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Whey Proteins as Source of Bioactive Peptides Against Hypertension

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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 substantiation 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	
Caseinomacropptide		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 dimer – 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 Phe₁₀₅-Met₁₀₆ 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 (IgG₁ and IgG₂) – with IgG₁ 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
<i>Whole whey protein</i>		Prevention of cancer	[74]
		Breast and intestinal cancer;	[14, 75]
		Chemically-induced cancer	[76-77]
		Increment of glutathione 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]
<i>β-Lactoglobulin</i>		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]
	Enzyme hydrolysis	Prevention of cancer	
<i>α-Lactalbumin</i>		Intestinal cancer	[14]
		Prevention of cancer	[119]
		Apoptosis of tumoral cells	[120-122]
		Lactose synthesis	[25, 123]
		Treatment of chronic stress-induced disease	[124]
		Antimicrobial (bactericidal)	
		<i>Streptococcus pneumonia</i>	[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	
<i>Bovine serum albumin</i>		Prostaglandin production	[130-132]
		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]
<i>Immunoglobulins</i>		Immunomodulation	[140]
		Disease protection through passive immunity	[141-142]
		Antibacterial	[143-145]
		Antifungal	[146]
		Opioid agonist	[147]
<i>Caseinomacropeptide</i>		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 through 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 opened 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 I₂ 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 CCK₁ and IP-receptor-dependent vasorelaxation; this peptide relaxes the mesenteric artery of SHR via prostaglandin I₂-IP receptor, followed by CCK-CCK₁ 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}))	References
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	DAQSAPLR VY ^c	12.2	10 (5)	[107, 117]
	Proteinase K	β-Lg f78-80	IPA ^c	141	31 (8)	[136]
	Cardosins	α-La f16-26	KGYGGVSL PEW ^c	0.7	20 (5)	[107, 117]
	Cardosins	α-La f97-103	DKVGINY ^c	99.9		[107]
	Cardosins	α-La f97-104	DKVGINY W ^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	LDAQSAPL R	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	LKGYGGVS LPEW	83		[237]
	Thermolysin	f18-26	YGGVSLPE W	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	YG ^c LF ^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]
<i>Bovine serum albumin</i>	Proteinase K	f208-216	ALKAWSV AR ^c	3		[238]
	Proteinase K	f221-222	FP	315	27 (8)	[136]
<i>Caseinomacrop eptide</i>	Trypsin	f106-112	MAIPPKK		28 (10)	[239]
<i>Lactoferrin</i>	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 Synthetic 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 (AT₁) 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, furanacrilol 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 PepT₁ 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 μ M) 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 ($IC_{50} > 1000$ μ M), 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 μ M. 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 μ M; 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 KVLPPVQ, 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 ($IC_{50} = 4.7 \mu M$) 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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