



Review

Strategies of producing bioactive peptides from milk proteins to functionalize fermented milk products



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ABSTRACT

Besides their basic nutritional role, dietary proteins contain bioactive peptides which are encrypted in their sequence and may modulate different body functions such as digestive, cardiovascular, immune and nervous systems, and therefore contribute in maintaining consumer health. Currently, milk proteins are considered to be the major source of bioactive peptides. The occurrence of these peptides has already been reported in fermented milk products such as yogurt, sour milk or kefir and some of them have been shown to confer health benefits. This review focuses on different strategies that could be employed to enhance the production of bioactive peptides from the milk proteins that will be consequently used to functionalize the fermented milk products. Three types of strategies are developed. The first exploits the proteolytic system of lactic acid bacteria (LAB) or food grade enzymes or combination of both to release the functional peptides from the milk proteins directly in the fermented milk products. The second concerns the supplementation of the fermented milk products with the bioactive peptides obtained outside of the product through the hydrolysis of the purified proteins by the same enzyme sources. Finally, the last consists in the production of the bioactive peptides, initially identified from the milk-proteins, by microorganisms using recombinant DNA technology.

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1. Introduction

Because of their increasing perception about the relationship between diet and health, consumers are interested in preventing diseases and improving their own wellness through the use of functional foods which could exert positive health effect when present in a normal diet (Ruttarattanamongkol, 2012). Proteins, one of the macronutrients of the bolus, can be used to develop such functional foods. Besides their basic nutritional role, the biofunctional properties of dietary proteins are mainly attributed to bioactive peptides, which are encrypted within sequence of certain proteins and that may have a positive effect on body function and health, when they are released (Kitts & Weiler, 2003).

Although potential bioactive peptides have been identified from various animal or plant proteins belonging to current diet, milk proteins remain the major source of bioactive peptides and these peptides display a wide range of biological activities. Casein and whey proteins are sources of peptides with biological activity containing 2–50 residues, which are released only after hydrolysis of these proteins *in vivo* or *in vitro*. Their activity depends on both their residue composition and sequence, and some of them are known to display multi-functional properties. Bioactive peptides can display, *inter alia*, antioxidant, hypotensive, immunomodulatory, antithrombotic or opioid properties (Fig. 1) and, consequently, appear to be capable to exert beneficial health effects by acting on the nervous, digestive, cardiovascular and immune systems. Research concerning them has made the subject of several reviews (Haque, Chand, & Kapila, 2009; Kamau et al., 2010; Nagpal et al., 2011).

In order to exploit their promising health effects for the development of novel functional foods, much progress has already been made in elucidating the formation of bioactive peptides, understanding the structure/activity relationship and bioavailability (for a recent review see Abd El-Salam & El-Shibiny, 2013; FitzGerald & Murray, 2006; Hernández-Ledesma, Contreras, & Recio, 2011). Among functional foods, dairy products are considered as a repertoire of bioactive peptides which originate from the hydrolysis of the milk proteins by proteolytic enzymes of starter bacteria. Thus, the existence of peptides with various bioactivities has been reported in the processed dairy products such as several cheese varieties and fermented milks like yogurt, sour milk, dahi or kefir (for a recent review see Choi, Sabikhi, Hassan, & Anand, 2012; Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011; Haque et al., 2009; Korhonen, 2009). Several fermented milk drinks containing bioactive peptides added and/or generated during the fermentation are currently being marketed. As example, milk with hypotensive effects such as “Calpis” or “Ameal S” (Calpis Co, Japan) and Evolus (Valio Oy, Finland) contains IPP and VPP peptides from β - and κ -casein (FitzGerald, Murray, & Walsh, 2004). Table 1 lists the occurrence of biological activities of some fermented milk products.

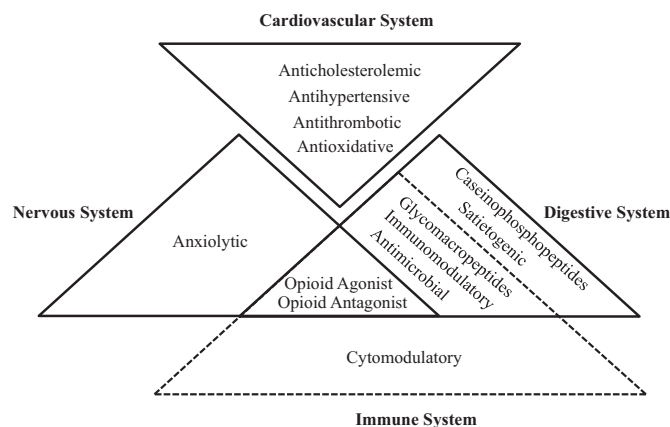


Fig. 1. Bioactivities of milk protein-derived peptides.

If we want to use the promising potential of bioactive peptides to maintain the health of the consumer, it is now interesting to increase the variety of fermented milks functionalized with specific bioactive peptides. For this purpose, different strategies can be employed and will be reviewed here with emphasis i) on the utilization of proteolytic system of lactic acid bacteria (LAB) to generate bioactive peptides, ii) in the manufacturing of the bioactive peptides outside the fermented milk product, i.e. *in vitro* followed by supplementation of milk, and finally iii) by exploiting the recombinant DNA technology for the production of bioactive peptides of interest.

2. Functionalization of fermented milk with bioactive peptides through proteolytic system of LAB

The functionalization of dairy products through the use of proteolytic system of LAB requires better understanding of this system, its regulation and its potentialities.

2.1. Presentation of the proteolytic system of LAB

Because of their inability to synthesize all of the amino-acids required for their growth, LAB have to degrade milk proteins into peptides and amino acids during the fermentation process. The free amino acids and small peptides are utilized by LAB as nutrient source essential for their growth (Juillard et al., 1995) whereas the generated peptides which are not consumed by the bacteria, can promote various physiological functions in consumers if they displayed certain bioactivities, as proven in numerous bioactive peptides which are found in various fermented milks (see below). The proteolytic system of LAB is mainly comprised of i) one or more cell wall protease(s) also referred to as Cell Envelope Proteinase (CEP; Laan & Konings, 1989), capable of hydrolyzing milk proteins to peptides containing 4–30 residues; ii) transport system of peptides that includes oligopeptide binding protein, two permeases forming a pore and two ATPases that provide energy to the system, and iii) a pool of intracellular peptidases necessary for the degradation of internalized peptides into amino acids (Savijoki, Ingmer, & Varmanen, 2006). Thus, the CEPs, responsible for the initial stages of the milk protein hydrolysis, represent the first and major actor of generation of bioactive peptides. They are synthesized as preproprotein of 1600–2000 residues (180–200 kDa) and are made from 6 to 8 distinct functional domains (Fig. 2; Fernandez-Espla, Garault, Monnet, & Rul, 2000; Siezen, 1999): the PP domain (pre-prodomain) which includes the signal peptide (~40 residues) and the propeptide (~150 residues); the PR domain (~500 amino acid residues) which contains the specific catalytic triad consisting of an aspartic acid, a serine and a histidine residue and an eventual insert domain I (~150 residues) which possibly modulates the substrate specificity of the CEP, when present; the A domain (~400 residues), less conserved than the previous domains, which might be involved in the regulation of proteolytic activity and/or could act on the specificity of the PR domain (Siezen, 1999); the B domain (~500 residues) which is found especially in CEPs of lactococci and lactobacilli (PrtB, PrtH, and PrtP) and possibly involved in the stabilization of the enzyme activity and specificity; the H helical domain (~200 residues) involved in the correct positioning of A and B domains outside the cell; the W domain (wall domain of about 100 residues) which functions as a cell wall spacer and can serve as the binding domain for proteases, particularly PrtB of *Lactobacillus delbrueckii* subsp. *bulgaricus*; and the AN domain (~35 residues) which is an anchor domain located after the W domain but not present in all cell wall proteases.

Five types of CEPs have been characterized in various LAB (Fernandez-Espla et al., 2000; Gilbert et al., 1996; Holck & Naes, 1992; Kok, Leenhouts, Haandrikman, Ledebor, & Venema, 1988; Pastar et al., 2003; Pederson, Mileski, Weimer, & Steele, 1999; Siezen, 1999) including PrtP from *Lactococcus (L) lactis* (also called Lactocipine) and *Lactobacillus (L) paracasei*, PrtS from *Streptococcus (S) thermophilus*, PrtH from *Lactobacillus helveticus*, PrtB from *L. delbrueckii* subsp.

Table 1
Examples of bioactivities of fermented milk products.

Type of product	Bioactivities	References
Commercial yogurt from local market	Antihypertensive properties	Kunda et al. (2012)
Yogurt (traditional and commercial)	Antioxidant activity	Aloglu and Oner (2011)
Yogurt (probiotic)	Antioxidant and antidiabetic activities	Ejtahed et al. (2012)
Yogurt (probiotic)	Antidiabetic activity	Ejtahed et al. (2012)
Plain yogurt (fat-free)	Antibacterial activity	Petti, Tarsitani, and Simonetti D'Arca (2008)
Freeze dried yogurt-based diets (with or without probiotics)	Antihypertensive properties	Ramchandran and Shah (2011)
Freeze dried yogurt-based diets (with or without probiotics)	Hypocholesterolemic properties	Ramchandran and Shah (2011)
Yogurt (probiotic enriched)	Inhibit demineralization and promote remineralization of enamel caries	Ferrazzano et al. (2008)
Yogurt beverage fortified with whey protein hydrolysate	ACE-inhibition	Lim, Lee, Park, Yoon, and Paik (2011)
Yogurt	Antihypertensive properties	FitzGerald and Murray (2006)
Yogurt	Antithrombotic activity	FitzGerald and Murray (2006)
Yogurt	Antimicrobial activity	FitzGerald and Murray (2006)
Yogurt	Antiamnesic	FitzGerald and Murray (2006)
Yogurt	ACE-inhibition	Meisel (2005)
Yogurt	Immunomodulation	Meisel (2005)
Sour milk	Antihypertensive properties	FitzGerald et al. (2004)
Sour milk	Phosphopeptides	Silva and Malcata (2005)
Fermented milk (<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> LA2)	ACE-inhibition	Regazzo et al. (2010)
Fermented milk (<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> LA2)	Immunomodulation	Regazzo et al. (2010)
Fermented milk (<i>E. faecalis</i> TH563)	ACE-inhibition	Regazzo et al. (2010)
Dahi	ACE-inhibition	Ashar and Chand (2004)
Plain kefir (Lifeway Foods, Inc.)	Improve lactose digestion	Hertzler and Clancy (2003)
Kephir	Antimicrobial activity	Chifiriuc, Cioaca, and Lazar (2011)
Kefir gel	Wound healing	Huseini, Rahimzadeh, Fazeli, Mehrazma, and Salehi (2012)
Kefir	Anti-allergic & anti-inflammatory	Lee et al. (2007)
Kefir (milk & soyamilk)	Hypocholesterolemic	Liu, Wang, Chen, Chen, Yueh, & Lin, 2006
Kefir	Anticarcinogenic and antimutagenic	Guzel-Seydim, Seydim, Greene, and Tas (2006), Guzel-Seydim et al. (2011)

bulgaricus and PrtR from *Lactobacillus rhamnosus*. These CEPs do not contain the same number of domains. For example, domain B is absent in PrtS, domain I in PrtR or domain AN in PrtH and PrtB. All are classified as subtilisin-like serine proteins, because of the homology existing between their catalytic domain and the subtilisin of *Bacillus subtilis*. *L. lactis* strains possess different forms of CEPs that are distinguished by their specificity of action (Exterkate, Alting, & Bruinenberg, 1993): the PI-type which preferentially hydrolyzes the β -casein into more than 100 oligopeptides with size ranging from 4 to 30 amino acid residues; the PIII-type capable of hydrolyzing the α_{s1} - and β -casein; and CEP displaying intermediate characteristics between the PI and PIII enzymes. Both types (I and III) of CEPs share 98% similarity with five substitutions in the substrate fixation region of these two proteases.

Several parameters, like enzyme/substrate ratio, composition of medium, heat treatment, temperature, pH, carbon/nitrogen ratio, may influence the expression as well as ability of CEPs to release bioactive peptides from the milk proteins. As an example, Miclo et al. (2012) showed that under non-growth conditions, it is possible to obtain different digestion profiles of various caseins with strains possessing the

same *prtS* allele. They attributed this to changes in the enzyme/substrate ratio, following a change in the expression/activity of CEP PrtS. It has also been reported that the increased peptide concentration in the culture medium reduced the expression of CEPs of *L. lactis* (Marugg et al., 1995; Miladinov, Kuipers, & Topisirovic, 2001). Similarly, decreased PrtP activity of *L. lactis* subsp. *lactis* MG1363 was described in the heated milks (Meijer & Hugenholtz, 1997).

Aggei, Potumarthi, and Danquah (2012) reported that the higher production of CEP of *L. delbrueckii* subsp. *lactis* can be achieved in the batch fermentation if the process is properly aerated with stable temperature, i.e. 45 °C and initial pH between 5.5 and 6.5. Furthermore, the type and concentration of nitrogen or carbon compounds in the culture medium are known to influence the production of the protease in lactobacilli and consequently proteolytic properties of the bacteria. It was reported that an increase in the nitrogen source enhances the growth of *L. delbrueckii* ssp. *lactis* and the production efficiency of the protease but to some extent, as if the medium is too rich in nitrogenous matter, the expression of the *prt* gene responsible for protease production decreases (Espeche Turbay, Savoy de Giori, & Hebert, 2009).

2.2. Influence of extracellular peptidases on bioactive peptide production/stability

Although proteins such as caseins are not their preferred substrates, peptidases may, by their ability to hydrolyze the peptides previously generated by CEP from casein, modify the profiles of peptides generated by these strains. In the absence of bacterial lysis, the peptides generated by the CEP from caseins have little possibility to be further hydrolyzed, except if extracellular peptidase(s) exists. To date, few extracellular peptidases have been described in LAB. Aminopeptidase N was found in *L. lactis* subsp. *cremoris* AM-2, *L. helveticus* LHE-511 and *L. acidophilus* R-26 (Miyakawa, Kobayashi, Shimamura, & Tomita,

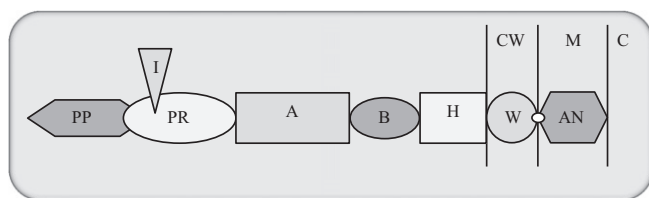


Fig. 2. Diagrammatic representation of CEP of *Lactococcus lactis*. CW: cell wall; M: membrane; C: cytoplasm; PP: preprodomain; PR: catalytic domain; I: insert domain; A: A domain; B: B domain; H: helix domain; W: cell wall spacer domain; white dot: sorting signal and AN: anchor domain.

1992; Neviani, Boquien, Monnet, Thanh, & Gripon, 1989), a tripeptidase in *L. lactis* subsp. *cremoris* IMN-C12, an X-prolyl dipeptidyl aminopeptidase in *L. lactis* subsp. *cremoris* P8-2-47 (Kiefer-Partsch, Boekelmann, Geis, & Teuber, 1989) and an aminopeptidase in *L. delbrueckii* subsp. *bulgaricus* CNRZ397 (Gilbert, Atlan, Blanc, & Portalier, 1994). Recently, the presence of cell wall-associated aminopeptidase and X-prolyl dipeptidyl peptidase activities has been reported in *S. thermophilus*. Moreover, it was shown that exposure of milk-derived bioactive peptides to the non-proliferating cells of *S. thermophilus* strains lacking or possessing CEP, i.e. PrtS resulted in their hydrolysis and in the release of shorter fragments (Hafeez et al., 2013).

2.3. Bioactive peptide production through microbial fermentation/ proteolytic system

The utilization of surface proteolytic system of LAB or their purified CEP provides a natural way of producing bioactive peptides in the milk products and thus allows their functionalization. These peptides can be generated either during the milk fermentation process or from hydrolysis of pure caseins outside the dairy product. In both cases, several types of bioactive peptides may be generated, and some of them may have antagonistic activities.

2.3.1. Bioactive peptide production in a non-proliferating medium

The ability of CEP of LAB to release bioactive peptides from the whole casein or from pure caseins has been evaluated in many studies. As an example, the hydrolysis of α_{s1} -, α_{s2} - and β -casein by 10 strains of the dairy starter *S. thermophilus*, each displaying varying levels of CEP activity (Galia, Perrin, Genay, & Dary, 2009), was studied in a non-proliferating medium. It was shown that after 4 h exposure, *S. thermophilus* strains were able to release peptides from the casein studied depending on their proteolytic activity. As expected, the highly proteolytic strains generated the highest number of peptides from the caseins in the following order β -casein > α_{s2} -casein > α_{s1} -casein. Among the peptides, 20 had been described elsewhere as bioactive, 15 had been reported as angiotensin I-converting enzyme (ACE)-inhibitor while the others had been classified as immunomodulatory and antimicrobial peptides (Miclo et al., 2012). Similarly, when α_{s1} - and β -casein were hydrolyzed with *L. delbrueckii* subsp. *lactis* CRL 581 in non-growth conditions, a great number of peptides were liberated from both caseins by the CEP of this bacterium. For example, the α_{s1} -casein peptide RPKHPKHKQ and two peptides present in the β -casein hydrolysate, i.e. IPP and DKHPP were potent ACE-inhibitor. It is important to note that in both studies, all the bioactivities were allocated based on the sequence identity with known bioactive peptides published in the literature or listed in the database of biologically active peptide sequences. Hence, many other unknown bioactivities could be present in the hydrolysates. Finally, in a recent study, casein medium (comprised of 3% casein, 5% lactose and 1.17% yeast carbon base) was fermented for 42 h with each of *L. casei* Shirota and *S. thermophilus* strains isolated from the commercial fermented milk Soful (Yakult®, Mexico). Both strains produced ACE-inhibitory peptides while the peptides with antithrombotic properties were released only by *L. casei* Shirota. Out of the seven identified thrombin inhibitory peptides, the most active peptide belongs to β -casein fragment 193–209 (YQEPVLGPVRGPFPIIV), which retained its activity even after in vitro degradation by digestive proteases, i.e. pepsin and trypsin (Rojas-Ronquillo et al., 2012).

2.3.2. Bioactive peptide production during fermentation

Many studies using very different lactic acid bacteria have demonstrated the ability of their surface proteolytic system, and mainly of their CEP, to generate bioactive peptides during fermentation. Many of these works concern the formation of ACE-inhibitor peptides, certainly because of the ease to detect their presence through the use of a simple enzymatic assay.

For example, 85 peptides were identified from a yogurt marketed as an antihypertensive functional food, and fifty of them confirmed as bioactive. Majority of the peptides were ACE-inhibitory whereas other peptides possessed bioactivities like antibacterial, antioxidant, immune or phagocytosis stimulation (Kunda et al., 2012). Likewise, ACE-inhibitory and immunomodulatory activities of the milks fermented with *Enterococcus faecalis* TH563 or *L. delbrueckii* subsp. *bulgaricus* LA2 (isolated from Italian dairy products) were determined. It was found that the peptide fraction obtained from the milk fermented with *L. delbrueckii* subsp. *bulgaricus* LA2 displayed both ACE-inhibitory and immunomodulatory activities (inhibited proliferation of bovine peripheral blood lymphocytes) in vitro, whereas only ACE-inhibitory activity was observed in the case of *E. faecalis* TH563 fermented milk (Regazzo et al., 2010). The isolation of an ACE-inhibitory peptide from a skim milk fermented for 72 h with a single dairy strain *L. helveticus* 130B4 isolated from traditionally fermented camel milk constitutes a third example. The identified κ -casein peptide fragment 107–115 (AIPPKKNQD) displayed ACE-inhibitory activity in vitro (IC_{50} = 19.9 μ M). Additionally, the peptide showed marked stability in vitro against digestive proteases (pepsin, trypsin and chymotrypsin) and heat treatment at 100 °C for 20 min (Shuang, Harutoshi, & Taku, 2008). Similarly, ACE-inhibitory activity of skim milk fermented with 25 LAB strains, either alone or in combination, was measured in vitro. All the bacteria displayed strain-dependent ACE-inhibitory activity which was closely connected to the bacterial growth and their proteolytic capacity. The isolation of two potent ACE-inhibitory peptides from a milk fermented with *Lactobacillus jensenii* constitutes a fifth example. The peptides correspond to the fragments 11–26 (LVYFPFGPIHNSLPQN) and 11–20 (LVYFPFGPIH) of β -casein and in vitro showed IC_{50} values of 71 μ M and 89 μ M, respectively. In spite of significant antihypertensive activity, Spontaneously Hypertensive Rats (SHR) only displayed a transient reduction from the baseline blood pressure after 7 days daily intake (200 mg/kg of whey fraction) of the *L. jensenii* fermented milk (Pihlanto, Virtanen, & Korhonen, 2010). As a sixth example, the detection of several novel ACE-inhibitory peptides in a skim milk fermented for 48 h with *E. faecalis* CECT 5727 isolated from raw milk can be cited. Two of the identified peptides derived from β -casein, i.e. fragments 133–138 (LHLPLP) and 58–76 (LVYFPFGPIPNSLPQNIPP) displayed ACE-inhibitory activity as low as 5 μ M in terms of IC_{50} values. Both peptides demonstrated antihypertensive activity when they were orally administered to SHR. In particular, LHLPLP significantly lowers the blood pressure in these animals at an oral dosage of 2 mg/kg of this peptide (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007). Finally, it must be noted that the caseins are not the sole source of ACE-inhibitory bioactive peptides. Thus, the hydrolysis of whey protein concentrate with crude proteinase of *L. helveticus* LB10 led to the isolation of a novel ACE-inhibitory peptide from the fraction displaying the highest ACE-inhibition. The peptide sequence RLSFNP corresponds to fragment (148–153) of β -lactoglobulin. It inhibited ACE activity in vitro with IC_{50} value of 177.39 μ M (Pan & Guo, 2010).

Other bioactivities have been detected among the peptides released from milk proteins during fermentation. Thus, the antioxidant activities of the bioactive peptide fractions isolated from the water soluble extracts of traditional and industrial yogurts were estimated. The traditional yogurt displayed a higher antiradical activity than commercial yogurts during storage, as determined in the water soluble extracts of these yogurts by the ABTS [2, 2', 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] method. Further, peptide fractions purified from the yogurts displayed higher ACE-inhibitory activity than the whole yogurt (Aloglu & Oner, 2011). Likewise, it has been shown that 26 h fermentation of skim milk with *L. helveticus* LH-2 yielded immunostimulatory activity in the supernatant fraction. Macrophages treated with cell-free supernatant of fermented milk showed phagocytosis and higher production of cytokines and nitric oxide. These observations were correlated to the four novel immune peptides present in the cell-free supernatant, three of which belong to β -CN-f143–154 WMHQPHQLPPT,

β -CN-f145–160 HQPHQLPPTVMFPQQ and β -CN-f148–154 HQPLPPT and one to α -lactalbumin (α -LA-f115–122 LDQWLCEK; Tellez, Corredig, Brovko, & Griffiths, 2010).

Besides yogurt, a variety of health benefits have been attributed to the consumption of kefir, a fermented milk beverage obtained by the fermentation of milk with kefir grains. Kefir grains are mixed group of microorganisms that include several LAB (lactobacilli, lactococci, leuconostoc), yeasts and acetic acid bacteria which coexist in a symbiotic association in a matrix of polysaccharides and proteins (Chen, Wang, & Chen, 2008; Garrote, Abraham, & De Antoni, 2001). The bacteria and yeasts in kefir grains hydrolyze proteins and other components from milk during the fermentation process and thus impart several functional/health promoting properties to kefir. Thus, kefir has been shown to display several bioactivities such as antimicrobial and anticarcinogenic properties, cholesterol lowering effects, improving lactose tolerance, immune system and gastrointestinal system or reducing blood pressure. These beneficial effects result from the probiotic properties of microbiota of kefir but also from the components released during the fermentation process (Table 1; for a recent review see Guzel-Seydim et al., 2011). For example, low molecular mass peptides released during the fermentation have been shown to display potency ACE-inhibitory properties and most particularly two of them (PYVRYL and LVYPFTGPIPN) exhibit the highest inhibitory effect (Quiros, Hernandez-Ledesma, Ramos, Amigo, & Recio, 2005).

2.4. Combination of proteolytic strain and commercial enzyme

An alternative strategy to functionalize the fermented products is to ferment the milk with LAB along with food grade enzymes. The combined action of the bacterial enzymes and additional proteases not only increases the peptide content of the product but it also diversifies the bioactivity/functionality generated.

For example, fermentation of fresh low-fat milk with a commercial starter culture mixture of five LAB accompanied by hydrolysis with microbial protease (prozyme 6) enhanced the ACE-inhibitory activity of the whey fraction. The diluted whey fraction (50 mL/day with peptide concentration 5 mg/mL) effectively reduced the systolic and diastolic blood pressure in SHR, when administered orally. ACE-inhibitory activity of the whey fraction was attributed to β -lactoglobulin fragment 17–19 (GTW) and α _{s1}-casein fragment 162–164 (GAW). The isolated peptides GTW and GAW exhibited ACE-inhibitory activity in vitro with IC₅₀ values of 464.4 μ M and 240.0 μ M, respectively (Chen, Tsai, & Pan, 2007). Flavourzyme, derived from *Aspergillus oryzae*, is another food grade protease used to facilitate milk fermentation in combination with LAB starter cultures. Flavourzyme-facilitated milk (consisting in 4.5% skim milk, 5.5% whole milk and 7% sucrose) fermentation was performed employing *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Here, again increased peptide concentration was reported in the whey fraction obtained by the combined action of these LAB and flavourzyme compared to the LAB alone. Moreover, the whey fraction displayed antihypertensive activity in SHR after 8 weeks of oral administration (150 mL/day of whey with peptide concentration 4.9 mg/mL). The peptide responsible for the highest hypotensive activity of the whey fraction corresponds to fragment 58–61 (YPYY) of κ -casein variant B, whose IC₅₀ value was 90.9 μ M, as determined in vitro. The isolated peptide showed stability against gastrointestinal (pepsin and pancreatin) digestion in vitro (Tsai, Chen, Pan, Gong, & Chung, 2008).

The functionalization of milk with bioactive peptides through the use of proteolytic system of LAB has been shown to be realistic, as demonstrated by the different publications. A great number of bioactive peptides can be generated by employing CEP of LAB or combination of CEP and other enzymes. Though a greater number of peptides have been observed in the latter case, certain peptides cannot be produced by these approaches. Indeed, some regions of milk proteins are always cut or conversely unreachable. One example is the anxiolytic peptide α -casozepine, α _{s1}-CN-(f91–100), obtained from the tryptic hydrolysis of

the α _{s1}-casein (Miclo et al., 2001). This peptide cannot be obtained easily by the CEP of *S. thermophilus* because it is present in a region which is often cut by this protease (Miclo et al., 2012). On the other hand, the inaccessibility of certain regions of the substrate to the CEP has been proposed by Miclo et al. (2012) to explain that the hydrophilic N-terminal region (1–50 residues) of β -casein is more resistant to hydrolysis than the remaining hydrophobic region and conversely in the case of the α _{s2}-casein. The hydrophilic and hydrophobic regions in α _{s2}-casein monomers could lead, in specific physico-chemical conditions, to the formation of oligomeric structures, making certain zones of the protein inaccessible for hydrolysis by the CEPs.

3. Functionalization of fermented milk with bioactive peptides by using food-grade enzymes

One of the alternate approaches to functionalize fermented products consists of supplementing them with the bioactive peptides, produced in vitro by hydrolysis of proteins with food-grade enzymes. Many gastrointestinal such as pepsin, trypsin, and chymotrypsin and microbial enzymes such as thermolysin and alcalase have been successfully employed to release biologically active peptides from the milk proteins (for a recent review see Choi et al., 2012; Korhonen, 2009; Phelan, Aherne, FitzGerald, & O'Brien, 2009). After developing some examples, the advantages and disadvantages will be discussed thereafter.

3.1. Examples of production of bioactive peptides through the use of food-grade enzymes

As shown below, the use of different food grade enzymes permits to obtain peptides with different bioactivities. For example, it has been shown that bovine casein hydrolysate prepared by AS1.398 neutral protease displayed dose-dependent (10, 100 and 300 mg/kg body weight) antihypertensive effects in SHR after oral administration. Two novel antihypertensive peptides, corresponding to the fragments 25–30 (RYPYSG) and 15–18 (DERF) of κ -casein, among others were isolated from this hydrolysate. Both peptides revealed ACE-inhibitory activity in vitro with IC₅₀ values of 54 ± 1.2 μ g/mL and 21 ± 0.8 μ g/mL, respectively (Jiang, Tian, Brodkorb, & Huo, 2010). Likewise, the pepsin hydrolysis of isoelectric casein (pH 4.6) generated ACE-inhibitory peptides of which two α _{s1}-casein fragments, i.e. 90–94 (RYLGY) and 143–149 (AYFYPEL), and an α _{s2}-casein peptide 89–95 (YQKFPQY) displayed higher activities in vitro (IC₅₀ values; 0.71 μ M, 6.58 μ M and 20.08 μ M, respectively). Moreover, all the three peptides also revealed antioxidant activities in vitro and antihypertensive potency in SHR after oral intake at a dose of 5 mg/kg body weight (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Similarly, antihypertensive and antioxidant peptides have been identified in the hydrolysate of various bovine milk protein-based infant formulas after digestion with pepsin and pancreatin (Hernández-Ledesma et al., 2007). In another study, the antioxidant activity of the bovine whey protein (α -lactalbumin and β -lactoglobulin variant A) hydrolysate obtained after digestion with different proteases of digestive (pepsin, trypsin, chymotrypsin, Corolase PP) and microbial (thermolysin) origins has been shown (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005). Antioxidant peptides were particularly detected in the Corolase PP digest of β -lactoglobulin variant A. The presence of iron binding peptides has also been reported in the heated whey and colostrum whey treated with papain, pepsin, flavourzyme, trypsin and alcalase. Among all, relatively greater peptides were quantified from the alcalase derived hydrolysate (Kim et al., 2007, 2010). Another food grade enzyme, the Protease N Amano, has been employed to release ACE-inhibitory peptides from the whey protein fraction β -lactoglobulin of which one peptide fragment 36–42 (SAPLRVY) displayed higher ACE-inhibitory activity in vitro with IC₅₀ value of 8 μ M (Ortiz-Chao et al., 2009; Welderufael & Jauregi, 2010).

3.2. Parameters to be considered for the addition of bioactive peptides during the fermentation

Although a variety of bioactive peptides can be produced by employing a single or different combinations of enzymes and be used to supplement fermented milk products to increase their functionality, they need to be purified by chromatographic and membrane separation techniques which are often costly. The addition of the bioactive peptides in the fermented milk products is limited, as they might be hydrolyzed by the surface CEP and/or peptidases of starter LAB. This problem can be overcome by fermenting the milk with peptidase-deficient strains of LAB. As example, it has been shown that the milk fermented with peptidase-negative mutant strain of *L. helveticus* displayed higher ACE-inhibitory activity as compared to the wild-type strain (Kilpi, Kahala, Steele, Pihlanto, & Joutsjoki, 2007), suggesting that some ACE-inhibitory peptides are not hydrolyzed by this strain. Secondly, the bioactive peptides should be added in the late phase of fermentation process where the CEP and/or peptidase activities may diminish due to lower pH. It has been reported that in yogurt simulated conditions, milk-derived bioactive peptides retain their integrity against cell-associated proteases/peptidases of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* at pH 4.5 but not at pH 7.0 (Paul & Somkuti, 2009; Somkuti & Paul, 2010). In a recent study, Hafeez et al. (2013) also found that the cell wall-associated exopeptidases of *S. thermophilus* degraded several milk-derived bioactive peptides upon incubation in phosphate/acetate buffer at pH ranging from 5.5 to 8.5, whereas no such peptide fragmentation was observed at pH 4.5 (Hafeez et al., 2013). Thus, the bioactive peptides can be added in the fermented milk products near or at the end of the fermentation process (pH 4.25–4.5) to impart specific functions to the finished product.

3.3. Protection of bioactive peptides in the food matrix by encapsulation

Encapsulation may offer an attractive alternate solution for keeping structural integrity and functionality of peptides in fermented milk matrices. In this regard, several procedures for encapsulation of bioactive components have been described and subjected to recent reviews (de Vos, Faas, Spasojevic, & Sikkema, 2010; Fathi, Mozafari, & Mohebbi, 2012; Moutinho, Matos, Teixeira, & Balcão, 2012). However, to our knowledge, the encapsulation of milk-derived bioactive peptides has not been reported so far, whereas some studies highlighted the encapsulation of peptides of other origins. For example, the antimicrobial peptide nisin was encased into nanovesicles (liposomes) prepared from soy lecithin (Malheiros, Daroit, de Silveira, & Brandelli, 2010). The authors reported that the application of liposome-encapsulated nisin in combination with refrigeration temperature (7 ± 1 °C) appeared to be effective in reducing the growth of *Listeria monocytogenes* in milk during storage. Similarly, encapsulation of nisin and ethylenediaminetetraacetic acid in phospholipid-based liposome displayed long-lasting inhibitory effects on the growth of both *Escherichia coli* and *L. monocytogenes* in a model system (Taylor, Bruce, Weiss, & Davidson, 2008). In another study, an antihypertensive peptide fraction from tuna cooking juice was entrapped in liposomes prepared with lecithin alone or mixed with cholesterol or phytosterols. This encapsulated antihypertensive peptide fraction lowered the blood pressure in SHR (Hwang, Tsai, & Hsu, 2010). More recently, bovine lactoferrin was encapsulated in lipid nanovesicles and revealed good stability during storage. Further the liposome-entrapped lactoferrin exhibited antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans* (Balcao et al., 2013).

4. Production of bioactive peptides through recombinant DNA technology

The use of recombinant DNA technology for the production of specific proteins, bioactive peptides or their precursors by microbial cells

constitutes another approach. It permits to increase the production yield of the specific bioactive peptides. Two types of strategies can be employed: the first consists of production of the bioactive peptide using a well-known bacterium such as *E. coli* followed by peptide addition to the food matrix; and the second, to use LAB to synthesize the bioactive peptide directly in the food product. In this latter case, this strategy implies the use of genetically modified organisms in food.

4.1. Production of bioactive peptides using *E. coli* as producer

As it has been very well genetically and biochemically characterized, easy to culture and that many tools are available for the production of recombinant proteins at high level, *E. coli* appeared to be an organism of choice for the production of bioactive peptides. Bioactive peptides can be produced either in monomer or in multimer form and/or as fusion proteins. In the latter case, the desired proteins/peptides are obtained after the hydrolysis of the fusion partner with suitable proteases. Numerous reports highlighted the production of bioactive peptides in *E. coli* by this approach. In particular, many anti-hypertensive peptides potentially able to lower blood pressure through the inhibition of vasoactive enzymes such as ACE have been produced successfully in *E. coli*. Three ACE-inhibitory peptides from bovine β -casein (A2 variant) along with flanked regions (indicated by the bold letters), i.e. **QTQSLVYPFPGPI****NS**, **LPQNIPPLTQTPVVVP** and **EDELQDKIHFAQTQS** were expressed in *E. coli* BL21-DE3-Star pLys, after cloning of their respective genes in fusion with that of the glutathione-S-transferase (GST) into the vector pGEX-4T1. The flanked regions ensured the correct proteolysis and release of active peptide sequences from the recombinant fusion proteins (GST with ACE-inhibitory peptides) by cleavage with partially purified proteinase of *L. helveticus* PR4. The purified recombinant active peptides, particularly SLVYPFPGPI, displayed ACE-inhibitory ($IC_{50} = 467.03$ μ M) activity in vitro comparable to other shorter β -casein derived peptides such as β -CN-f60–66 YPFPGPI (Losacco, Gallerani, Gobbetti, Minervini, & De Leo, 2007). Likewise, a novel procedure to produce and enhance the yield of another ACE-inhibitory peptide by genetic engineering has been established. A casein-derived antihypertensive peptide (β -CN-f169–174 KVLPPV) was tandemly multimerized (6-copies) with an additional R residue at the C-terminal, as the clostripain specific cleavage site. The synthetic gene encoding dotetracontapeptide was cloned in frame downstream the GST fusion protein into the vector pGEX-4T-2. After expression in *E. coli* BL21, the purified fusion protein was sequentially subjected to clostripain and carboxypeptidase B hydrolysis. The original antihypertensive peptides released from N- and C-terminals without additional amino acid residues were further tested for their bioactivity. In vitro measured IC_{50} of recombinant antihypertensive peptide was 4.6 μ M, and it remarkably reduced the systolic blood pressure in SHR after 4 h administration, when given orally (Liu, Sun, Zhang, Li, & Qin, 2007). In another study, an antihypertensive peptide multimer (VWIS **QR** RPLKPW **QER** RIY LW **QK** LKPNM AINPSK IKW **QR** RPLKPW **QY** VWIS **QR** RIY LKPNM **W** IPP) was designed in which 11 different antihypertensive peptide monomers from different food sources (milk, rapeseed, fish, egg, etc...) were tandemly linked up according to the restriction sites of gastrointestinal proteases (bold letters). The synthetic gene encoding the antihypertensive peptide multimer was introduced in fusion with GST into the vector pGEX-3X and expressed in the *E. coli* BL21 strain. The potent antihypertensive (ACE-inhibitory) peptide monomers were released effectively after simulated gastrointestinal digestion (pepsin followed by pancreatic enzymes) of the purified antihypertensive peptide multimer (Rao, Su, Li, Xu, & Yang, 2009). More recently, Rao et al. (2011) redesigned another antihypertensive peptide multimer according to the same design principles but this time the multimer was composed of 15 low molecular weight antihypertensive peptides. The optimized polypeptide synthetic gene was inserted downstream of fusion protein thioredoxin into the plasmid pET32a for expression in *E. coli* Origami (DE3). The simulated gastrointestinal

digestion analysis of the fusion protein revealed that the antihypertensive peptide fragments were successfully released from the precursor polypeptide and the hydrolysate displayed potent antihypertensive activity ($IC_{50} = 4.5 \mu\text{g/mL}$) in vitro (Rao et al., 2011).

Like antihypertensive peptides, a little number of antimicrobial peptides of milk origin has also been produced in *E. coli*. The overexpression of bovine lactoferricin B-W10 (LfcinB-W10), a novel derivative of cationic antimicrobial peptide lactoferricin Lf-(f17–41) was studied in *E. coli*. The gene encoding LfcinB-W10 was synthesized in such a way that the methionine at position 10 of the peptide was replaced with tryptophan (to enhance the antimicrobial activity) and contains an added enterokinase cleavage site upstream of the peptide. Followed by ligation of LfcinB-W10 into the vector pGEX-4T-2 downstream of the GST fusion protein, the recombinant fusion protein was successfully expressed in *E. coli* BL21 (DE3). The recombinant LfcinB-W10 released from purified fusion protein by the enterokinase digestion effectively inhibited the growth of *S. aureus* ATCC25923 displaying potent antimicrobial activity (Feng et al., 2010). Likewise, expression of LfcinB15-W4,10, a modified shorter N-terminal fragment of bovine lactoferricin – Lf-(f17–31) –, in which residue of arginine 4 and residue of methionine 10 were substituted with residues of tryptophan was investigated. The synthetic gene encoding LfcinB15-W4,10 or its different oligomers containing tandem repeats was inserted individually into the expression vector pET32a downstream of the fusion partner thioredoxin and expressed in BL21 (DE3). Among the recombinant monomer and different multimeric peptides, the tetramer was produced at the highest level. The antimicrobial activity of the recombinant tetramer against *S. aureus* ATCC25923 was close to that of the synthetic LfcinB15-W4,10 monomer (Tian et al., 2007). Recently, a hybrid antimicrobial peptide LFT33 was developed by combining bovine lactoferricin and thanatin (an inducible insect antimicrobial peptide) from *Podisus maculiventris*. The LFT33 synthetic gene was designed by gathering the first 15 residues of lactoferricin, where the residue of methionine 10 was replaced by a residue of tryptophan, with the residues 4–21 of thanatin. The synthetic gene carrying an upstream enterokinase cleavage sequence was cloned into the vector pET32a in fusion with thioredoxin. After successful expression in *E. coli* BL21, the fusion tag was removed from the hybrid peptide after exposure of the fusion protein to the enterokinase cleavage. The purified recombinant LFT33 demonstrated improved antimicrobial activity against different strains over parent peptides (Feng et al., 2012). Similarly, expression of another antimicrobial peptide, lactophorin, corresponding to residues 113–135 of component 3 of proteose peptone, a phosphorylated glycoprotein of bovine milk, was studied in *E. coli* C41 (DE3). The genes encoding lactophorin or its tandem repeats were ligated downstream of the fusion partner ketosteroid isomerase (steroid δ -isomerase, EC 5.3.3.1) into the vector pET31b. The recombinant lactophorin as tandem repeats was successfully expressed and released from fusion protein after cyanogen bromide hydrolysis (Park, Kim, Choi, & Kim, 2009).

The expression of bovine β -lactoglobulin derived hypocholesterolemic peptide IIAEK, β -LG-(f71–75), was conducted in *E. coli*. It was reported that the higher yield of the peptide multimers was achieved when soybean proglycinin A1aB1b was used as carrier protein. The peptide monomers were efficiently released from the insoluble protein after trypsin hydrolysis (Prak & Utsumi, 2009).

4.2. Production of bioactive peptides using recombinant LAB or probiotic bacteria

Although several milk-derived bioactive peptides have been successfully produced in *E. coli* and the bioactivity of some of them has been shown either in vitro or in vivo (animal models), the recombinant *E. coli* strains (non-food grade) cannot be included in the fermented milk products. Consequently, it is necessary to purify to homogeneity the recombinant peptide (or the multimer) which will then be added to the milk-based products.

Thus, there is a need to construct food grade host bacteria like LAB expressing bioactive peptides in order to develop novel foods. Numerous recombinant lactococci strains expressing therapeutic proteins including cytokines, antigens and enzymes have been reported for their prophylactic and therapeutic effects in different animal models (for recent reviews see Bermúdez-Humarán, Kharrat, Chatel, & Langella, 2011; Morello et al., 2008). However, studies regarding the expression of bioactive peptides or proteins in the food grade host like LAB are lacking. To the best of our knowledge only β -lactoglobulin, a cow's milk protein which is a strong allergen, and a peanut allergen has been successfully expressed in LAB. The biological effects of such engineered strains were tested only by delivering in vivo the recombinant bacteria, without being applied in milk fermentation. In this context, cloning and expression of β -lactoglobulin were reported in *L. casei* (Hazebrouck et al., 2007) and *L. lactis* (Cortes-Perez et al., 2009) using nisin inducible expression system. It was shown that the mice treated with a recombinant *L. lactis* strain expressing β -lactoglobulin displayed reduced levels of β -lactoglobulin-induced allergy. The intranasal administration (5×10^9 CFU) of this recombinant *L. lactis* strain in mice, either after sensitization or before challenge to β -lactoglobulin, favored the development of β -lactoglobulin-specific T-helper type 1 immune response which is associated with increased production of IFN- γ . As a consequence, T-helper type 2 response is down-regulated resulting in decreased IgG1 production (Cortes-Perez et al., 2009). The formation of the peanut allergen Ara h2 was studied in *L. lactis*, where the synthetic codon optimized truncated *ara h2* gene (signal sequence removed) was cloned into lactococcal expression vector (Glenting et al., 2007). The purified recombinant Ara h2 displayed comparable immunological response as determined by ELISA to that of native Ara h2.

Concerning the bioactive peptides, only limited studies regarding their expression in LAB or other probiotic bacteria are available and all the recombinant peptides or strains were not tested in animal studies. Thus, the expression of the three ACE-inhibitory peptides from bovine β -casein (variant A2) SLVYPFPGPI, NIPPLTQTPV, DKIHPP and their pro-active precursors FAQTQSLVYPFPGPIPNSLP, NSLPQ-NIPPLTQTPVVPPF and EDELQDKIHPPFAQTQS respectively, was described in a probiotic strain *Bifidobacterium pseudocatenulatum* M115. The synthetic genes encoding the peptides and their precursors were cloned into the vector pAM1 and introduced into *B. pseudocatenulatum* M115. Among the recombinant strains, strain harboring the ACE-inhibitory precursor EDELQDKIHPPFAQTQS or the peptide NIPPLTQTPV displayed a 50% higher ACE-inhibitory activity than that of the wild type strain when 50 μg cell lysate of each was tested in vitro (Losurdo et al., 2013). Finally, the overproduction of an 11-mer antimicrobial peptide (RRWQWR MKKLG; Lf-(f20–30)) and a hypotensive peptide (FFVAPFPEVFGK; α_5 -CN-(f23–34)) was studied (Renyé & Somkuti, 2008). The synthetic genes based on streptococcal preferred-codons encoding each peptide were cloned into the vector pG341Pa carrying green fluorescent protein as selection marker. The *S. thermophilus* ST128 transformants having antimicrobial or hypotensive peptide were identified with the aid of green fluorescent protein while the expression of hypotensive peptide was detected by reverse transcriptase-polymerase chain reaction.

5. Concluding remarks

There is no doubt that the hydrolysis of milk proteins gives rise to a diversity of peptides, some of them displaying remarkable functionalities relevant to the maintenance of human health. The knowledge about new bioactive peptides from milk proteins and about their potent functionalities in the fermented milk products is consistently increasing. They offer an exciting opportunity in the area of the development of novel functional foods which in turn could contribute to the prevention and management of certain diseases such as hypertension, type 2 diabetes or obesity and more broadly metabolic syndrome. Among the different food matrices, fermented milk appeared to be attractive since it is

consumed regularly by a large number of consumers, it possesses an adequate nutritional profile and it does not change the balance of the diet.

The functionalization of fermented milk with bioactive peptides can be achieved through different ways which have been discussed in this review. The first consists of using the surface proteolytic system of LAB to liberate bioactive peptides or their precursors from the milk proteins. This activity can also be combined with that of food-grade proteases purified from suitable microorganisms. If this way has been shown to be effective, as evidenced by numerous studies published in the field, it faces limitations. Firstly, the proteolytic system of LAB combined or not with food-grade proteases will generate hydrolysates of milk proteins constituting by a mixture of peptides. Certain peptides will be bioactive peptides but other could be antagonist. Secondly, the production of these peptides is strongly influenced by the expression level of the surface protease of LAB, i.e. the main actor of the formation of bioactive peptides from milk proteins. Many parameters that are able to influence the level of expression/activity of CEP have been identified and are difficult to control. The consequence could be a variation of the bioactive peptides concentration depending on fermentation reproducibility. Finally, the bioactive peptides can be internalized by the bacterium for its own growth or further hydrolyzed by extracellular peptidases when they exist. Consequently, in both cases their stability in the food product will be affected. Apart from these, another important factor that the breakdown of milk proteins might also produce off-flavors in the fermented milk products should also be considered. In fact, during cheese ripening certain peptides are generated that lead to off-flavor and bitterness that arise from the accumulation of hydrophobic peptides such as proline rich peptides. Nevertheless, this phenomenon is dependent on the protease/peptidase capacity of starter strains used, as some strains degrade the bitter peptides and consequently do not impart bitterness to the fermented dairy products.

The addition in the product of specific bioactive peptides isolated from milk protein hydrolysate seems to provide a practical approach to enhance the functionality of the fermented milk products. In this case, the initial concentration of the peptide added can be checked, but the peptide remains exposed to the proteolytic system of the starter LAB, except if extracellular protease/peptidase mutants are used during the fermentation step or peptides are encapsulated. Further, given the cost of purification of peptides, it is easier to add a hydrolysate keeping in mind that peptides with antagonist activities can be present in this hydrolysate.

The use of recombinant LAB seems to be a promising approach to increase the functionality of the fermented milk products and to deliver targeted health benefits to consumer. This also permits to produce into the fermented milk, bioactive peptides initially identified in food proteins not belonging to milk. As in the previous case, if the peptides are not produced directly into the milk product, but added to it, the problem of their susceptibility to hydrolysis by bacterial extracellular proteases/peptidases remains. Despite the risks of uncontrolled product expression and transfer of the transgene into a commensal bacterium, the production of bioactive peptides during milk fermentation using genetically modified organisms could be an area of interest for future research. Moreover, peptides with modified sequences can be designed in a view to increase their stability, functionality and duration of action.

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