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Antimicrobial Peptides Derived from Milk: A Review

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ABSTRACT: Milk proteins provide a natural source of bioactive peptides with potential health benefits and applications in the food industry. The release of these peptides from milk proteins is achieved either by hydrolysis using digestive proteases or by lactic acid bacteria fermentation. Peptides, particularly those derived from milk proteins, can exert a wide range of nutritional, functional and biological activities. Bioactive peptides are inactive within the sequence of the parent protein molecule and can be liberated by gastrointestinal digestion of milk, fermentation of milk with proteolytic starter cultures or hydrolysis by proteolytic enzymes. They should be from 3 to 20 amino acids in size and many of them have multifunctional properties. Bioactive peptides have been isolated from several dairy products including cheese, kefir, milk, and yoghurt. Milk-derived peptides with antimicrobial activity have huge industrial potential, as they have the advantage of being derived from a safe and economical source. The aim of this review study is to introduce bioactive peptides with antimicrobial activities that have been produced from milk proteins.

Keywords: *Antimicrobial Peptide, Bioactive Peptide, Milk.*

Introduction

Many food proteins can exert a physiological action, either directly or, after their degradation, in the form of fragments (Tidona *et al*., 2009). Milk is also a known food source of potent bioactive peptides with various properties including antihypertensive, antimicrobial, antioxidant, opioid and anti-thrombiotic (Pritchard, 2012). Bioactive peptides have been defined as specific protein fragments that have a positive impact on food properties and conditions and may ultimately influence the health (Kitts & Weiler, 2003). Bioactive peptides are inactive within the sequence of the parent protein molecule and can be liberated by gastrointestinal digestion of milk, fermentation of milk with proteolytic starter cultures or hydrolysis by proteolytic enzymes. Biologically active peptides released from caseins and whey proteins contain three to twenty amino acids per molecule (Korhonen & Pihlanto-Leppälä, 2004). Bioactive peptides activity is based on their inherent amino acid composition and sequence and many peptides are known to reveal multifunctional properties (Meisel & FitzGerald, 2003). α_{s2}-casein f (203–208) is a good example of a multifunctional peptide because it exhibited not only antimicrobial activity, but also, potent antihypertensive and antioxidant activity (Recio *et al*., 2005).

A simple schematic representation of major bioactive functional compounds derived from milk is presented in Figure 1 (Korhonen & Pihlanto-Leppälä, 2004).

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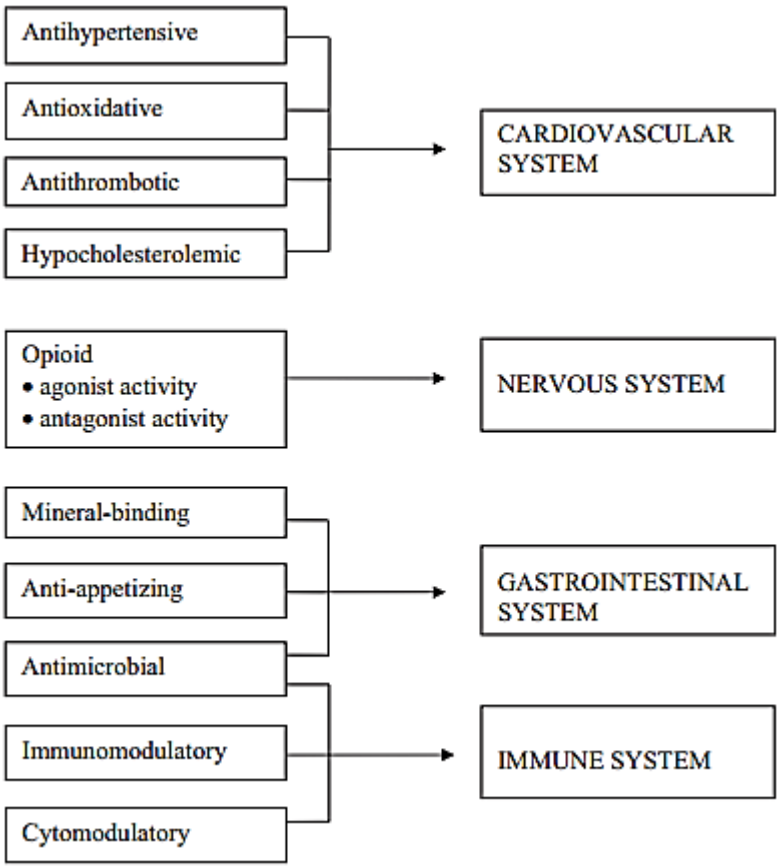

Fig. 1. Physiological functionality of milk-derived bioactive peptides

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. Many of the known bioactive peptides have been produced using gastrointestinal enzymes, usually pepsin and trypsin. Angiotensin-converting enzyme (ACE)-inhibitory peptides (FitzGerald *et al*., 2004) and calcium-binding phosphopeptides (CPPs) (Meisel &FitzGerald, 2003), for example, are most commonly produced by trypsin. Enzymatic hydrolysis is seen as a favorable method to generate milk bioactive peptides due to the lack of residual organic solvents or toxic compounds in the end products (Zambrowicz *et al*., 2013). On the other hand, bioactive peptides can be generated by the starter and non-starter bacteria used in the manufacture of fermented dairy products. The proteolytic system of lactic acid bacteria (LAB), e.g. *Lactococcus lactis*, *Lactobacillus helveticus* and *Lb. delbrueckii ssp. bulgaricus*, are 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 (Christensen *et al*., 1999).

Although, it is well documented that bioactive peptides can be generated during milk fermentation with the starter cultures (Gobbetti *et al*., 2002; Matar *et al*., 2003) but The use of enzymatic hydrolysis to produce natural bioactives may be preferential to microbial fermentation in many cases due to ease of scalability and predictability. The majority of dairy-derived antimicrobial peptides reported to date are liberated via hydrolysis with gastric enzymes such as trypsin and pepsin (Akalın, 2014; Benkerroum, 2010). Sometimes, these

processes may overlap since the protelytic action can start in food and continue in the organism (Kitts & Weiler, 2003).

Biologically active peptides are produced from several dietary proteins during gastrointestinal digestion and fermentation, but milk proteins are considered as the main source of biopeptides, with specific nutritional, sensorial and functional properties (Tidona *et al*., 2009). Antimicrobial peptides derived from food proteins constitute a new field in the use of antimicrobial agents in food (Clare *et al*. 2003; Hayes *et al*. 2007). These peptides have been found to be active against broad range of pathogenic organisms, such as Escherichia, Helicobacter, Listeria, Salmonella and Staphylococcus, yeasts, and filamentous fungi (Lahov & Regelson 1996). The antimicrobial properties of milk have been widely acknowledged for many years. Before discovering the properties of biopeptides some milk proteins such as immunoglobulins, lactoferrin, lactoperoxidase and lysozyme were known to have antimicrobial activities but recently, bioactive peptides displayed broad antimicrobial activities in food industry. These peptides can exert antimicrobial activities comparable to antibiotics, with potential application as natural alternatives (Hayes *et al*., 2007). Antimicrobial peptides either eradicate or suppress the growth of microorganisms. They have been derived from a variety of milk proteins including β-lactoglobulin, α-lactalbumin, α_{s1}-casein, α_{s2}-casein and κ-casein (Pritchard, 2012). This review focuses on the antimicrobial peptides that are originated from milk proteins.

- *Bioactive peptide derived from casein*

Caseins, digested by enzymes *in vitro*, *in vivo* or during food processing may be a source of numerous bioactive peptides. Isracidin was the first peptide with antimicrobial properties identified into the sequence of bovine α_{s1}-casein (Hill *et al*., 1974). Isracidin (α_{s1}-casein f (1-23) obtained from chymosin hydrolysis has been shown to have antibacterial activity against *Satphylococcus aureus* and *Candida albicans* (Lahov & Regelson, 1996). α_{s1}-casein f (99-109) is a cationic peptide (with a theoretical pI 10.46) which was obtained by hydrolysis with pepsin of bovine sodium caseinate and subsequent purification by several steps of preparative RP-HPLC. This peptide has an MIC of 125mg/mL against the Gram-positive bacteria *B. Subtilis* and *Listeria innocua*. With respect to Gram-negative bacteria, f (99–109) presented activity against *Salmonella typhimurium* (MIC 125mg/mL), *E. coli* (MIC 250mg/mL), *Salmonella. Enteritidis* (MIC 125mg/mL) and *Citrobacter freundii* (MIC 500mg/mL) (McCann *et al*., 2006).

In addition, some α_{s1}-casein-derived peptides have demonstrated a cytotoxic action against several cell lines. These cytotoxic peptides are seen to be valuable materials for the development of antiseptic and carcinostatic drugs. Otani and Hata (1995) reported that bovine α_{s1}-casein digested with bovine trypsin produced cytotoxic activity towards mouse spleen cells.

Caseicin A α_{s1}-casein f (21e29) and caseicin B α_{s1}-casein f (30-37), two casein-derived antimicrobial peptides were identified and isolated from bovine α_{s1}-casein, with potential applications as dairy-based protectants against pathogens in powdered foods. Both peptides were found to inhibit the neonatal gram negative pathogen *Cronobacter sakazakii* in culture broth and when included as part of fermentates in powdered infant formula trials (Hayes *et al*., 2006; Hayes *et al*., 2009). Further studies demonstrated their ability to inhibit other important Gram-negative pathogens, including Salmonella and Klebsiella and the Gram-positive pathogen *Staphylococcus aureus* (McDonnell *et al*., 2012; Norberg *et al*.,

2011). Kent and co-workers (2012) show that a conserved feature of the *Bacillus thuringiensis* and *Bacillus cereus* group have the ability to cleave casein to liberate the caseicin A (IKHQGLPQE) and B (VLNENLLR) peptides.

Casocidin-I is the first described antimicrobial peptide derived from α_{s2}-casein (Zucht *et al*., 1995). It is a 39 amino-acid fragment, corresponding to residues 150–188. It was obtained from boiled and acidified milk and purified by cation exchange chromatography combined with three different RP-HPLC gradients. Chemically synthesized casocidin-I displayed antibacterial effect against *E. coli*, *S. carnosus* and the latter was the most sensitive to the action of the peptide (Bradshaw, 2003). In addition, α_{s2}-casein f (164–179) and α_{s2}-casein f (183–207) showed an important antibacterial activity against Gram positive and Gram-negative bacteria with MIC values ranging from 25 to 100mM in the case of f (164–179), and from 8 to 16mM in f (183–207) (Bargeman *et al*., 2002; Recio & Visser, 1999). α_{s2}-casein f (181–207), f (175–207) and f (164–207) are also several peptides with activity against a wide variety of Gram-positive and Gram-negative bacteria with MIC ranging from 21–168mg/mL for f (181–207), 10.7–171.2mg/mL for f (175–207) and 4.8–76.2mg/mL for f (164–207). It should be considered that depending on the target bacterial strains, inhibitory concentrations of peptides vary. These peptides derived from α_{s2}-casein were identified from a chymosin digest of sodium caseinate (McCann *et al*., 2006). Chantaysakorn and Richter (2000) believe that presence of metal cations in certain foods reduces the antibacterial activity of these cationic antibacterial peptides, which could limit their application as food preservatives.

The search for antibacterial activity from α_{s2}-casein has been extended to milk from other species. Four antibacterial peptides could be identified from a pepsin hydrolysate of ovine α_{s2}-casein (Lopez-Exposito *et al.*, 2006). The peptides corresponded to sequences α_{s2}-casein f (165–170), f (165–181), f (184–208) and f (203–208), and fragments f (165–181) and f (184–208) were homologous to those previously identified in the bovine protein. Peptides from ovine α_{s2}-casein showed less potent antibacterial activity than those of bovine origin against Gram-negative bacteria (Recio *et al*., 2005).

Kappacin is another example of an antimicrobial peptide derived from k-casein. Kappacin corresponds to the non-glycosylated, phosphorylated form of caseino-macropeptide (CMP). k-casein A (138–158) was the active form with antimicrobial activity against *Str. mutans*, *E. coli* and *Porphyromonas gingivalis*. It is important to emphasize that the active form is the phosphorylated and non-glycosylated form, since it has been demonstrated that non-phosphorylated and glycosylated forms do not reveal any activity against *Str. Mutans* (Malkoski *et al*., 2001).

- *Bioactive peptide derived from whey*

Lactoferricin (Lfcin) is perhaps the most well-known multifunctional peptide derived from lactoferrin. This peptide is generated by pepsin digestion of lactoferrin (Lopez-Exposito & Recio 2008; Vogel *et al*., 2002). Lactoferricin B was isolated from the N-terminal region of bovine lactoferrin (Bellamy *et al*., 1992). Lactoferricin B consists of 25 amino acid residues having the sequence Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe (Bellamy *et al*., 1993).

Lfcin elicits a more potent bactericidal effect than the parent molecule that may be due in part to its smaller size, which facilitates access to the target site on the microbial surface (Lopez-Exposito & Recio 2008; Vogel *et al*., 2002). Several in vivo

studies have been undertaken to examine the effects of lactoferricin. It has been reported to have protective effects against *Staphylococcus aureus* and infections caused by *Toxoplasma gondii* (Isamida *et al*., 1998; Recio & Visser 1999b). The *in vivo* properties of lactoferricin are controversial, as it has been shown that the addition of five percent cow's milk or increasing the concentration of mucin reduced the antimicrobial effects (Rutherfurd-Markwick and Moughan, 2005). Lactoferrampin (LFampin), is another peptide corresponding to this domain of LF, LF f (268–284) that has been chemically synthesised and has demonstrated candidacidal activity and antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (van der Kraan *et al*., 2004).

Proteolytic digestion of α-lactalbumin by pepsin, trypsin and chymotrypsin yielded three polypeptide fragments with bactericidal properties. Two fragments were obtained from the tryptic digestion. One was a pentapeptide with the sequence EQLTK (residues 1-5) and the other, GYGGVSLPEWVCTTF ALCSEK (residues (17-31) S-S (109-114)), was composed of two polypeptide chains held together by a disulfide bridge. Fragmentation of α-lactalbumin by chymotrypsin yielded CKDDQNPH ISCDKF (residues (61-68) S-S (75-80)), also a polypeptide composed of two polypeptide chains held together by a disulfide bridge. All of polypeptides were found to exert antimicrobial activities. The polypeptides were mostly active against Gram-positive bacteria. Gram-negative bacteria were only poorly susceptible to the bactericidal action of the polypeptides (Pellegrini *et al*., 1999).

VAGTWY (residues 15-20), AASDISLLDAQSAPLR (residues 25-40), IPAVFK (residues 78-83) and VLVLDTDYK (residues 92-100), four peptide fragments with bactericidal activity have been produced as the result of bovine β-lactoglobulin digestion by trypsin. The four peptides were synthesized and found to exert bactericidal effects against the Gram-positive bacteria only. In order to understand the structural requirements for antibacterial activity, the amino acid sequence of the peptide VLVLDTDYK was modified. The replacement of the Asp (98) residue by Arg and the addition of a Lys residue at the C-terminus yielded the peptide VLVLDTRYKK which enlarged the bactericidal activity spectrum to the Gram-negative bacteria *Escherichia coli* and *Bordetella bronchiseptica* and significantly reduced the antibacterial capacity of the peptide toward *Bacillus subtilis* (Pellegrini *et al*., 2001).

- *Antimicrobial mechanism*

The mode of action of antimicrobial peptides has been extensively investigated and it has been shown that an amphiphilic, mostly α-helical formation, and an overall net positive charge is proposed to initiate the interaction with the bacterial surface to enter the membrane (Floris *et al*., 2003). Cationic peptides are thought to inhibit Gram-negative bacteria through a variety of mechanisms including interacting with the lipopolysaccharides and electrostatic interactions with the negatively charged lipid head groups in the membrane leading to leakage of essential nutrients (Pritchard & Kailasapathy, 2011). Lactoferricin, one of the multifunctional peptides shows antimicrobial, antifungal, antitumor, and antiviral properties due to tryptophan/arginine rich proportion of the peptide, and anti-inflammatory and immune-modulating properties because of its positively charged region of the molecule (Vogel *et al*., 2002). It is recognized that the antibacterial activity of LFcin starts with the electrostatic interaction with the negatively charged membranes of bacteria (Bellamy *et al.,* 1993). In this initial binding, lipopolysaccharide (LPS) and teichoic acid

have been identified as binding sites in Gram negative and Gram-positive bacteria, respectively (Vorland, 1999). It has been demonstrated that once the peptide reaches the cytoplasm the bacterial protein synthesis is inhibited (Ulvatne *et al*., 2004).

- *Isolation method*

There are several ways that bioactive peptides can be released from milk proteins, by enzymatic hydrolysis with digestive enzymes such as trypsin and chymotrypsin (*in vivo)*, during digestion by microbial enzymes(*in vivo)*, endogenous enzymes present in milk (*in vitro),*proteolysis with enzymes derived from microorganisms or plants (*in vitro)* and during food processing or ripening by proteolytic starter cultures or enzymes isolated from proteolytic microorganisms(*in vitro)* (Yamamoto *et al*., 1999; Hebert *et al*., 2008). Commercial production of bioactive peptides from milk proteins has been limited by the lack of suitable large-scale technologies. Until now, membrane separation techniques have provided the best technology available for the enrichment of peptides with a specific molecular weight range (Korhonen & Pihlanto, 2003).

Conclusion

Bioactive peptides have attracted increasing interest as the prominent candidates for various health-promoting functional foods. These can be incorporated in the form of ingredients in the functional and novel foods, dietary supplements and even pharmaceuticals with the purpose of delivering specific health benefits. presently, milk proteins are the best known source of such ingredients but until recently the commercial production of milk derived bioactive peptides has been limited by the lack of suitable large-scale technologies. Nevertheless, it seems bioactive peptides will have special place in food industry as harmless preservatives in the near future.

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