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Lactic Fermentation and Bioactive Peptides

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<http://dx.doi.org/10.5772/51692>

1. Introduction

Fermented milk products have naturally high nutritional value, and as an extra benefit many health-promoting effects, such as improvement of lactose metabolism, reduction of serum cholesterol and reduction of cancer risk [1]. The beneficial health effects associated with some fermented dairy products may, in part, be attributed to the release of bioactive peptide sequences during the fermentation process. Numerous peptides and peptide fractions, having bioactive properties have been isolated from fermented dairy products. These activities include immunomodulatory, cytomodulatory, hypocholesterolemic, antioxidative, antimicrobial, mineral binding, opioid and bone formation activities. Many recent articles and book chapters have reviewed the release of various bioactive peptides from milk proteins through microbial proteolysis [2-5].

Many industrially utilized dairy starter cultures are highly proteolytic. The use of bioactive peptides producers microbial cultures (starter and non-starter) may allow the development new fermented dairy products. The proteolytic system of lactic acid bacteria e.g. *Lactococcus (L.) lactis*, *Lactobacillus (Lb.) helveticus* and *Lb. delbrueckii ssp. bulgaricus*, is already well characterized. This system consists of a cell wall-bound proteinase and a number of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases [6]. *Lb. helveticus* are known to have high proteolytic activities [7], causing the release of oligopeptides from digestion of milk proteins [8]. These oligopeptides can be a direct source of bioactive peptides following hydrolysis by gastrointestinal enzymes. Rapid progress has been made in recent years to elucidate the biochemical and genetic characterization of these enzymes. The fact that the activities of peptidases are affected by growth conditions makes it possible to manipulate the formation of peptides to a certain extent [9].

Cardiovascular disease (CVD) is the single leading cause of death for both males and females in technologically advanced countries in the world. In lesser-developed countries it generally ranks among the top five causes of death. The World Health Organization

estimates that by 2020, heart disease and stroke will have surpassed infectious diseases to become the leading cause of death and disability worldwide [10]. Consequently, there has been an increased focus on improving diet and lifestyle as a strategy for CVD risk reduction.

Elevated blood pressure is one of the major independent risk factors for CVD [11]. Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II as well as inactivates the vasodilator bradykinin. By inhibiting these processes, synthetic ACE inhibitors (ACEI) have long been used as antihypertensive agents. In recent years, some food proteins have been identified as sources of ACEI peptides and are currently the best-known class of bioactive peptides [12, 13]. These nutritional peptides have received considerable attention for their effectiveness in both the prevention and the treatment of hypertension.

Oxidant stress, the increased production of reactive oxygen species (ROS) in combination with outstripping endogenous antioxidant defense mechanisms, is another significant causative factor for the initiation or progression of several vascular diseases. ROS can cause extensive damage to biological macromolecules like DNA, proteins and lipids. Specifically, the oxidative modification of LDL results in the increased atherogenicity of oxidized LDL. Therefore, prolonged production of ROS is thought to contribute to the development of severe tissue injury [14]. Some peptides derived from hydrolyzed food proteins exert antioxidant activities against enzymatic (lipoxygenase-mediated) and nonenzymatic peroxidation of lipids and essential fatty acids [15]. The antioxidant properties of these peptides have been suggested to be due to metal ion chelation, free radical scavenging and singlet oxygen quenching.

This review centers on liberation during fermentation, of bioactive peptides with properties relevant to cardiovascular health including the effects on blood pressure and oxidative stress. The focus is mainly to those peptides with *in vivo* blood pressure lowering effects. Moreover, bioavailability of peptides and aspects of necessary further information is given.

2. Release and identification of peptides

2.1. Peptides in cheese

Proteolysis in cheese has been linked to its importance for texture, taste and flavour development during ripening. Changes of the cheese texture occur due to breakdown of the protein network. It contributes directly to taste and flavour by the formation of peptides and free amino acids as well as by liberation of substrates for further catabolic changes and thereby formation of volatile flavour compounds. Besides sensory quality aspects of proteolysis, formation of bioactive peptides as a result of proteolysis during cheese ripening has been reported. Cheese contains phosphopeptides as natural constituents [16, 17], and secondary proteolysis during cheese ripening leads to the formation of other bioactive peptides, such as those with ACEI activity. The findings by Meisel et al. [18] showed that inhibitory activity increased as proteolysis developed, however, the bioactivity decreased

when proteolysis during ripening exceeded a certain level. Another link between potential antihypertensive peptides and proteolysis was found in Parmesan cheese [19]. A bioactive peptide derived from α_{s1} -casein was isolated from 6-month old cheese, but it was degraded further during maturation and was not detectable after 15 month of ripening. ACEI peptide fractions having different potencies have been isolated from various Italian cheeses, e.g. Crescenza (37% inhibition), mozzarella (59% inhibition), Gorgonzola (80% inhibition) and Italic (82% inhibition) [20]. ACEI peptides have also been found in enzyme-modified cheeses [21], in a low-fat cheese made in Finland [22] and Manchego cheeses manufactured with different starter cultures [23]. Mexican Fresco cheese manufactured with *Enterococcus faecium* or a *L. lactis* ssp. *lactis*-*Enterococcus faecium* mixture showed the largest number of fractions with ACEI activity among tested lactic acid strains [24]. Pripp et al. [25] investigated the relationship between proteolysis and ACE inhibition in Gamalost, Castello, Brie, Pultost, Norvegia, Port Salut and Kesam. The traditional Norwegian cheese Gamalost had per unit cheese weight higher ACE inhibition potential than Brie, Roquefort and Gouda-type cheese. However, ACE inhibition expressed as IC_{50} per unit peptide concentration from ethanol soluble fraction assessed by the OPA-assay was highest for Kesam, a Quark-type cheese with a low degree of proteolysis.

When β -casomorphins were looked from commercial cheese products, no peptides were found or their concentration in the cheese extract was below 2 $\mu\text{g/ml}$ [26]. They further noted that the enzymatic degradation of β -casomorphins was influenced by a combination of pH and salt concentration at the cheese ripening temperature. Therefore, if formed in cheese, β -casomorphins may be degraded under conditions similar to Cheddar cheese ripening. Precursors of β -casomorphins, on the other hand, have been identified in Parmesan cheese [19]. β -Casomorphins were found at a higher level in the mould cheeses (166–648 mg/100 g), whereas the opioid peptides with antagonistic activity (casoxin-6) were identified at a higher level in the semi-hard cheeses (136–276 mg/100 g) and a low quantity of casomorphins (4–100 mg/100 g) [27]. Immunomodulating properties in water-soluble extracts from traditional French Alps cheeses, Abondance and Tomme de Savoie have been observed [28]. However, no correlation between peptide composition and *in vitro* immunomodulation of T-lymphocyte cells could be established.

A limited number of bioactive peptides have been isolated and identified in Gouda, Manchego, Festivo and Crescenza cheeses (Table 1). Several ACEI peptides have been identified from N-terminal of α_{s1} -casein of Gouda, Festivo, Cheddar and Fresco cheeses [22, 24, 29, 30]. In addition, peptides from β -casein, Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (β -cn, f(60–68)); and Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Gln-Pro-Phe (β -cn, f(109–119)) from Gouda [29] and Tyr-Gln-Glu-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (β -cn, f(193–209)) from Cheddar [30] have been identified. Antihypertensive peptides Val-Pro-Pro (VPP) (β -cn, f(84–86)) and Ile-Pro-Pro (IPP) (β -cn, f(74–76) and κ -cn, f(108–110)), have also been identified and quantified in different cheese varieties [31–33]. In some varieties physiologically relevant amounts was observed, however, a large variation exists between samples of the same cheese variety, as well as between different varieties. The concentrations of VPP and IPP were in the range of 0–224 mg/kg and 0–95 mg/kg,

respectively, indicating that some cheese varieties contain similar concentrations of VPP and IPP to fermented milk products. Milk pretreatment, cultures, scalding conditions, and ripening time were identified as the key factors influencing the concentration of these two naturally occurring bioactive peptides in cheese. Thus, it is necessary to develop a reproducible cheese-making process with selected cultures to produce higher concentrations of these peptides that could be used for clinical trials.

Cheese variety	Milk protein fragment	Peptide sequence	ACE-inhibition IC ₅₀ μM	Ref
Gouda	α _{s1} -cn f(1-9)	RPKHPIKHQ	13.4	29
	α _{s1} -cn f(1-13)	RPKHPIKHQGLPQ	ND	
	β-cn f(68-66)	YFPGPIP	14.8	
	β-cn f(109-119)	MPFPKYPVQPF	ND	
Manchego	ovine α _{s1} -cn f(102-109)	KKYNVPQL	77.2	23
	ovine α _{s1} -cn f(205-208)	VRYL	24.1	
Cheddar (with probiotics)	α _{s1} -cn f(1-9)	RPKHPIKHQ	ND	30
	α _{s1} -cn f(1-7)	RPKHPIK		
	α _{s1} -cn f(1-6)	RPKHPI		
	α _{s1} -cn f(24-32)	FVAPFPEVFGK		
	β-cn f(193-209)	YQEPVLGPVRGPFPIIV		
Swiss cheese varieties	β-cn, f(84-86)	VPP	9	31-
	β-cn, f(74-76) and	IPP	5	34
	κ-cn, f(108-110)			
Fresco cheese	α _{s1} -cn f(1-15)	RPKHPIKHQGLPQEV	ND	24
	α _{s1} -cn f(1-22)	RPKHPIKHQGLPQEVLNEN		
	α _{s1} -cn f(14-23)	LLR		
	α _{s1} -cn f(24-34)	EVLNENLLRF		
	β-cn f(193-205)	FVAPFPEVFGK		
	β-cn f(193-207)	YQEPVLGPVRGPF		
	β-cn f(193-209)	YQEPVLGPVRGPFPI		
	YQEPVLGPVRGPFPIIV			

ND: Not described

IC₅₀: Peptide concentration that shows 50% inhibition of ACE activity

One letter amino acid codes used

Table 1. Examples of identified bioactive peptides in different cheese varieties

2.2. Fermented milk

During fermentation process, lactic acid bacteria hydrolyze milk proteins, mainly caseins, into peptides and amino acids which are used as nitrogen sources necessary for their growth. Hence, bioactive peptides can be generated by starter and non-starter bacteria used in the manufacture of fermented dairy products (Table 2). Proteolytic system of *Lb. helveticus*, *Lb. delbrueckii ssp bulgaricus*, *L. lactis ssp. diacetylactis*, *L. lactis ssp. cremoris*, and *Streptococcus (Str.) salivarius ssp. thermophilus* strains have demonstrated to hydrolyze milk proteins and release ACEI peptides. Among lactic acid bacteria, *Lb. helveticus* has high

extracellular proteinase activity and the ability to release large amount of peptides in fermented milk. As a result, among various kinds of fermented milk, antihypertensive effect related to ACEI peptides were found in milk produced by *Lb. helveticus*. Two ACEI peptides have been purified from sour milk and identified as VPP and IPP [34].

Organisms	ACE-inhibition	Identified peptides		Dose	Response (Δ SBP mmHg)	Ref.
	IC ₅₀ mg/ml	Sequence	IC ₅₀ μ M			
<i>Lb. helveticus</i> and <i>Str. thermophilus</i>	ND	VPP	9	5 ml/kg	-21.8 \pm 4.2 after 6 h	34
<i>Lb. helveticus</i>		IPP	5			
		VPP	9	27 ml/day	-21 after 4 weeks	67
		IPP	5			
<i>Lb. helveticus</i> CPN4	ND	YP	720	10 ml/kg	32.1 \pm 7.4 after 6 h	42
<i>Lb. helveticus</i> CHCC637	0.16			10ml/kg	-12 after 4-8 h	37
<i>Lb. helveticus</i> CHCC641	0.26				-11 after 4-8 h	
<i>Lact. delbrueckii</i> ssp. <i>bulgaricus</i>		SKVYFPFGPI	1.7		ND	43
<i>Str. salivarius</i> ssp. <i>thermophilus</i> and <i>L. lactis</i> biovar <i>diacetylactis</i>		SKVYP	mg/ml			
			1.5			
			mg/ml			
<i>Lb. jensenii</i>	0.52	LVYFPFGPIHNSLP	71	0.2 kg/kg	approx -12 after 2 h	38
		QN	89			
		LVYFPFGPIH				
<i>Enterococcus faecalis</i> CECT 5727	0.053	LHLPLP	5.5	2 mg/kg	-21.87 \pm 4.51 after 4h ¹⁾	44
		LVYFPFGPIPNSLP	5.2	6 mg/kg	approx -15 after 4 h	
		QNIPP				
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> SS1	ND	NIPPLTQTPV	173.3		ND	36
		LNVPGEIVE	300.1			
<i>L. lactis</i> subsp. <i>cremoris</i> FT4		DKIHPF	256.8			
Mixed lactic acid bacteria (<i>Lb. casei</i> , <i>acidophilus</i> , <i>bulgaricus</i> , <i>Str. thermophilus</i> , <i>Bifidobacterium</i>) and protease	0.24	GTW	464.4	5 mg/ml	SBP -22 after 8 weeks	76
		GVW	240.0			

One letter amino acid codes used

ND Not described

1) Pure synthetic peptides were used in the study

Table 2. ACE-inhibitory and antihypertensive activity in spontaneously hypertensive rats of peptides produced by fermentation of milk

Pihlanto-Leppälä et al. [35] studied the potential formation of ACEI peptides from cheese whey and caseins during fermentation with various commercial dairy starters used in the manufacture of yogurt, ropy milk and sour milk. No ACEI activity was observed in these hydrolysates. Further digestion of the above samples with pepsin and trypsin resulted in the release of several strong ACEI peptides derived primarily from α_{s1} -casein and β -casein. The formation of ACEI peptides was demonstrated in two dairy strains, *Lb. delbrueckii* ssp. *bulgaricus* and *L. lactis* ssp. *cremoris*, after fermentation of milk separately with each strain for 72 hours [36]. The most inhibitory fractions of the fermented milk mainly contained β -casein-derived peptides with inhibitory concentration (IC₅₀) values ranging from 8.0 to 11.2 μ g/ml. Fuglsang et al. [37] tested a total of 26 strains of wild-type lactic acid bacteria, mainly belonging to *L. lactis* and *Lb. helveticus*, for their ability to produce a milk fermentate with ACEI activity. All tested strains produced ACEI substances in varying amounts, and two of the strains exhibited high ACE inhibition and a high OPA index, which correlates well with peptide formation. In another study 25 lactic acid strains of *Lactobacillus*, *Lactococcus* and *Leuconsotoc* were used [38]. The strains were tested alone or in combination and the highest activities were observed in *Lb. jensenii*, *Lb. acidophilus* and *Leuc. mesenteroides* strains and all strains showed correlation between ACE inhibition and degree of proteolysis. In a recent study, milk was fermented to defined pH values with 13 strains of lactic acid bacteria. The highest ACEI activity was obtained with two highly proteolytic strains of *Lb. helveticus* and with the *Lactococcus* strains. Fermentation from pH 4.6 to 4.3 with these strains slightly increased the ACEI activity, whilst fermentation to pH 3.5 with *Lb. helveticus* reduced the ACEI activity [39]. Moreover, four different *Enterococcus faecalis* strains, isolated from raw milk, produced fermented milk with potent ACEI activity [40]. In a recent research it was found that *L. lactis* strains isolated from artisanal dairy starters or commercial starter cultures are potential for the production of fermented dairy products with ACEI properties. Especially, a strain isolated from artisanal cheese presented the lowest IC₅₀ (13 μ g/ml) [41].

Bioactive peptides isolated from skim milk and whey fermented using a range of organisms are summarized in Table 2. The majority of identified peptides are casein-derived ACEI peptides having IC₅₀ values ranging from 5 to 500 μ M. The best characterized ACEI and antihypertensive peptides liberated with *Lb. helveticus* alone or in combination with *Saccharomyces cerevisiae* are the tripeptides IPP, and VPP. Yamamoto et al. [42] identified an ACEI dipeptide (Tyr-Pro) from a yogurt-like product fermented with *Lb. helveticus* CPN4 strain. This peptide sequence is present in all major casein fractions, and its concentration was found to increase during fermentation, reaching a maximum concentration of 8.1 μ g/ml in the product. Ashar and Chand [43] identified an ACEI peptide from milk fermented with *Lb. delbrueckii* ssp. *bulgaricus*. The peptide showed the sequence Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile from β -casein with an IC₅₀ value of 1.7 mg/ml. In combination with *Str. salivarius* ssp. *thermophilus* and *L. lactis* biovar. *diacetylactis*, a peptide structure with a sequence of Ser-Lys-Val-Tyr-Pro was obtained from β -casein with an IC₅₀ value of 1.4 mg/ml. Both peptides were markedly stable to digestive enzymes, acidic and alkaline pH, as well as during storage at 5 and 10 °C for four days. Two β -casein-derived peptides were identified from water soluble fraction of milk fermented with *Lb. jensenii*. The identified peptides were Leu-Val-Try-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu-Pro-Gln-Asn, and

Leu-Val-Try-Pro-Phe-Pro-Gly-Pro-Ile-His [38]. Quirós et al. [44] identified two peptides in fermented milk with *Enterococcus faecalis* that corresponded to β -casein fragments Lys-His-Leu-Pro-Leu-Pro and Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro, with potent ACEI activity.

Many kinds of proteolytic enzymes have been reported from lactic acid bacteria, and have been reviewed extensively [6, 45]. The components of the proteolytic systems of lactic acid bacteria are divided into three groups, including the extracellular proteinase that catalyzes casein breakdown to peptides, peptidases that hydrolyze peptides to amino acids and a peptide transport system. The extracellular proteinase activity was almost correlated with ACEI activity in the fermented milk, suggesting that the proteolysis of casein by the extracellular proteinase is the most important parameter in the processing of active components [46]. The importance of the proteinase was also supported by the fact that a proteinase negative mutant was not able to generate antihypertensive peptides in the fermented milk, whereas the wild-type strain had the ability to release strong antihypertensive peptides in the fermented milk [47]. The enzymatic process generating the antihypertensive peptides VPP and IPP in *Lb. helveticus* has been elucidated. By the proteolytic action of the extracellular proteinase long peptide with amino acid residue including VPP and IPP sequences were generated. Next the long peptide would be hydrolyzed to shorter peptides by intracellular peptidases. A key enzyme that can catalyze C-terminal processing of Val-Pro-Pro-Phe-Leu and Ile-Pro-Pro-Leu-Thr to VPP and IPP has been purified from *Lb. helveticus* CM4. The endopeptidase has sequence homology in amino terminal sequence to a previously reported pepO-gene product [48]. Kilpi et al. [49] found out higher ACE inhibition in milk fermentation using peptidase-deletion mutants compared to the wild-type of *Lb. helveticus* strain. Unlike with the wild type strain, ACEI remained constant during the course of fermentation with the proline-specific peptidase mutant. The mutant strains had also different peptide profiles than the wild-type strain.

2.3. Other

Various types of fermented soybean foods are consumed in Asian countries such as Korea, China, Japan, Indonesia and Vietnam. Soybeans are traditionally fermented primarily by *Bacilli* species during the early stage of fermentation followed by *Aspergillus* species, which predominate during the remaining fermentation period [50]. ACEI peptides have been found in many traditional Asian fermented soy foods, such as soybean paste, soy sauce, natto and tempeh. ACEI peptide His-His-Leu was isolated from Korean fermented soybean paste [51]. Rye gluten sourdoughs fermented with *Lb. reuteri* and added protease were found to contain the lactori-peptides VPP, IPP [52]. Moreover, our recent studies showed that fermentation of rapeseed or flaxseed meals with *Bacillus subtilis* or *Lb. helveticus* strains produced ACEI activity [53].

2.4. Other activities

It is reasonable to expect that lactic acid bacteria produce scavengers for hydroxyl radical, which can be metabolic compounds produced by bacteria or degradation products of milk

proteins. The results have demonstrated that the antioxidant production is commonly higher within the group of obligately homofermentative lactobacilli, than within the facultatively or obligately heterofermentative strain groups. Also heterofermentative *Lactobacillus* sp. have been reported to exhibit antioxidative activity. *Lb. acidophilus*, *Lb. bulgaricus*, *Str. thermophilus* and *Bifidobacterium longum* exhibited antioxidative activity by various mechanisms, like metal ion chelating capacity, scavenging of reactive oxygen species (ROS), reducing activity and superoxide dismutase activity [54, 55]. Peptides liberated during fermentation can be partially responsible for the reported antioxidative properties. An antioxidative peptide derived from κ -casein was detected in milk after fermentation with *Lb. delbrueckii* subs. *bulgaricus* [56]. Moreover, Hernández-Ledesma et al. [57] found a moderate ABTS radical scavenging capacity in commercial fermented milk from Europe. Further studies of this radical scavenging activity in different HPLC fractions showed low TEAC values. Virtanen et al. [58] found that fermentation with *Leuc. mesenteroides* ssp. *cremoris*, *Lb. jensenii* and *Lb. acidophilus* strains produced compounds that showed both radical scavenging activity and inhibition of lipid peroxidation.

Inflammation plays a key role in the development of cardiovascular disease. It often begins with inflammatory changes in the endothelium, which begins to express the adhesion molecule VCAM-1. VCAM-1 attracts monocytes, which then migrate through the endothelial layer under the influence of various proinflammatory chemoattractants [59]. Accordingly, fermentation by lactic acid may be able to release components that possess immunomodulatory properties. Most of the studies have been done with synthetic peptides derived from enzymatic treatment of milk proteins using different *in vitro* models. Leblanc et al. [60] investigated the effect of peptides released during the fermentation of milk by *Lb. helveticus* on the humoral immune system and on the growth of fibrosarcomas. The study showed that bioactive components were released during fermentation that contributed to the immunoenhancing and antitumor properties. Antimutagenic compounds were produced during fermentation by *Lb. helveticus*, and release of peptides is one possible explanation [61]. The permeate fraction obtained from milk fermented by *Lb. helveticus* was able to modulate the *in vitro* proliferation of lymphocytes by acting on the production of cytokines [62]. Tompa et al. [63] found that peptide fractions from *Lb. helveticus* BGRA43 fermented milk have anti-inflammatory potential. Matar et al. [64] fed milk fermented with a *Lb. helveticus* strain to mice for three days and detected significantly higher numbers of IgA secreting cells in their intestinal mucosa, compared with control mice fed with similar milk incubated with a non-proteolytic variant of the same strain. The immunostimulatory effect of fermented milk was attributed to peptides released from the casein fraction.

3. Antihypertensive effects in vivo

The search for *in vitro* ACEI is the most common strategy followed in the selection of potential antihypertensive peptides derived from food proteins. *In vitro* ACEI activity is generally measured by monitoring the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. The antihypertensive effects have been assessed by *in vivo* experiments using spontaneously hypertensive rats (SHR) as an animal model to study

human essential hypertension [7]. Following a positive response in animal studies human studies may be carried out to ascertain the ACEI potential

3.1. Animal studies

A great number of studies have addressed the effects of both short-term and long-term administration of potential antihypertensive peptides using this animal model. Fermented milks with different IC_{50} -values ranging from 0.08 to 1.88 mg/ml have been shown to decrease blood pressure in SHR from 10 to 32 mmHg (Table 2).

The first antihypertensive effect of milk casein-derived peptides was first demonstrated by casein hydrolysate formed by purified proteinase from *Lb. helveticus* CP790 and milk fermented with the same bacteria [65]. The authors concluded that peptides liberated from casein by extracellular proteinases were responsible for the antihypertensive effect. The active substances were liberated during fermentation of milk with *Lb. helveticus* and *Saccharomyces cerevisiae* and were identified to be IPP and VPP. Oral administration of fermented milk or pure tripeptides were shown to produce strong antihypertensive effect in SHR after single-dose [34, 66]. Thereafter, several animal studies have been conducted to characterize the long-term effects of lactotriptides or fermented milk containing them. These studies were mainly conducted with SHR but also Goto-Kakizaki (GK) rats and double transgenic rats (dTGR) with malignant hypertension have been used. The development of hypertension was attenuated significantly in rats receiving fermented milk product containing lactotriptides, attenuation in systolic blood pressure was 12-21 mmHg in SHR, 10 mmHg in high salt-fed GK rats and 19 mmHg in dTGR in comparison to control group [67-69]. Pure tripeptides did not produce as strong antihypertensive effect as the milk products containing them. In addition, minerals alone did not attenuate the development of blood pressure as much as the fermented milk products [68]. These studies indicate that the bioavailability of peptides may be better from milk in comparison of water or is improved by other milk components.

After the blood pressure monitoring has been completed the effect of long-term intake of lactotriptides on vascular function has been assessed [68,70,71]. Jauhiainen et al. [70], showed improved endothelium-dependent relaxation in mesenteric arteries and aortas of rats that had received minerals and lactotriptide. Endothelial function of mesenteric arteries was strongly impaired in all groups of salt-loaded GK rats, and significantly improved endothelium-dependent relaxations were observed after treatment with different fermented milk products [68]. Protection of endothelial function after incubation with tripeptides IPP and VPP for 24 h was found in a study with isolated SHR mesenteric arteries [71].

Evidence from ACE inhibition was gained by Masuda et al. [72], who found that after receiving a single-dose of Calpis™ sour milk, ACE activity was decreased in SHR aorta. The lactotriptides were detected in solubilized fraction from the abdominal aorta of SHR but not from WKY given the sour milk. Moreover, in SHR, plasma rennin activity increased after long-term treatment of fermented milk product containing the lactotriptides [67]. In addition, treatment with fermented milk containing lactotriptides and plant sterols

decreased serum ACE activity [73]. In salt-loaded GK rats, fermented milk with lactotripeptides decreased serum ACE and aldosterone levels [68].

Besides the most extensively studied lactotripeptides, also other fermented milk products and peptides have been found. Different strains of lactic acid bacteria, such as *Lb. helveticus* CPN4, *Lb. bulgaricus*, *Lb. jensenii* and *Str. thermophilus*, have been also shown to provoke liberation of peptides with antihypertensive activity in SHR [36, 37, 41]. Two peptides, corresponding to β -casein fragments Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro and Leu-His-Leu-Pro-Leu-Pro, have been isolated in fermented milk with *Enterococcus faecalis* and their antihypertensive effect in SHR, after acute and long-term administration has been proved. The administration of 2 mg/kg of peptide Leu-His-Leu-Pro-Leu-Pro resulted in a significant decrease of the SBP in SHR 4 h post-administration [74,75]. Fermentation of milk with one or more lactic acid bacteria strains followed by hydrolysis using food-grade enzymes liberated tripeptides (Gly-Thr-Trp and Gly-Val-Trp). Oral administration of this fermented whey lowered significantly SBP in SHR from 9 to 15 weeks of age. Bioactive substances, tripeptides and γ -aminobutyric acid (GABA), contributed to lowering blood pressure of SHR [76].

Some of ACE-inhibitory peptide fractions from cheese have shown *in vivo* activities. A water-soluble peptide preparation isolated from Gouda ripened for 8 months was found to have the most potent antihypertensive activity (maximum decrease in SBP = 24.7 (\pm 0.3) mmHg ($P \leq 0.01$) after 6 h) when administered to SHR by gastric intubation at doses between 6.1 and 7.5 mg/kg body weight. Three peptide fractions were isolated from water-soluble extract by hydrophobic chromatography using different concentrations of acetonitrile. The fractions eluting between 15% and 30%, 30–45% and 60–75% acetonitrile decreased SBP in SHR by 15.0, 29.3 and 18.8 mmHg ($P \leq 0.01$), respectively, 6 h after gastric intubation. The peptide fraction eluting between 30% and 45% acetonitrile was shown to contain the sequences (α 1-cn f(1–9)) Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln and (β -cn f(60–68)) Tyr-Pro-Phe-Gly-Pro-Ile-Pro-Asn (Table 1), which, respectively, decreased SBP in SHR by 9.3 (\pm 4.8) and 7.0 (\pm 3.8) mmHg 6 h after gastric intubation [29].

Several sequences have been proposed as responsible for the antihypertensive activity of soy protein hydrolysates and fermented products, but only the peptide His-His-Leu derived from fermented soy paste was assayed in pure form in SHR, where a decrease of 32 mm Hg of SBP was reached at a dose of 100 mg/kg. Moreover, the synthetic tripeptide His-His-Leu resulted in a significant decrease of ACE activity in the aorta [77]. Soybean-derived products contain isoflavones, which are thought to possess a favourable effect in reducing cardiovascular risk factors as well as vascular function [78]. However, on the basis of *in vitro* results and literature review, Wu and Muir [79] have indicated that the contribution of isoflavones to a blood-pressure-lowering effect in soybean ACEI peptides may be negligible. Similarly, it has been reported that the reduction of hypertension of a fermented product from soy milk was contributed mainly by peptides of 800–900 Da but it could be also attributable to GABA [80]. Moreover, fermented soy product, miso, with added tripeptides

(VPP and IPP) from casein was reported to act as antihypertensive agents in SHR [81]. Recently, Nakahara et al. [82] used the Dahl salt-sensitive rats as a model of salt-sensitive hypertension to evaluate the antihypertensive effect of a peptide-enriched soy sauce-like seasoning. The results of these tests have highlighted an important lack of correlation between the *in vitro* ACEI activity and the *in vivo* action. This fact has provided doubts on the use of the *in vitro* ACEI activity as the exclusive criteria for potential antihypertensive substances, since physiological transformations may occur *in vivo*, and because other mechanisms of action than ACE inhibition might be responsible for the antihypertensive effect.

3.2. Effects in clinical studies

Evidence of the beneficial effects of bioactive peptides has to be based on clinical data. Most research has been focused in lactotriptides, VPP and IPP, and their antihypertensive properties. About twenty human studies have been published linking the consumption of products containing lactotriptides with significant reductions in both SBP and DBP. Oral administration of these tri-peptides included in different formulas, fermented milk, dried product, fruit juice, etc., products. However, recent studies have provided some conflicting results. Most clinical trials have assessed BP-lowering effects at multiple points over time. Most of the BP studies with lactotriptides have been done in Japanese subjects, and several studies have been done in Finnish subjects [83-88]. Generally, maximum duration of treatment was 8 weeks at doses between 3 and 52 mg/day (Table 3). From these data, it becomes apparent that the largest part of the total BP reduction takes place in the first 1–2 weeks of treatment. Thereafter, a further gradual lowering is seen, but to a lesser extent than in the first period [84-86]. The first significant effects of lactotriptides on BP in hypertensive subjects were observed after 1–2 weeks of treatment with dosages as low as 3.8 mg/d. Maximum BP-lowering effects of lactotriptides approximate 13 mmHg SBP and 8 mmHg DBP active treatment *v.* placebo, and are likely reached after 8–12 weeks of treatment. Lactotriptides exert a gradual effect on BP lowering after start of intake and return of BP after end of treatment as well [85, 86, 89]. The highest effective dosage of lactotriptides was evaluated in a safety study, and consisted of 52.5 mg/d [88]. After 10 weeks of active treatment, mean SBP in subjects with hypertension decreased by 4.1 mmHg and DBP by 1.8 mmHg. The next highest dose of lactotriptides that was tested amounted to 13.0 mg/d [89]. After 4 weeks of active treatment, SBP in subjects with mild hypertension decreased by 11.2 mmHg compared to placebo, and DBP tended to decrease by 6.5 mmHg. In none of the trials with normotensives were statistically significant BP changes found [90-92]. Even at the highest dosage of lactotriptides used in normotensives, which included a total of 29.2 mg/d during a period of 7 d, no BP lowering effects by lactotriptides were observed [93]. Thus lactotriptides only seem to be active at elevated BP values. Evidence indicates that effectiveness is positively associated with BP level, which is in line with existing data for BP-lowering medication [94].

Design	Duration (weeks)	Study population	Treatment				BP changes mmHg		Ref.
			IPP mg/d	VPP mg/d	Source of peptides	Formula	SBP	DBP	
R, p-c, s- bld, parallel	8	30 elderly hypertensive patients	1.1	1.5	<i>Lb. helv</i> + <i>Str. cer.</i>	1 x 95 ml milk drink	-14.1	-6.9	83
R, p-c, d- bld, parallel	8	64 subjects with SBP 140-159 and DBP 90-99 mmHg	1.58	2.24	<i>Lb. helv</i> + <i>Str. cer.</i>	2 x 150 g milk drink	-13	-8.4	84
R, p-c, d- bld, parallel	8	32 subjects with SBP 140 - 180 and DBP 90-105 mmHg	1.60	2.66	<i>Lb. helv</i> + <i>Str. cer.</i>	1 x 120 g milk drink	-12.1	-5.8	85
R, p-c, d- bld, parallel	8	18 hypertensive and 26 normotensive subjects	1.1	1.5	<i>Lb. helv</i> + <i>Str. cer.</i>	2 x 100 g milk drink	-7.6	-2	91
R, p-c, d- bld, parallel	8	30 subjects with SBP 140-180 and DBP 90-105 mmHg	1.52	2.53	<i>Lb. helv</i> + <i>Str. cer.</i>	2 x 160 g milk drink	-13.2	-7.8	92
R, p-c, d- bld, parallel ¹⁾	21	39 subjects with SBP 133-176 and DBP 86-108 mmHg	2.25	3.0	<i>Lb. helv</i> LBK-16H	2 x 150 ml milk drink	-6.7	-3.6	86
R, p-c, d- bld, parallel	10	60 Finnish subjects with SBP 140-180 and DBP 90-110 mmHg	2.4-2.7	2.4- 2.7	<i>Lb. helv</i> LBK-16H	1 x 150 ml milk drink	-2.3	-0.5	87
Cross- over ²⁾	7						-12.3	-3.7	
R, p-c, d- bld, parallel	10	94 hypertensive patients	30	22.5	<i>Lb. helv</i> LBK-16H	2 x 150 ml milk drink	4.1	1.8	88
R, p-c, d- bld, parallel	1	20 healthy volunteers normal blood pressure (<130 mmHg SBP and <85 mmHg DPB).	11.5	17.7	<i>Lb. helv</i> CM4	1 x 14 tablets	2.6	2	93
R, p-c, d- bld, parallel	8	135 Dutch subjects with untreated high-normal BP or mild hypertension	4.2	5.8	Fermentation	1 x 200 ml yoghurt drink	-0.5	-1.2	97
R, p-c, d- bld, crossover	4	70 Caucasian subjects with prehypertension or stage 1 hypertension	15	-	Hydrolysis by endopeptidase	2 x 7.5 mg capsules	-3.8	-2.3	102

1) Results reported as changes in SBP and DBP after each month of treatment for all subjects (intention-to-treat analysis), and as mean changes over the total intervention period among subjects who had BP measurements for each month (per protocol analysis); 2) First part of the study was carried out in parallel design and second part of the study was carried out in crossover design.

Table 3. Hypotensive effects of fermented milks with bioactive peptides in humans

The results have been included in two meta-analysis [95, 96], which described decreases around 5 mmHg for SBP and 2.3 mmHg for DBP. In general, the effects described in Japanese studies on lactotriptides are larger than those reported in Finnish studies. However, it is unlikely that genetic differences can account for these differential effects. Moreover, clinical trials in Dutch and Danish subjects have described controversial results since no effect on blood pressure was found [97, 98]. In a recent meta-analysis with a total of 18 trials, it was found a reduction of 3.73 mm Hg for SBP and 1.97 mm Hg for DBP but it was highlighted that the effect was more evident in Asian subjects than in Caucasian ones [99]. The relevance of these findings in genetics or dietary patterns should be further investigated. Comparative studies on antihypertensive medication in different races/ethnic groups have demonstrated that pharmacokinetic parameters and haemodynamic effects are essentially the same in Chinese and Japanese subjects compared with Caucasian subjects [100].

Hypertension is a complex multifactor disorder that is thought to result from an interaction between environmental factors and genetic background. Subject characteristics such as age and race/ethnicity can affect BP, including the BP response to specific antihypertensive medication. For certain antihypertensive drugs, it has been reported that a polymorphism found in humans can affect the clinical effectiveness, and similarly, these differences could be also affecting clinical trials of functional ingredients [101]. Although ACE inhibition has been postulated as the underlying mechanism of these lactotriptides, results about the inhibition of this enzyme are not conclusive in humans. Several studies have shown that rennin or ACE activity was not affected by the oral administration of the tripeptides [95, 102]. Therefore, other mechanisms could be implicated in the observed blood pressure reduction. It has been found that the intake of fermented milk containing these peptides may decrease sympathetic activity, leading to a diminished heart rate variability, heart rate and total peripheral resistance, although differences did not reach statistical significance [98].

4. Bioavailability

Bioavailability of bioactive peptides is an important target to establish the relationship between *in vitro* and *in vivo* activities. The likelihood of any bioactive peptide released during fermentation mediating a physiological response is dependent on the ability of that peptide to reach an appropriate target site. Therefore, peptides may need to be resistant to further degradation by proteolytic and peptidolytic enzymes in digestive tract. Thereafter peptides should be absorbed and enter systemic circulation. Resistance to hydrolysis is one of the main factors influencing the bioavailability of bioactive peptides. The effects of digestive enzymes on bioactive peptides, in particular ACEI peptides derived from different food matrices, have been evaluated *in vitro* gastrointestinal simulated systems. The common purpose of these experiments was to assess the effects of the peptidases of the stomach and the pancreas on the preservation of the ACEI activity of different hydrolysates. Studies have shown that the ACEI is low after fermentation but increases during hydrolysis that simulates gastrointestinal digestion [35,103]. The ACEI peptides in rapeseed hydrolysate exhibited good stability in an *in vitro* digestion model using human gastric and duodenal fluids [104]. The digestion of some peptides have been reported. For example, Ile-Val-Tyr

was hydrolysed by pepsin, trypsin and chymotrypsin alone or in combination and IC_{50} -value did not change significantly during digestion [105]. Proline- and hydroxyproline-containing peptides are usually resistant to degradation by digestive enzymes. Tripeptides containing C-terminal proline-proline are generally resistant to proline-specific peptidases [106]. In some cases, pancreatic digestion is needed to produce active peptide. For instance, the active form of peptide Lys-Val-Leu-Pro-Val-Pro-Glu is generated by hydrolysis of the glutamine residue at the C-terminal during pancreatic digestion [107]. The results are not completely predictive of the resistance of the bioactive peptides because they do not mimic all the physiological factors affecting food digestion, as pH variations, the relative amounts of the enzymes, the interactions with other molecules, and the ratio peptidase/tested compound. These variations may affect the rate of enzymatic degradation of the bioactive peptides under study, therefore affecting the estimated bioavailability of these bioactive peptides. Moreover, commercial enzymes appear to digest whey proteins more efficiently compared with human digestive juices when used at similar enzyme activities [108]. This could lead to conflicting results when comparing human *in vivo* protein digestion with digestion using purified enzymes of non-human species.

Peptides have been reported to have poor permeation across biological barriers (e.g. intestinal mucosa) [109]. Peptides can be transported by active transcellular transport or by passive processes. Although substantial amino acid absorption occurs in the form of di- and tripeptides at the apical side of enterocytes, efflux of intact peptides via the basolateral membrane into the general circulation seems to be negligible [110]. The intestinal absorption of peptides have been performed using *in vitro* tests with monolayer of intestinal cell lines, simulating intestinal epithelium, as well as analysis of peptides and derivatives in blood samples after animal and clinical studies. Foltz et al. [111] investigated the transport of IPP and VPP by using three different absorption models and demonstrated that these tripeptides are transported in small amounts intact across the barrier of the intestinal epithelium. The major transport mechanisms of IPP and VPP were demonstrated to be paracellular transport and passive diffusion [112]. Another ACEI peptide, Leu-His-Leu-Pro-Leu-Pro resisted gastrointestinal simulation but was degraded to His-Leu-Pro-Leu-Pro by cellular peptidases before crossing Caco-2 cell monolayer. The pentapeptide was rapidly transported through Caco-2 cell monolayers through paracellular route [113].

Vascular endothelial tissue peptidases and soluble plasma peptidases further contribute to peptide hydrolysis. As a consequence, for most peptides, the plasma half-life is limited to minutes as shown for endogenous peptides such as angiotensin II and glucagon-like peptide 1 [114]. In order to exert antihypertensive effect ACEI peptides need to resist different peptidases such as ACE. In this regard ACEI peptides can be classified into three groups: the inhibitor type, of which the IC_{50} -value is not affected by preincubation with ACE; the substrate type, peptides that are hydrolysed by ACE to give peptides with a weaker activity; the pro-drug type inhibitor, peptides that are converted to true inhibitors by ACE or other proteases/peptidases. Only peptides belonging to pro-drug or inhibitor type exert antihypertensive properties after oral administration. There are some examples showing that peptides are absorbed and can exert *in vivo* activities. As regard to casein-derived IPP,

Jauhiainen et al. [115] used radiolabelled tripeptide and showed that it absorbed partly intact from the gastrointestinal tract after a single oral dose to rats. Considerable amounts of radioactivity were found from several tissues, e.g., liver, kidney and aorta. The excretion of IPP was slow; even after 48 hours the radiolabelled peptide had not been completely excreted. IPP did not bind to albumin or other plasma proteins *in vitro*. Considering this and the long-lasting retention of the radioactivity in the tissues, accumulation of IPP may occur in sufficient concentrations to cause blood pressure lowering effects e.g., by ACE-inhibition in the vascular wall. In another study the absolute bioavailability of the tripeptides in pigs was below 0.1%, with an extremely short elimination half-life ranging from 5 to 20 min [116]. In humans, maximal plasma concentration did not exceed picomolar concentration [117].

The improvement of limited absorption and stability of peptides has been a goal when evaluating their effectiveness. For example, some carriers interact with the peptide molecule to create an insoluble entity at low pH which later dissolves and facilitates intestinal uptake, by enhancing peptide transport over the non-polar biological membrane [118]. Bioavailability of bioactive tripeptides (VPP, IPP, LPP) was improved by administering them with a meal containing fiber, as compared to a meal containing no fiber. High methylated citrus pectin was used as a fiber [119]. Ko et al. [120] applied emulsification, microencapsulation and lipophilization to enhance the antihypertensive activity of a hydrolysate of tuna cooking juice. Among these treatments, lipophilization was the most effective, followed by microencapsulation and lecithin emulsification, getting for each of them a stronger effect than the obtained with the double untreated dosage. Antihypertensive effect of ovokinin (Phe-Arg-Ala-Asp-Pro-Phe-Leu) increased four-times compared to the untreated dosage after administration with egg yolk [121]. In this case, phospholipids were identified as responsible for enhancing the antihypertensive effect, particularly phosphatidylcholine, that could improve intestinal absorption or by protecting ovokinin of peptidases. Among drug delivery systems, emulsions have been used to enhance oral bioavailability or promoting absorption through mucosal surfaces of peptides and proteins [118]. Individually, various components of emulsions have been considered as candidates for improving bioavailability of peptides.

5. General conclusions

The interest on foods possessing health-promoting or disease-preventing properties has been increasing. An increasing number of foods sold in developed countries bears nutrition and health claims. Fermented milk with putative antihypertensive effect in humans could be an easy applicable lifestyle intervention against hypertension. In fact, much work has been done with dietary antihypertensive peptides and evidence of their effect in animal and clinical studies. Moreover, there are numerous available patents of products containing antihypertensive bioactive peptides. However, certain aspects, such as identification of the active form in the organism and the different mechanisms of action that contribute in the antihypertensive effect still need to be further investigated. Recent advances on specific

analytical techniques able to follow small amounts of the peptides or derivatives from them in complex matrices and biological fluids will allow performing these kinetic studies in model animals and humans. Similarly, advances in new disciplines such as nutrigenomic and nutrigenetic will open new ways to follow bioactivity in the organism by identifying novel and more complex biomarkers of exposure and/or of activity. There is still poor knowledge on the resistance of peptides to gastric degradation, and low bioavailability of peptides has been observed. This reinforces the need of various strategies to improve the oral bioavailability of peptides.

More emphasis has been put on the legal regulation of the health claims attached to the products. Authorities around the world have developed systematic approaches for review and assessment of scientific data. Evidence on the beneficial effects of a functional food product should be enough detailed, extensive and conclusive for the use of a health claim in the product labeling and marketing. Besides being based on generally accepted scientific evidence, the claims should be well understood by the average consumer. First, it is necessary to identify and quantify the active sequences. Antihypertensive peptides are only minor constituents in highly complex food matrices and, therefore, a monitoring of the large-scale production by hydrolytic or fermentative industrial process is mandatory. Second, extensive investigations to prove the antihypertensive effect in humans as well as the minimal dose to show this effect are necessary to fulfill the requirements of the legislation concerning functional foods. Japan was the pioneer with the Foods for Special Health Use (FOSHU) legislation in 1991. Europe adopted a joint Regulation on Nutrition and Health Claims made on Foods in 2006 being the European Food Safety Authority (EFSA). At present, EFSA have concludes that the evidence is insufficient to establish a cause and effect relationship between the consumption of the tripeptides VPP and IPP and the maintenance of normal blood pressure. Bearing in mind that 'essential hypertension' consists of disparate mechanisms that ultimately lead to elevations in systemic BP, it is most probably that that products containing lactotripeptides offer a valuable option as a non-pharmacological, nutritional treatment of elevated blood pressure for some groups of people.

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