ali F, Samanen J. Demonstration of Ac-Arg-Gly-Asp-Ser-NH_2 as an antiaggregatory agent in the dog by intracoronary administration. Journal of Thrombosis and Haemostasis 1989; 61, 183-188.

[151] Chabance B, Marteau P, Rambaud JC, Migliore-Samour D, Boynard M, Perrotin P, Guillet R, Jollès P, Fiat AM. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. Biochimie 1998; 80, 155-165.

[152] Rutherford KJ, Gill H. Peptides affecting coagulation. British Journal of Nutrition 2000; 84, 99-102.

[153] Manso MA, Escudero C, Alijo M, López-Fandiño R. Platelet aggregation inhibitory activity of bovine, ovine and caprine κ-casein macropeptides and their tryptic hydrolyzates. Journal of Food Protection 2002; 65, 1992-1996.

[154] Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S, Takano T. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. Journal of Dairy Science 1995; 78, 777-783.

[155] Manso MA, López-Fandiño R. Angiotensin I converting enzyme-inhibitory activity of bovine, ovine, and caprine κ-casein macropeptides and their tryptic hydrolysates. Journal of Food Protection 2003; 66, 1686-1692.

[156] Mizuno S, Matsuura K, Gotou T, Nishimura S, Kajimoto O, Yabune M, Kajimoto Y, Yamamoto N. Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. British Journal of Nutrition 2005; 94, 84-91.

[157] Neeser JR, Chambaz A, del Vedovo S, Prigent MJ, Guggenheim B. Specific and nonspecific inhibition of adhesion of oral actinomyces and streptococci to erythrocytes and polystyrene by caseinoglycopeptide derivatives. Infection and Immunity 1988; 56, 3201-3208.

[158] Kawasaki Y, Isoda H, Tanimoto M, Dosako S, Idota T, Ahiko K. Inhibition by lactoferrin and κ-casein glycomacropeptide of binding of cholera toxin to its receptor. Bioscience, Biotechnology and Biochemistry 1992; 56, 195-198.

[159] Schupbach P, Neeser JR, Golliard M, Rouvet M, Guggenheim B. Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutant streptococci. Journal of Dental Research 1996; 75, 1779-1788.

[160] Oh S, Worobo RW, Kim B, Rheem S, Kim S. Detection of cholera toxin-binding activity of κ-casein macropeptide and optimization of its production by the response surface methodology. Bioscience, Biotechnology and Biochemistry 2000; 64, 516-522.

[161] Bouhallab S, Favrot C, Maubois JL. Growth-promoting activity of tryptic digest of caseinomacropeptide of *Lactococcus lactis* subsp. *lactis*. Le Lait 1993; 73, 73-77.

[162] Beucher S, Levenez F, Yvon M, Corring T. Effects of gastric digestive products from casein on CCK release by intestinal-cells in rat. Journal of Nutritional Biochemistry 1994; 5, 578-584.

[163] Yvon M, Beucher S, Guilloteau P, le Huerou-Luron I, Corring T. Effects of caseinomacropeptide (CMP) on digestion regulation. Reproduction, Nutrition and Development 1994; 34, 527-537.

[164] Pederson NLR, Nagain-Domaine C, Mahe S, Chariot J, Roze C, Tome D. Caseinomacropeptide specifically stimulates exocrine pancreatic secretation in the anesthetized rat. Peptides 2000; 21, 1527-1535.

[165] Dartey C, Leveille G, Sox TE. (2003). Compositions for appetite control and related methods. US Patent 0,059,495 A1.

[166] Hasler C. A new look at an ancient concept. Chemistry and Industry 1998; 2, 84-89.

[167] Pihlanto-Leppälä A. Antioxidative peptides derived from milk proteins. International Dairy Journal 2006; 16, 1306-1314.

[168] Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: production, processing, and potential health benefits. Journal of Food Science 2012; 77, R11–R24.

[169] Korhonen H. Milk-derived bioactive peptides: from science to applications. Journal of Functional Foods 2009; 1, 177-187.

[170] Ustunol Z, Zeckzer T. Relative proteolytic action of milk-cloting enzyme preparations on bovine β and κ casein. Journal of Food Science 1996; 61, 1136-1138.

[171] Fox PF. Exogeneous enzymes in dairy technology. A review. Journal of Food Biochemistry 1993; 17, 173-199.
[172] Simões IIG. Caracterização molecular da acção das cardosinas A e B sobre caseínas β- e κ-bovinas. Tese de Mestrado, Universidade de Coimbra; 1998.
[173] López-Fandiño R, Otte J, van Camp J. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. International Dairy Journal 2006; 16, 1277-1293.
[174] Schmidt DG, Meijer RJ, Slangen CJ, van Beresteijn EC. Raising the pH of the pepsin-catalysed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolyzates. Clinical and Experimental Allergy 1995; 25, 1007-1017.
[175] Barros RM, Malcata FX. Molecular characterization of peptides released from β-lactoglobulin and α-lactalbumin via cardosins A and B. Journal of Dairy Science 2006; 89, 483-494.
[176] Lamas EM, Barros RM, Balcão VM, Malcata FX. Hydrolysis of whey proteins by proteases extracted from *Cynara cardunculus* and immobilized onto highly activated supports. Enzyme and Microbial Technology 2000; 28, 642-652.
[177] Barros RM, Malcata FX. Modelling the kinetics of whey protein hydrolysis brought about by enzymes from *Cynara cardunculus*. Journal of Agricultural and Food Chemistry 2002; 50, 4347-4356.
[178] Barros RM, Malcata FX. A kinetic model for hydrolysis of whey proteins by cardosin A extracted from *Cynara cardunculus*. Food Chemistry 2004; 88, 351-359.
[179] Saboya LV, Maubois JL. Current developments of microfiltration technology in the dairy industry. Le Lait 2000; 80, 541-553.
[180] Muller A, Daufin G, Chaufer B. Ultrafiltration modes of operation for the separation of α-lactalbumin from acid casein whey. Journal of Membrane Science 1999; 153, 9-21.
[181] Cheang B, Zydney AL. Separation of α-lactalbumin and β-lactoglobulin using membrane ultrafiltration. Biotechnology and Bioengineering 2003; 83, 201-209.
[182] Cheang B, Zydney AL. A two-stage ultrafiltration process for fractionation of whey protein isolate. Journal of Membrane Science 2004; 231, 159-167.
[183] Brans G, Schroën CGPH, van der Sman RGM, Boom RM. Membrane fractionation of milk: state of the art and challenges. Journal of Membrane Science 2004; 243, 263-272.
[184] Díaz O, Pereira CD, Cobos A. Functional properties of ovine whey protein concentrates produced by membrane technology after clarification of cheese manufacture by-products. Food Hydrocolloids 2004; 18, 601-610.
[185] Atra R, Vatai G, Bekassy-Molnar E, Balint A. Investigation of ultra- and nanofiltration for utilization of whey protein and lactose. Journal of Food Engineering 2005; 67, 325-332.
[186] Zydney AL. Protein separations using membrane filtration: new opportunities for whey fractionation. International Dairy Journal 1998; 8, 243-250.
[187] Rektor A, Vatai G. Membrane filtration of Mozzarella whey. Desalination 2004;162, 279-286.
[188] Smithers GW, Ballard FJ, Copeland AD, Silva KJ, Dionysius DA, Francis GL, Goddard C, Grieve PA, Mcintosh GH, Mitchell IR, Pearce RJ, Regester GO. New opportunities

from the isolation and utilization of whey proteins. Journal of Dairy Science 1996; 79,1454-1459.

[189] Wong CW, Seow HF, Husband AJ, Regester GO, Watson DL. Effects of purified bovine whey factors on cellular immune functions in ruminants. Veterinary Immunology and Immunopathology 1997; 56, 85-96.

[190] Tavares TG, Amorim M, Gomes D, Pintado ME, Pereira CD, Malcata FX. Bioactive peptide-rich concentrates from whey: pilot process characterization. Journal of Food Engineering 2012; 110, 547-552.

[191] Eastern Stroke and Coronary Heart Disease Collaborative Research Group. Blood pressure, cholesterol and stroke in Eastern Asia. Lancet 1998; 352, 1801-807.

[192] Chalmers J. Blood pressure burden: vascular changes and cerebrovascular complications. Journal of Hypertension 2000; 18, S1-S2.

[193] Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jones DW, Materson BJ, Oparil S, Wright JT, Roccella EJ. The seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. JAMA 2003; 289, 2560-2571.

[194] Tsai JS, Chen TJ, Pan BS, Gong SD, Chung MY. Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk. Food Chemistry 2008; 106, 552-558.

[195] Murray CJL, Lopez AD (eds.). The global burden of disease: a comprehensive assessment of mortality and disability from disease, injuries and risk factors in 1990 and projected to 2020. Global Burden of Disease and Injury Series, vol 1. Cambridge: Harvard School of Public Health; 1996.

[196] Geisterfer AA, Peach MJ, Owens GK. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circulation Research 1988; 62, 749-756.

[197] Daemen MJ, Lombardi DM, Bosman FT, Schwartz SM. Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. Circulation Research 1991; 68, 450-456.

[198] Geerlings A, Villar IC, Zarco FH, Sánchez M, Vera R, Gomez AZ, Boza J, Duarte J. Identification and characterization of novel angiotensin-converting enzyme inhibitors obtained from goat milk. Journal of Dairy Science 2006; 89, 3326-3335.

[199] Hong F, Ming L, Yi S, Zhanxia L, Yongquan W, Chi L. The antihypertensive effect of peptides: a novel alternative to drugs? Peptides 2008; 29, 1062-1071.

[200] Scholz-Ahrens K, Schrezenmeir J. Effects of bioactive substances in milk on mineral and trace element metabolism with special reference to casein phosphopeptides. British Journal of Nutrition 2000; 84, S147-S153.

[201] Jauhiainen T, Korpela R. Milk peptides and blood pressure. Journal of Nutrition 2007; 137, 825S-829S.

[202] Sipola M, Finckenberg P, Korpela R, Vapaatalo H, Nurminen ML. Effect of long-term intake of milk products on blood pressure in hypertensive rats. Journal of Dairy Research 2002; 69, 103-111.

[203] Fujita H, Usui H, Kurahashi K, Yoshikawa M. Isolation and characterization of ovokinin, a bradykinin b-1 agonist peptide derived from ovalbumin. Peptides 1995; 16, 785-790.

[204] Matoba N, Usui H, Fujita H, Yoshikawa M. A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery. FEBS Letters 1999; 452, 181-184.

[205] Erdmann K, Grosser N, Schipporeit K, Schröder H. The ACE inhibitory dipeptide Met-Tyr diminishes free radical formation in human endothelial cells via induction of heme oxygenase-1 and ferritin. Journal of Nutrition 2006; 136, 2148-2152.

[206] Maes W, Van Camp J, Vermeirssen V, Hemeryck M, Ketelslegers JM, Schrezen-meir J, Van Oostveldt P, Huyghebaert. Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells. Regulatory Peptides 2004; 118, 105-109.

[207] Hernández-Ledesma B, Miralles B, Amigo L, Ramos M, Recio I. Identification of antioxidant and ACE-inhibitory peptides in fermented milk. Journal of the Science of Food and Agriculture 2005; 85, 1041–1048.

[208] Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B. Antihypertensive peptides from food proteins: a review. Food and Function 2012; 3, 350-361.

[209] Sánchez D, Kassan M, Contreras MM, Carrón R, Recio I, Montero MJ, Sevilla MA. Long-term intake of a milk casein hydrolysate attenuates the development of hypertension and involves cardiovascular benefits. Pharmacological Research 2011; 63, 398-404.

[210] Quirós A, Ramos M, Muguerza B, Delgado MA, Miguel M, Aleixandre A, Recio I. Identification of novel antihypertensive peptides in milk fermented with *Enterococcus faecalis*. International Dairy Journal 2007; 17, 33-41.

[211] Miguel M, Contreras MM, Recio I, Aleixandre A. ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. Food Chemistry 2009; 112, 211-214.

[212] Hernández-Ledesma B, Contreras MM, Recio I. Antihypertensive peptides: production, bioavailability and incorporation into foods. Advances in Colloid and Interface Science 2011; 165, 23-35.

[213] Gobetti M, Minervini F, Grizzello C. Angiotensin-I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. International Journal of Dairy Technology 2004; 57, 173-188.

[214] Meisel H. Biochemical properties of peptides encrypted in bovine milk proteins. Current Medicinal Chemistry 2005; 12, 1905-1919.

[215] Silva SV, Malcata FX. Caseins as source of bioactive peptides. International Dairy Journal 2005; 15, 1-15.

[216] Vermeirssen V, Verstraete W, van Camp J. Bioavailability of angiotensin I converting enzyme inhibitory peptides. British Journal of Nutrition 2004; 92, 357-366.

[217] Pihlanto-Leppälä A. Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. Trends in Food Science and Technology 2001; 11, 347-356.

[218] Lee SH, Song KB. Isolation of an angiotensin-converting enzyme inhibitory peptide from irradiated bovine blood plasma protein hydrolysates. Food Chemistry and Toxicology, 2003; 68, 2469-2472.

[219] Mine Y, Kovacs-Nolan J. New insights in biologically active proteins and peptides derived from hen egg. World Poultry Science Journal 2006; 62, 87-96.

[220] Matsumura N, Fujii M, Takeda Y, Shimizu T. Isolation and characterization of angiotensin I-converting enzyme-inhibitory peptides derived from bonito bowels. Bioscience, Biotechnology and Biochemistry 1993; 57, 1743-1744.

[221] Wung J, Ding X. Hypotensive and physiological effects of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. Journal of Agricultural and Food Chemistry 2001; 49, 501- 506.

[222] Takayanagi T, Yokotsuka K. Angiotensin I converting enzyme-inhibitory peptides from wine. American Journal of Enology and Viticulture 1999; 50, 65-68.

[223] Miyoshi S, Ishikawa H, Kaneko, Fukui F, Tanaka H, Maruyama S. Structures and activity of angiotensin-converting enzyme inhibitors in α-zein hydrolysate. Agricultural and Biological Chemistry 1991; 55, 1313-1318.

[224] Maruyama S, Suzuki H. A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. Agricultural and Biological Chemistry 1982; 46, 1393-1394.

[225] Maruyama S, Mitachi H, Tanaka H, Tomizuka N, Suzuki H. Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. Agricultural and Biological Chemistry 1987; 51, 1581-1586.

[226] Mullally MM, FitzGerald RJ, Meisel H. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β-lactoglobulin. FEBS Letters 1997; 402, 99-101.

[227] FitzGerald RJ, Meisel H. Lactokinins, whey protein derived ACE inhibitory peptides. Nährung-Food 1999; 43, 165-167.

[228] Hernández-Ledesma B, Recio I, Ramos M, Amigo L. Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. International Dairy Journal 2002; 12, 805-812.

[229] Ondetti MA, Rubin B, Cushman DW. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. Science 1977; 196, 441-444.

[230] Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, Corvol P. Molecular basis of human hypertension: role of angiotensinogen. Cell 1992; 71, 169-180.

[231] Timmermans PBMWM, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JAM, Smith RD. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacological Reviews 1993; 45, 205-251.

[232] Nelson KM, Yeager BF. What is the role of angiotensin-converting enzyme inhibitors in congestive heart failure and after myocardial infarction? Annals of Pharmacotherapy 1996; 30, 986-993.

[233] Pihlanto-Leppälä A, Koskinen P, Piilola K, Tupasela T, Korhonen H. Angiotensin-I converting enzyme inhibitory properties of whey proteins digests: concentration and characterization of active peptides. Journal of Dairy Research 2000; 67, 53-64.

[234] Schlothauer RC, Schollum LM, Reid JR, Harvey SA, Carr A, Fanshawe RL. Improved bioactive whey protein hydrolyzate. Patent PCT/NZ01/00188 (WO 02/19837 A1), New Zealand 2002.

[235] Ortiz-Chao P, Gómez-Ruiz JA, Rastall RA, Mills D, Cramer R, Pihlanto A, Korhonen H, Jauregi P. Production of novel ACE inhibitory peptides from β-lactoglobulin using Protease N Amano. International Dairy Journal 2009; 19, 69-76.

[236] Hernández-Ledesma B, Miguel M, Amigo L, Aleixandre MA, Recio I. Effect of simulated gastrointestinal digestion on the antihypertensive properties of synthetic β-lactoglobulin peptide sequences. Journal of Dairy Research 2007; 74, 336-339.

[237] Otte J, Shalaby S, Zakora M, Nielsen MS. Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. International Dairy Journal 2007; 17, 1460-1472.

[238] Chiba H, Yoshikawa M. Bioactive peptides derived from food proteins. Kagaku to Seibutsu (in Japanese) 1991; 29, 454-458.

[239] Miguel M, Manso MA, López-Fandiño R, Alonso MJ, Salaices M. Vascular effects and antihypertensive properties of κ-casein macropeptide. International Dairy Journal 2007; 17, 1473-1477.

[240] Ruiz-Giménez P, Ibáñez A, Salom JB, Marcos JF, López-Díez JJ, Vallés S, Torregrosa G, Alborch E, Manzanares P. Antihypertensive properties of lactoferricin B-derived peptides. Journal of Agricultural and Food Chemistry 2010; 58, 6721-6727.

[241] Kang DG, Kim YC, Sohn EJ, Lee YM, Lee AS, Yin MH, Lee HS. Hypotensive effect of butein via the inhibition of angiotensin-converting enzyme. Biological and Pharmaceutical Bulletin 2003; 26, 1345-1347.

[242] Miguel M, López F, Ramos M, Aleixandre A. Short-term effect of egg-white hydrolysate products on the arterial blood pressure of hypertensive rats. British Journal of Nutrition 2005; 94, 731-737.

[243] Okamoto K, Aoki K. Development of a strain of spontaneously hypertensive rats. Japanese Circulation Journal 1963; 27, 282-293.

[244] Nakamura Y, Yamamoto N, Sakai K, Takano T. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. Journal of Dairy Science 1995; 78, 1253-1257.

[245] Fujita H, Yokoyama K, Yoshikawa M. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. Journal of Food Science 2000; 65, 564-569.

[246] Muguerza B, Ramos M, Sánchez E, Manso MA, Miguel M, Aleixandre A, Delgado MA, Recio I. Antihypertensive activity of milk fermented by *Enterococcus faecalis* strains isolated from raw milk. International Dairy Journal 2006; 16, 61-69.

[247] Contreras MM, Carrón R, Montero MJ, Ramos M, Recio I. Novel casein-derived peptides with antihypertensive activity. International Dairy Journal 2009; 19, 566-573.

[248] Matsui T, Matsufugi HY, Osajima Y. Colorimetric measurement of angiotensin I-converting enzyme inhibitory activity with trinitrobenzene sulfonate. Bioscience, Biotechnology and Biochemistry 1992; 56, 517-518.

[249] Friedland J, Silverstein E. A sensitive fluorometric assay for serum angiotensin converting enzyme. American Journal of Clinical Pathology 1976; 66, 416-424.

[250] Shihabi ZK. Analysis of angiotensin-converting enzyme by capillary electrophoresis. Journal of Chromatography A 1999; 853, 185-188.

[251] Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochemical Pharmacology 1971; 20, 1637-1648.

[252] Vermeirssen V, van Camp J, Verstraete W. Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. Journal of Biochemical and Biophysical Methods 2002; 51, 75-87.

[253] Kohmura M, Nio N, Kubo K, Minoshima Y, Munekata E, Ariyoshi Y. Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. Agricultural and Biological Chemistry 1989; 53, 2107-2114.

[254] Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. American Journal of Clinical Nutrition 2003; 77, 326-330.

[255] Lv GS, Huo GC, Fu XY. Expression of milk-derived antihypertensive peptide in *Escherichia coli*. Journal of Dairy Science 2003; 86, 1927-1931.

[256] Ondetti MA, Cushman DW. Enzymes of the renin-angiotensin system and their inhibitors. Annual Reviews of Biochemistry 1982; 51, 283-308.

[257] Cushman DW, Cheung HS, Sabo EF, Ondetti MA. Development and design of specific inhibitors of angiotensin-converting enzyme. American Journal of Cardiology 1982; 49, 1390-1394.

[258] Cheung HS, Wang FL, Ondetti MA, Sabo EH, Cushman DW. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Journal of Biological Chemistry 1980; 25, 401-407.

[259] Stevens RL, Micalizzi ER, Fessler DC, Pals DT. Angiotensin I converting enzyme of calf lung. Method of assay and partial purification. Biochemistry 1972; 11, 2999-3007.

[260] Kim SK, Byun HG, Park PY, Fereidoon S. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. Journal of Agricultural and Food Chemistry 2001; 49, 2992-2997.

[261] Gómez-Ruiz JA, Ramos M, Recio I. Angiotensin-converting enzyme-inhibitory activity of peptides isolated from Manchego cheese. Stability under simulated gastrointestinal digestion. International Dairy Journal 2004; 14, 1075-1080.

[262] Pripp AH, Isaksson T, Stepaniak L, Sørhaug T. Quantitative structure-activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. European Food Research and Technology 2004; 219, 579-583.

[263] Wu JP, Aluko RE, Nakai S. Structural requirements of antiotensin I-converting enzyme inhibitory peptides. Quantitative structure-activity relationship study of di- and tripeptides. Journal of Agricultural and Food Chemistry 2006; 54, 732-738.

[264] Gobbetti M, Stepaniak L, de Angelis M, Corsetti A, Cagno RD. Latent bioactive peptides in milk proteins, proteolytic activation and significance in dairy processing. Critical Reviews in Food Science and Nutrition 2002; 42, 223-239.

[265] Meisel H. Biochemical properties of regulatory peptides derived from milk proteins. Biopolymers 1997; 43, 119-128.

[266] Georgiadis D, Beau F, Czarny B, Cotton J, Yiotakis A, Dive V. Roles of the two active sites of somatic angiotensin-converting enzyme in the cleavage of angiotensin-I and bradykinin, insights from selective inhibitors. Circulation Research 2003; 93, 148-154.

[267] Saito T. Antihypertensive peptides derived from bovine casein and whey proteins. In: Bösze, Z (ed.). Advances in experimental medicine and biology: bioactive components of milk, vol. 606. New York, USA: Springer 2008; 295-317.

[268] Shimizu M. Food-derived peptides and intestinal functions. Biofactors 2004; 21, 43-47.

[269] Yang CY, Dantzig AH, Pidgeon C. Intestinal peptide transport systems and oral drug availability. Pharmaceutical Research 1999; 16, 1331-1343.

[270] Gardner MLG. Intestinal assimilation of intact peptides and proteins from the diet – a neglected field. Biological Reviews of the Cambridge Philosophical Society 1984; 59, 289-331.

[271] Roberts PR, Burney JD, Black KW, Zaloga GP. Effects of chain length on absortion of biologically active peptides from the gastrointestinal tract. Digestion 1999; 60, 332-337.

[272] Roufik S, Gauthier SF, Turgeon SL. *In vitro* digestibility of bioactive peptides derived from bovine β-lactoglobulin. International Dairy Journal 2006; 16, 294-302.

[273] Kim YS, Bertwhistle W, Kim YW. Peptide hydrolyses in the brush borde rand soluble fractions of small intestinal mucosa of rat and man. Journal of Clinical Investigation 1972; 51, 1419-1430.

[274] Masuda O, Nakamura Y, Takano T. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides in spontaneously hypertensive rats. Journal of Nutrition 1996; 126, 3063-3068.

[275] Matsui T, Yukiyoshi A, Doi S, Sugimoto H, Yamada H, Matsumoto K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. Journal of Nutritional Biochemistry 2002; 13, 80-86.

[276] Langley-Danysz P. Des hydrolysats protéiques pour développer des aliments santé. RIA Technology Veille 1998; 581, 38-40.

[277] Miguel M, Recio I, Ramos I, Delgado MA, Aleixandre MA. Antihypertensive effect of peptides obtained from *Enterococcus faecalis*-fermented milk in rats. Journal of Dairy Science 2006; 89, 3352-3359.

[278] Vermeirssen V, van Camp J, Decroos K, van Wijmelbeke L, Verstraete W. The impact of fermentation and *in vitro* digestion on the formation of angiotensin-I-converting enzyme inhibitory activity from pea and whey protein. Journal of Dairy Science 2003; 86, 429-438.

[279] Hernández-Ledesma B, Amigo L, Ramos M, Recio I. Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. Journal of Agricultural and Food Chemistry, 2004; 52, 1504-1510.

[280] Hernández-Ledesma B, Amigo L, Ramos M, Recio I. Release of angiotensin converting enzyme-inhibitory peptides by simulated gastrointestinal digestion of infant formulas. International Dairy Journal 2004; 14, 889-898.

[281] Quirós A, Contreras MM, Ramos M, Amigo L, Recio I. Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties. Peptides 2009; 30, 1848-1853.

[282] Maeno M, Yamamoto N, Takano T. Identification of an antihypertensive peptide from casein hydrolyzate produced by a proteinase from *Lactobacillus helveticus* CP790. Journal of Dairy Science 1996; 79, 1316-1321.

[283] Yamamoto N, Maeno M, Takano T. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. Journal of Dairy Science 1999; 82, 1388-1393.

[284] Gómez-Ruiz JA, Ramos M, Recio I. Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 2004; 1054, 269-277.

[285] Miguel M, Aleixandre MA, Ramos I, López-Fandiño R. Effect of simulated gastrointestinal digestion on the antihypertensive properties of ACE-inhibitory peptides derived from ovalbumin. Journal of Agricultural and Food Chemistry 2006; 54, 726-731.