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REVIEW ARTICLE

Lactic Acid Bacteria as Cell Factories for the Generation of Bioactive Peptides

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Abstract: There is a growing interest in the incorporation of functional foods in the daily diet to achieve health promotion and disease risk reduction. Numerous studies have focused on the production of biologically active peptides as nutraceuticals and functional food ingredients due to their health benefits. These short peptides, displaying antihypertensive, antioxidant, mineral binding, immunomodulatory and antimicrobial activities are hidden in a latent state within the primary sequences of food proteins requiring enzymatic proteolysis for their release. While microbial fermentation is one of the major and economically most convenient processes used to generate bioactive peptides, lactic acid bacteria (LAB) are widely used as starter cultures for the production of diverse fermented foods. This article reviews the current knowledge on LAB as cell factories for the production of bioactive peptides from a variety of food protein sources. These microorganisms depend on a complex proteolytic system to ensure successful fermentation processes. In the dairy industry, LAB containing cell envelope-associated proteinases (CEPs) are employed as biocatalysts for the first step of casein breakdown releasing bioactive peptides during milk fermentation. A better understanding of the functionality and regulation of the proteolytic system of LAB opens up future opportunities for the production of novel food-derived compounds with potential health-promoting properties.

Keywords: Lactic acid bacteria, bioactive peptides, proteinases, proteolytic system, antihypertensive peptides, milk fermentation.

1. INTRODUCTION

The role of proteins as physiologically active components in the diet has being increasingly acknowledged. In recent years, a considerable amount of research has focused on the study of bioactive peptides as nutraceuticals and functional food ingredients aimed at health maintenance [1]. Bioactive peptides are generally short peptides (3–20 amino acids) derived from proteins that may exert biological activities beyond their expected nutritional value. Peptides displaying antihypertensive, antioxidant, immunomodulatory and antimicrobial effects have been reported in several food products including milk, wheat, soybean, peanut meal, meat and marine shrimp (*Acetes chinensis*) hydrolysates generated by proteinases from lactic acid bacteria (LAB) [2-5]. Undoubtedly, milk proteins are recognized as a primary source of bioactive peptides, which are inactive within their protein sequence, requiring proteolysis for their release and activation. Since there is a growing interest in the therapeutic applications of natural compounds, fermentation of milk proteins using proteinases of LAB was proposed as a novel and attractive alternative for the production of functional foods naturally enriched in bioactive peptides [6]. Thus, this review focuses on the use of LAB as cell factories for the production of bioactive peptides derived from food proteins.

2. RELEASE OF ENCRYPTED BIOACTIVE PEPTIDES FROM FOOD PROTEINS

Bioactive peptides are encrypted within the sequence of the primary protein and are functionally inactive. However, they can be released by enzymatic proteolysis *in vitro, in vivo* or a combination of both to achieve their specific "bioactive" roles (Figure **1**) [5]. The *in vitro* generation of bioactive peptides includes the enzymatic hydrolysis of food proteins by endogenous enzymes present in the food matrix as well as proteolysis occurring during food processing or ripening by starter cultures or exogenous proteolytic enzymes (e.g., enzymes derived from microorganisms or plants). On the other hand, the *in vivo* release involves the gastrointestinal digestion where series of digestive enzymes breakdown food proteins to hydrolysates and peptide fractions. Some of the enzymes involved in this process include pepsin, trypsin, chymotrypsin and peptidases from the intestinal brush border membranes as well as enzymes derived from the human mi-

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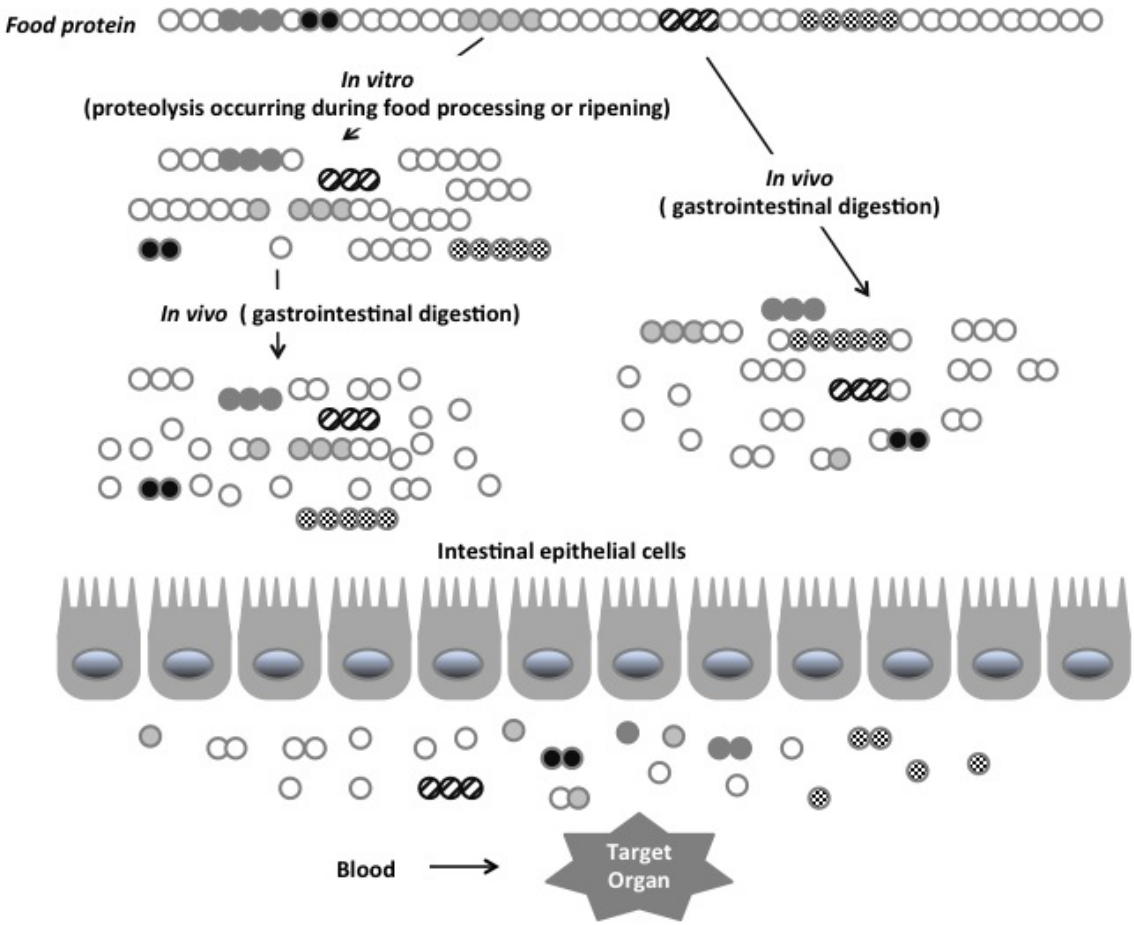

Figure 1. *In vitro* hydrolysis of food proteins by the proteolytic system of the lactic acid bacteria and/or *in vivo* by several digestive enzymes. Encrypted bioactive peptides are indicated

crobiota (Figure **1**). Furthermore, a combination of enzymatic hydrolysis by gastrointestinal digestion and milk fermentation with proteolytic starter cultures or proteolysis using microbial and plant origin enzymes has been demonstrated to be effective in producing short functional peptides [7]. In addition, preparation of bioactive peptides can be obtained using recombinant DNA technology or chemical synthesis [8].

Microbial fermentation is one of the major and cheapest processes used to generate bioactive peptides. The dairy industry stands out among others, due to the use of LAB cell envelope-associated proteinases (CEPs) that release bioactive health-beneficial peptides during milk fermentation [5, 9, 10]. Thus, fermentation of milk proteins using LAB constitutes an attractive alternative for generation of functional foods enriched in bioactive peptides due to the low cost and positive nutritional image associated with fermented dairy products.

3. LACTIC ACID BACTERIA IN FERMENTED PRODUCTS

LAB are defined as Gram-positive, non-sporulating, catalase-negative, and facultative anaerobic bacteria with a fermentative metabolism [11]. LAB constitute a heterogeneous group of phylogenetically closely related microorganisms that produce lactic acid as the major or sole product from carbohydrate fermentation. These bacteria have been associated with food and feed fermentation not only because of their contribution to raw-material preservation due to acidification, but also because of their capacity to contribute to food organoleptic characteristics such as flavor and texture. The most relevant LAB in fermented foods belong to the genera *Lactococcus* (*L.*), *Streptococcus* (*S.*), *Pediococcus* (*P.*), *Leuconostoc* (*Leuc.*) and *Lactobacillus* (*Lb.*). Their long history of safe use in food production earned most LAB species the GRAS (Generally Regarded As Safe) designation by the US Food and Drug Administration (FDA), and the Qualified Presumption of Safety (QPS) classification by the European Food Safety Authority (EFSA) [12]. LAB occupy a diverse set of ecological niches ranging from dairy, meat and plant material fermentation to the oral cavity and the genital and gastrointestinal tracts of humans and animals [13]. However, the most important application of LAB is their use as starter cultures in the manufacturing processes of various fermented food (dairy) products. In particular, *S. thermophilus*, *Lb. helveticus, Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* are widely used as dairy starters and

are of major economic importance [14]. Nowadays, the benefits of fermented dairy products in the human diet are well accepted, and the science of food has evolved towards nutrition for health promotion and disease risk reduction [10]. The beneficial effects of LAB can be exerted by two main mechanisms; (i) a probiotic effect which is related with specific live microbial cells, and (ii) a biogenic effect that involves the production of secondary metabolites with health-promoting properties [10]. In the latter case, LAB act as cell factories for the generation of such bioactive metabolites.

4. THE PROTEOLYTIC SYSTEM OF LAB

Comparative genome analysis of lactobacilli strains revealed that dairy LAB have lost the majority of their amino acid biosynthetic genes and therefore depend on exogenous nitrogen sources for optimal growth [15, 16]. As milk contains only small amounts of amino acids and short peptides, LAB depend on a complex proteolytic system to obtain essential amino acids from caseins during growth in this medium, thereby ensuring successful fermentation. This proteolytic system also contributes to the flavor and texture development of fermented products [14], and can release bioactive health-beneficial peptides during milk fermentation [5, 10]. Therefore, to enhance bioactive peptides generation through the LAB proteolytic system, a better understanding of the functionality, regulation and potentialities of this system is necessary. Among LAB, the proteolytic system of *Lactococcus* has received considerably more attention than that of *Lactobacillus* [14]. *L. lactis* is the most extensively studied LAB organism, and the second most studied Gram-positive bacterium with respect to its genetics, physiology, and molecular biology [14]. However, in the last few years, the proteolytic system of lactobacilli has gained relevance because of their ability to generate bioactive peptides from casein during the milk fermentation process [5, 10, 17].

The proteolytic system of LAB consists of a CEP, specialized transport systems to allow uptake of the resulting peptides, and several intracellular peptidases, which degrade peptides to amino acids (Figure **2**) [14, 18, 19]. The CEP plays a key role in this process since it is responsible for the first step of casein breakdown [14, 18]. Furthermore, gene deletion studies have demonstrated that LAB are not able to grow in milk in the absence of a functional CEP [20].

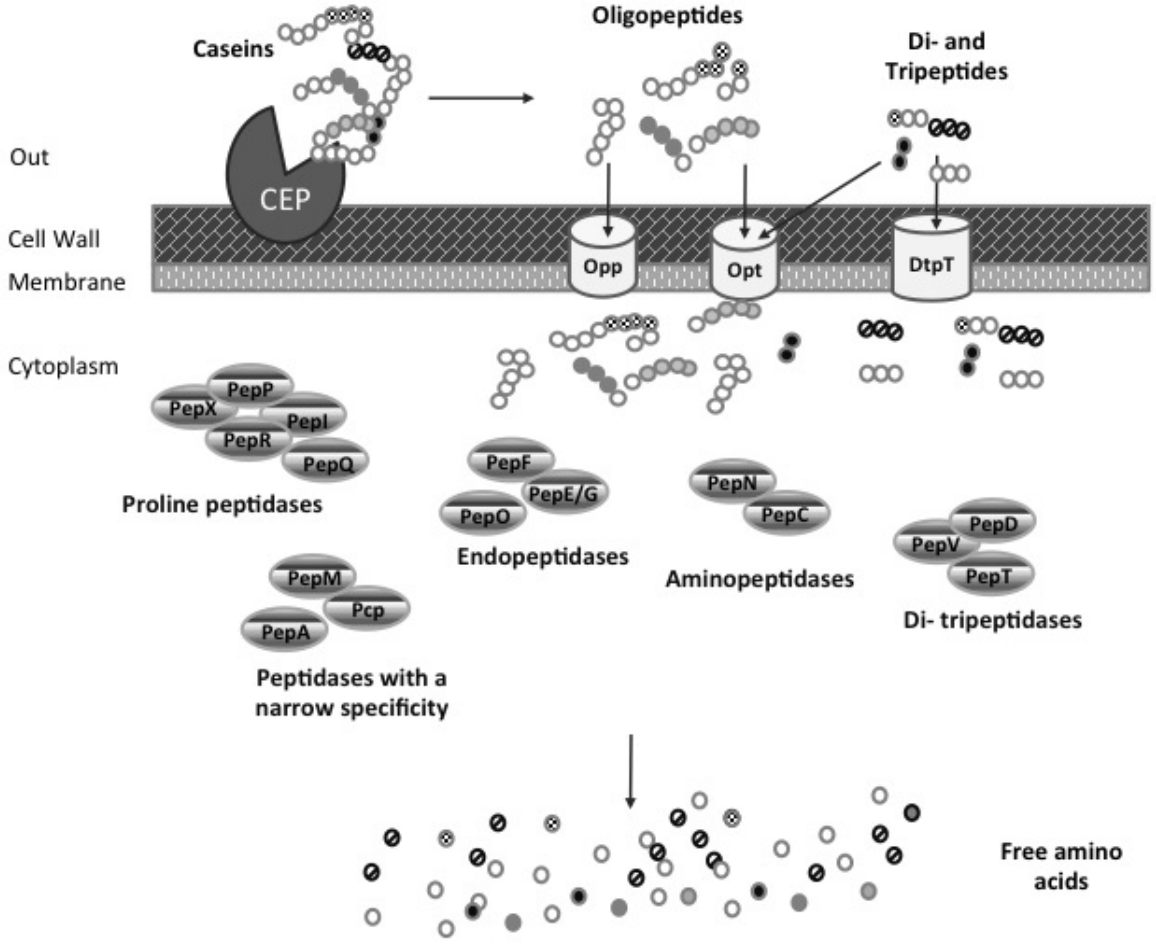

Figure 2. Schematic representation of the proteolytic system of lactic acid bacteria [5, 13, 35]. Cell-envelope associated proteinase (CEP), peptide transporters (Opp, Opt and DtpT), intracellular endopeptidases (PepO, PepF, PepE/PepG), general aminopeptidases (PepN, PepC), unique aminopeptidases (PepM, PepA, Pcp), peptidases with a specificity limited to proline-containing peptides (PepX, PepP, PepI, PepQ, PepR), tripeptidase (PepT), and dipeptidases (PepD and PepV) are indicated.

5. BIOCHEMICAL AND GENETIC CHARACTERISTICS OF PROTEINASES OF LAB

As mentioned before, bioactive peptides are encrypted within the sequence of the parent protein and can be released through hydrolysis by proteolytic enzymes from microbial starters such as LAB to promote health benefits to the consumer.

5.1. Genes Encoding CEPs

To date, eight types of CEPs from LAB have been characterized including PrtP from *L. lactis* and *Lb. paracasei*, PrtS from *S. thermophilus*, PrtH and PrtH2 from *Lb. helveticus*, PrtB from *Lb. delbrueckii* subsp. *bulgaricus*, PrtL from *Lb. delbrueckii* subsp. *lactis* and PrtR from *Lb. rhamnosus* [17, 19, 21-27]. In addition, a genome-wide comparative genomic analysis of proteolytic system components (i.e., CEPs, peptide transporters and peptidases) in 22 sequenced LAB strains showed that the CEP gene was only found in the chromosome of *Lb. acidophilus, Lb. johnsonii, Lb. delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. rhamnosus* and *S. thermophilus* strain LMD9, as well as in the plasmid of *L. lactis* subsp. *cremoris* SK11 [18]. Most of LAB possess only one CEP, although at least two distinct CEPs differing in their substrate specificity have been reported for *Lb. rhamnosus, Lb. casei, Lb. paracasei* and *Lb. helveticus* strains [18, 28]. In *L. lactis, Lb. johnsonii, Lb. rhamnosus, Lb. casei* and *Lb. paracasei*, the proteinase gene (*prtP*) is preceded by a divergently transcribed gene encoding a membrane-bound lipoprotein (PrtM) that was shown to be essential for autocatalytic maturation of the proteinase [18, 29]. Interestingly, the *prtM* encoding a maturation protein of 300 amino acid residues is present in the chromosome of the strains *Lb. helveticus* CNRZ 32 and DPC4571 but no requirement of this PrtM-like chaperon for CEP maturation has been reported [17]. Contrariwise, in *Lb. delbrueckii* subsp. *lactis* CRL 581 as well as in strains of *Lb. acidophilus, Lb. helveticus, S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* no *prtM* gene was identified in the flanking regions of proteinase genes [18, 19].

5.2. The Multi-Domain CEPs of LAB

In general, CEPs are synthesized as pre-proteins of approximately 2000 residues. Comparative sequence analyses allowed the prediction of several functional domains (Figure **3**) [26]. From the N-terminus, these domains include: i) a pre-pro domain (PP) corresponding to a typical signal sequence (~40 residues) followed by a pro-sequence (~150 residues) that is removed by autocatalytic processing; ii) a catalytic protease domain (PR, ~500 residues) that contains the catalytic triad with an eventual insert (I) domain (~150 residues); iii) a domain A (~400 residues) of unknown function; iv) a domain B (~500 residues) with probably a stabilizing role; v) a helical spacer (H) domain (~200 residues) involved in positioning the A and B domains outside the bacterial cell; vi) a hydrophilic cell wall spacer or attachment domain (W, ~100 residues); and finally vii) a cell wall anchor domain (AN, ~35 residues) characterized by a sorting signal (LPxTG) that covalently anchors the proteinase to the cell envelope [26]. However, not all mentioned domains are present in each CEP. For instance, differences in the C-terminal region among several CEPs raise the possibility of another anchoring mechanism to the cell envelope. PrtP, PrtR and PrtS possess the AN domain, suggesting a covalent attachment of the proteinase to the cell wall [14]. On the other hand, this AN domain is absent in PrtB, PrtL, PrtH and PrtH2, which seem to be attached to the cell envelope through their W domain [14, 26]. The C-terminal end of PrtH and PrtH2 is homologous to that of S-layer proteins, and is possibly attached to the cell wall via a mechanism resembling that utilized by S-layer proteins [17]. The W domain of PrtB and PrtL are rich in lysine residues (~32%) suggesting that binding occurs through electrostatic interactions with the negatively charged teichoic acids of the cell wall [19, 21]. This electrostatic interaction was confirmed for PrtL, demonstrating that it can be modulated by changing the protonation/deprotonation state of charged residues via environmental pH [19, 30].

5.3. Activity and Specificity of LAB CEPs

On the basis of degradation patterns of α_{s1}-, β-, and κ-caseins, two proteinase specificity classes have initially been described in lactococci, namely CEP_I and CEP_{III} [31]. The primary substrate for the CEP_I-type enzymes is β-casein and, to a lesser extent, κ-casein while CEP_{III}-type enzymes hydrolyze α_{s1}-, β-, and κ-casein [32]. Furthermore, lactococcal CEPs are further classified into seven groups (from a to g) according to their specificities toward the α_{S1}-casein frag-

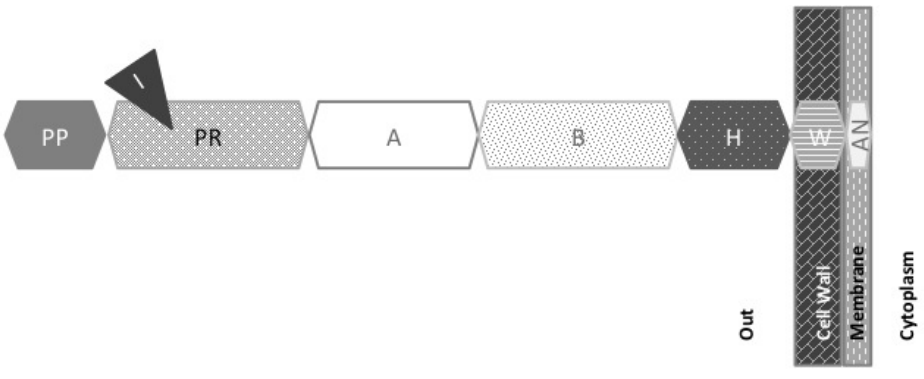

Figure 3. Schematic representation of the predicted domains of CEPs of lactic acid bacteria: preprodomain (PP), catalytic proteinase domain (PR), insert domain (I), A-domain (A), B-domain (B), helical domain (H), cell-wall domain (W) and anchored domain (AN).

ment comprising residues 1 to 23 (f1–23) [32]. It is worth mentioning that the caseinolytic specificity of the lactobacilli CEPs did not fit into the criteria for any group distinguished so far among the lactococcal CEP variants [4, 17, 19, 32].

Protein engineering studies employing hybrid Wg2 (CEP_I) –SK11 (CEP_{III}) proteinases of *L. lactis* identified the N-terminal fragment of the mature proteinase (particularly positions 131, 138, 142, 144, 166, 177, and 222 of the *L. lactis* subsp. *cremoris* SK11 PrtP), as well as the large C-terminal containing residues 747 to 748 as sites that significantly affect casein cleavage specificity [32]. These residues are different among PrtL, PrtB, PrtH, PrtP, PrtR, and PrtS, showing that caseinolytic specificity of the mentioned CEPs are distinct [19, 32].

5.4. Mechanisms Controlling the Regulation of CEP Biosynthesis

A better knowledge on the functionality and regulation of the proteolytic system of LAB is essential to fully understand the production and processing of bioactive peptides. In *L. lactis*, the main regulation controlling biosynthesis of proteolytic components takes place at transcriptional levels and involves the global pleiotropic CodY repressor in response to branched-chain amino acid (BCAA) availability [33, 34]. However, no CodY-like homologs have been reported in lactobacilli so far and information about the regulation of proteinase biosynthesis is still scarce. For instance, it was demonstrated that the production of bioactive peptides in *Lb. delbrueckii* subsp. *lactis* CRL 581 by PrtL was repressed by the peptide content of the growth medium [15]. Furthermore, the CEP activity of strains of *Lb. helveticus* as well as *Lb. delbrueckii* subsp. *bulgaricus* was inhibited after bacterial growth in a peptide rich medium like MRS broth [4, 17, 35]. In *Lb. helveticus* CM4 the production of the antihypertensive peptides VPP and IPP in fermented milk was repressed by peptone addition [36]. Thereafter, Wakai and Yamamoto [37] described a novel type of BCAA-responsive transcriptional regulator (BCARR) in *Lb. helveticus* CM4 that negatively acts on the proteolytic system. In *Lb. helveticus* CNRZ 32 microarrays experiments confirmed the CEP biosynthesis regulation at transcriptional levels; the expression of both *prtH* and *prtH2* genes were up-regulated during bacterial growth in milk compared to MRS medium [38] Similarly, *prtR* expression was Casitone-dependent, emphasizing that nitrogen depletion elevates its transcription [24].

6. PRODUCTION OF MILK-DERIVED BIOACTIVE PEPTIDES BY PROTEINASES OF LAB

Theoretically, any food protein can be used as potential source of bioactive peptides [5]; however, milk proteins have been the main source of bioactive peptides obtained through microbial fermentation. CEPs of LAB are highly specific since the sites of cleavage on the caseins differ from one strain to another depending on the type of casein (α- or β-casein) used as substrate [19, 39]. Thus, the strain selection has a crucial impact on the composition of the released peptides during dairy fermentation [5]. Bioactive peptides can be generated either during milk fermentation processes or through hydrolysis of pure milk proteins by using partially purified CEP [19]. In both cases, several types of bioactive peptides exhibiting diverse activities may be generated depending on their amino acid sequences. In fact, several immunomodulatory, hypocholesterolemic, antimicrobial, mineral-binding, opiod, and antihypertensive bioactive peptides have been isolated from fermented dairy products [5, 10, 39, 40]. Taking into account the large number of existing literature related to the presence of bioactive peptides in fermented milks, in this review we will focus only on those peptides released by the action of LAB proteinases, which are shown in Table **1**.

The antihypertensive effect is the main studied activity of a bioactive peptide produced during milk fermentation [5, 39-41]. Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure. ACE increases blood pressure by converting angiotensin I into the potent vasoconstrictor angiotensin II, which induces the release of aldosterone with the concomitant increase in the sodium concentration and consequently, the blood pressure. In addition, ACE degrades bradykinin, which has vasodilatatory properties. By inhibiting these processes, synthetic ACE inhibitors have long been used as antihypertensive agents. Since many drugs display side effects, research has been devoted toward producing foods with ACE inhibitory peptides, which are beneficial for individuals who have high blood pressure [42]. ACE- inhibitory peptides present mainly an antihypertensive effect but can also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity [43]. These are short peptides with only two to nine amino acids although mostly are di- or tri-peptides, which are resistant to the action of digestive-tract endopeptidases [43, 44]. In milk proteins, low molecular weight peptides containing Pro residues exhibit strong ACE inhibitory activity [45]. The YP sequence is frequently present at the C-terminal region of bovine caseins (α_{s1}-casein f146-147 and f159-160; β-casein f114-115; and κ-casein f58-59). ACE-inhibitory peptides have been mainly isolated from dairy products fermented with *Lb. helveticus* strains [5, 17, 39, 41]. For example, the IPP and VPP β-casein derived tripeptides produced during milk fermentation by *Lb. helveticus* CP790 are able to reduce blood pressure in spontaneously hypertensive rats (SHR) after a single or long - term oral administration [46-48]. Contrariwise, milk fermented with *Lb. helveticus* CP791, a variant lacking CEP activity, did not affect the systolic blood pressure of SHR. The antihypertensive effect of these biopeptides was also demonstrated in several clinical trials [46, 49, 50]. Two commercial antihypertensive milk products, Ameal S™ and Evolus™, which contain the antihypertensive peptides IPP and VPP produced by *Lb. helveticus* are currently available on the market [5]. These tripeptides were also identified in β-casein hydrolysates generated by the strains *Lb. helveticus* CRL 1062, *Lb. helveticus* CRL 1177 and *Lb. helveticus* CRL 1179 as well as in *Lb. delbrueckii* subsp. *lactis* CRL 581, being CRL 581 the strain that produced the highest amount [51]. Minervini, Algaron, *et al.* [52] demonstrated that sodium caseinate hydrolysates prepared from bovine, ovine, caprine, pig, buffalo or human milk using a partially purified CEP of *Lb. helveticus* PR4 produced various ACE-inhibitory and antimicrobial peptides. One of these antibacterial peptide (β-CN f184-210),

Table 1. Bioactive peptides produced from hydrolysis of milk proteins by CEP of lactic acid bacteria.

Strain	Precursor Protein	Peptide Sequence	Bioactivity
Lb. helveticus CP790	Bovine β-casein	DELQDKIHPFAQTQSLVYPFPGPIPNS	ACE inhibitor[a]
	Bovine β-casein Bovine β-casein	LLYQQPVLGPVRGPFPIIV	ACE inhibitor[a]
		PPQSVLSLSQSKVLPVPE	ACE inhibitor[a]
	Bovine β-casein	SKVLPVPE	ACE inhibitor[a]
Lb. helveticus PR4	Bovine α_{S1}-casein	FVAPFPEVFGKEKVNELSKDIGSE	ACE inhibitor[b]
	Bovine α_{S1}-casein	LGTQYTDAPSFSDIPNPIGSENSEK	ACE inhibitor[b]
	Bovine β-casein	LVYPFPGPIPNSLPQNIPP	ACE inhibitor[b]
	Ovine α_{S1}-casein	RPKHPI	ACE inhibitor[b]
	Ovine α_{S1}-casein	RPKH	ACE inhibitor[b]
	Ovine α_{S1}-casein	HPIKH	ACE inhibitor[b]
	Ovine α_{S2}-casein	TVDQ	ACE inhibitor[b]
	Ovine α_{S2}-casein	HQK	ACE inhibitor[b]
	Caprine α_{S2}-casein	TVDQHQ	ACE inhibitor[b]
	Caprine β-casein	LVYPFPGP	ACE inhibitor[b]
	Buffalo β-casein	LVYPFPGPI	ACE inhibitor[b]
	Human β-casein	QPQ	ACE inhibitor[b]
	Human β-casein	VPQ	ACE inhibitor[b]
	Human β-casein	IPQ	ACE inhibitor[b]
	Human β-casein	QELLLNPTHQYPVTQPLAPVHNPISV	Antimicrobial[b]
Lb. helveticus LB10	Bovine β-lactoglobulin	RLSFNP	ACE inhibitor[c]
Lb. helveticus R389	Milk proteins	No sequence available	Immunomodulating[d]
Lb. helveticus LH-2	Bovine β-casein	WMHQPHQPLPPT	Immunomodulating[e]
	Bovine β-casein	HQPHQPLPPTVMFPPQ	Immunomodulating[e]
	Bovine β-casein	HQPLPPT	Immunomodulating[e]
	α-lactalbumin	LDQWLCEK	Immunomodulating[e]
Lb. acidophilus ATCC 4356	Bovine casein	3 kDa-ultrafiltered casein hydrolysates	Immunomodulating[f]
Lb. delbrueckii subsp. *lactis* CRL 581	Bovine α_{S1}-casein	KEDVPSE	ACE inhibitor[g]
	Bovine α_{S1}-casein	QGIH	ACE inhibitor[g]
	Bovine α_{S1}-casein	GLPQE	ACE inhibitor[g]
	Bovine α_{S1}-casein	VEQKHIQKE	ACE inhibitor[g]
	Bovine α_{S1}-casein	EIVPNSAEERLH	ACE inhibitor[g]
	Bovine α_{S1}-casein	QAMEDIKQMEAEZ-	Mineral binding (CPP)[h]
		IZZZEEIVPNZVEQKHIQKEDVPSERYLGYLE	Mineral binding (CPP)[h]
	Bovine α_{S1}-casein	EIVPNZAEERLHSM	Mineral binding (CPP)[h]
	Bovine α_{S1}-casein	ZAEERLHSM	Mineral binding (CPP)[h]
	Bovine α_{S1}-casein	ZAEERLH	Mineral binding (CPP)[h]
	Bovine α_{S1}-casein	LZZZEESITRINKKI	ACE inhibitor[g]
	Bovine β-casein	FPPQS	ACE inhibitor[g]
	Bovine β-casein	QEPVLGPVRGPFP	ACE inhibitor[g]
	Bovine β-casein	LGPVRGPFP	ACE inhibitor[g]
	Bovine β-casein	IPP	Anti-inflammatory[i]

(Table 1) Contd….

Strain	Precursor Protein	Peptide Sequence	Bioactivity
	Bovine β-casein	No sequence available LZZZEESITRINKKIEK-FQZEEQQ	Mineral binding (CPP)[h]
	Bovine β-casein	KFQZEEQQQTEDELQNKIHPFAQTQ	Mineral binding (CPP)[h]
	Bovine β-casein	KFQZEEQQQTEDELQNKIHPF	Mineral binding (CPP)[h]
	Bovine β-casein	KFQZEEQQQTEDELQ	Mineral binding (CPP)[h]
	Bovine β-casein	KFQZEEQQQTED	Mineral binding (CPP)[h]
	Bovine β-casein	KFQZEEQQQTE	Mineral binding (CPP)[h]
Lb. delbrueckii subsp. *bulgaricus* IFO13953.	Bovine κ-casein	ARHPHPHLSFM	Antioxidant[j]
L. lactis subsp. *lactis* GR5	Bovine casein	3 kDa-ultrafiltered casein hydrolysates	Immunomodulating[f]
S. thermophilus 4F44	Bovine β-casein	VKEAMAPK	Antioxidant[k]
	Bovine β-casein	SKVLPVPQ	ACE inhibitor[k]
	Bovine β-casein	DKIHPF	ACE inhibitor[k]
	Bovine β-casein	NIPPLTQTPV	ACE inhibitor[k]
	Bovine β-casein	EMPFPK	ACE inhibitor[k]
	Bovine β-casein	HLPLPLL	ACE inhibitor[k]
	Bovine β-casein	SQSKVLPVPQ	ACE inhibitor[k]
	Bovine β-casein	QEPVLGPVRGPFPIIV	ACE inhibitor[k]
S. thermophilus 4F44, ATE19P88, Y4, LMD-9, PB302, PB385	Bovine β-casein	KVLPVPQ	ACE inhibitor[k]
	Bovine β-casein	RDMPIQAF	ACE inhibitor[k]
	Bovine β-casein	LLYQEPVLGPVRGPFPIIV	ACE inhibitor and immunomodulating[k]
	Bovine β-casein	LYQEPVLGPVRGPFPIIV	Mitogene[k]
	Bovine β-casein	YQEPVL	ACE inhibitor[k]
	Bovine β-casein	YQEPVLGPVR	ACE inhibitor[k]
	Bovine β-casein	YQEPVLGPVRGPFPIIV	Immunomodulating and antimicrobial[k]
	Bovine β-casein	GPVRGPFPIIV	ACE inhibitor[k]
	Bovine β-casein	GPFPIIV	ACE inhibitor[k]
	Bovine α_{S1}-casein	RPKHPIKHQ	ACE inhibitor[k]
S. thermophilus 4F44, ATE19P88, Y4, LMD-9	Bovine β-casein	KVLPVPQK	Lipoxigenase inhibitor[k]
S. thermophilus Y4, LMD-9	Bovine β-casein	VRGPFPIIV	ACE inhibitor[k]
S. thermophilus PB385	Bovine α_{S2}-casein	PYVRYL	ACE inhibitor and antioxidant[k]
S. thermophilus 4F44, PB302, PB385	Bovine α_{S2}-casein	AMKPWIQPK	ACE inhibitor[k]
		MKPWIQPK	ACE inhibitor[k]
S. thermophilus 4F44, Y4, PB302	Bovine α_{S2}-casein	TKVIP	ACE inhibitor[k]
S. thermophilus 4F44, Y4, LMD-9, PB385	Bovine α_{S1}-casein	RPKHPIKHQGLPQEVLNENLLRF	Immunomodulating and antimicrobial[k]
S. thermophilus 4F44, PB385	Bovine α_{S2}-casein	FALPQYLK	ACE inhibitor[k]

Zs are phosphoserines.

[a][48] [b][52] [c][63] [d][59] [e][60] [f][64] [g][51] [h][15] [i][9] [j][62] [k][54, 63]

identified in human sodium caseinate hydrolysate, showed a broad spectrum of inhibition against Gram-positive and Gram-negative bacteria including species of potential clinical interest such as *Enterococcus faecium, Bacillus megaterium, Escherichia coli, Listeria innocua, Salmonella* spp., *Yersinia enterocolitica* and *Staphylococcus aureus* (Table **1**). Furthermore, a bovine sodium caseinate fermentate by the strain *Lb. animalis* DPC6134 contained an array of antithypertensive, antioxidant and antimicrobial peptides [53].

Besides lactobacilli, the capacity of *S. thermophilus* strains expressing various levels of PrtS to generate bioactive peptides from bovine caseins has been reported [54, 55]; the number and type of released peptides being strain-dependent. Among the reported biopeptides, 13 produced from β-casein and 7 from α-casein have been claimed to be bioactive while 15 of them showed ACE inhibitory activity (Table **1**). It is worth mentioning that in this study, 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.

In addition to the ACE inhibitory activity, some lactobacilli strains are able to release bioactive peptides affecting mineral absorption. Casein phosphopeptides (CPPs) are released from α_{s1}-, α_{s2}-, β- , and κ-caseins by enzymatic hydrolysis either during fermentation or in the gastrointestinal tract. As a consequence of the high number of negative charges, these peptides efficiently bind divalent cations such as Ca^{++}, Mg^{++}, Fe^{++}, Zn^{++}, Cu^{++}, Mn^{++}, Ni^{++}, Co^{++}, Se^{++}, and Cr^{++} acting as biocarriers for these elements [44]. Calcium has many important functions in the human body, including bone development and recalcification, and the prevention of hypertension and colon cancer [56]. The use of CPPs for the prevention of dental caries has also been proposed because CPPs inhibit caries lesions through recalcification of the dental enamel [57]. Five and six CPPs were identified by mass-spectrometric analysis in the α- and β-casein hydrolysates generated by the CEP of the strain *Lb. delbrueckii* subsp. *lactis* CRL 581, respectively (Table **1**) [15].

Bovine milk also contains several immunoregulatory peptides that affect the immune system via cellular functions [58]. Three kDa-ultrafiltered casein hydrolysates produced after digestion with CEPs of *Lb. acidophilus* ATCC 4356 and *L. lactis* subsp. *lactis* GR5, significantly decreased the basal nuclear factor (NF)-kβ activity in Caco-2 cells demonstrating immunomodulatory activity [58]. In addition, the β-casein hydrolysate generated by PrtL of *Lb. delbrueckii* subsp. *lactis* CRL 581 exerted a beneficial effect on acute intestinal inflammation by increasing interleukin 10 and decreasing IFN-γ [9]. Bioactive peptides released from milk proteins by the proteolytic strain *Lb. helveticus* R389 were able to stimulate the immune system and inhibited the growth of an immunodependent fibrosarcoma in a mouse model [59]. On the other hand, fermentation of milk by *Lb. helveticus* LH-2 resulted in the production of specific peptides capable of modulating macrophage activity [60]. These β-casein (f143-154, f145-160, and f148-154) and α-lactalbumin (f115-122) derived bioactive peptides upregulated cytokines and nitric oxide production by macrophages and stimulated the activity of phagocytic cells [60].

Dietary intake of antioxidant compounds can reinforce the body's oxidant status and help to maintain a balanced condition in terms of oxidant/antioxidant in the body. Therefore, natural antioxidant compounds attracted the attention of many food manufacturers to produce healthy foods. Recently, there has been a particular focus on milk-derived peptides as source of antioxidants. Once released, certain peptides have been shown to possess radical scavenging, metal ion chelation properties and the ability to inhibit lipid peroxidation [61]. Milk-derived antioxidative peptides consist of 5–11 amino acids including hydrophobic amino acids, proline, histidine, tyrosine or tryptophan in their sequence [62]. Antioxidant activity of the hydrolysates seems to be inherent to the characteristic amino acid sequences of the derived peptides, depending on the proteinase specificity [62]. To date, only a few antioxidant peptides have been identified in milk protein hydrolysates generated by CEPs from LAB. A κ-casein derived peptide (F96-106) with radical scavenging activity has been found in milk fermented with *Lb. delbrueckii* subsp. *bulgaricus* [62]. Furthermore, hydrolysis of β-casein by the proteolytic system of *S. thermophilus* 4F44 resulted in the release of the peptide β-CN (f98−105) displaying antioxidant activity [55].

CONCLUSIONS

Considering the direct correlation between diet and health, nowadays consumers are interested in improving their lifestyle through the consumption of functional foods that exert positive health effects when present in a normal diet. To date, there are countless studies on the synthesis of biologically active peptides from a variety of food protein sources. Milk proteins are recognized as the most important source of bioactive health-beneficial peptides that can be released through hydrolysis by proteolytic enzymes from LAB. As there is growing interest in the therapeutic applications of natural compounds, hydrolysis of milk proteins by proteinases of LAB constitutes an attractive approach to generate dietary supplements or functional foods naturally enriched in bioactive peptides with diverse health benefits. New food protein sources for the release of bioactive peptides displaying interesting properties may be the next challenging studies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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