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Milk Proteins-derived antibacterial peptides as novel functional food ingredients

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ABSTRACT

Milk protein can be regarded not only for its nutritive value but also as a possible resource to increase the natural defence of the organism against invading pathogens. The antimicrobial activity of milk is mainly attributed to immunoglobulins, and to non-immune proteins, lactoferrin, lactoperoxidase and lysozyme. In addition to the naturally occurring antimicrobial proteins present in milk, there are also a variety of antibacterial peptides encrypted within the sequence of milk proteins that are released upon suitable hydrolysis of the precursor protein. Antibacterial peptides have been derived from milk proteins, lactoferrin, α_{s1} -casein, α_{s2} -casein, κ -casein, α -lactalbumin, β -lactoglobulin, and lysozyme. These peptides have been found to be active against a broad range of pathogenic organisms. With the rise of consumer concerns about the deleterious effects of chemical preservatives and the increasing preference for natural components, bioactive peptides have the potential to be used in the formulation of health enhancing nutraceuticals for food preparations. Addition of these peptides to food products could improve consumer safety as a result of their antimicrobial properties.

This article reviews the antibacterial activities of milk proteins-derived peptides as milk – based nutraceuticals and briefly discusses the effect of these peptides on pathogens in the light of recent studies.

Keywords: bioactive, functional foods, antibacterial peptides.

INTRODUCTION

Antimicrobial activity of milk is mainly attributed to immunoglobulins, and non-immune proteins, such as lactoferrin, lactoperoxidase and lysozyme. One of the most potent antimicrobial peptides corresponds to a fragment of lactoferrin, named lactoferricin. More recently, other whey proteins such as α -Lactalbumin and β -lactoglobulin have also been considered as potential precursors of bactericidal fragments. Similarly, antibacterial fragments have also been derived from α_{s1} -, α_{s2} - β - and κ -casein. These peptides have been found to be active against a broad range of pathogenic organisms (e.g. Escherichia, Helicobacter, Listeria, Salmonella and Staphylococcus), yeast and filamentous fungi [7].

The bioactivities of peptides encrypted in major milk proteins are latent until released and activated by enzymatic proteolysis, e.g. during gastrointestinal digestion or food processing [15].

Despite the chemical and structural heterogeneity within the group of antimicrobial peptides, a few common features can be distinguished. Most, if not all, antimicrobial peptides contain a positively charged domain and are able to adopt an amphipathic conformation allowing their presence at hydrophilic / hydrophobic interfaces. These properties

are supposed to be essential for antimicrobial activity, the amphipathic character causes disruption of the negatively charged microbial membrane and cell death [25].

This article reviews the antibacterial activities of milk proteins-derived peptides as milk-based nutraceuticals and briefly discusses the effect of these peptides on pathogens in the light of recent studies.

2- Whey-protein-derived antibacterial peptides

2-1 Lactoferrin-derived antibacterial peptides

2-1-1 Cationic peptides

Typically, antimicrobial peptides are relatively short (less than 100 amino acids), positively charged, and amphiphilic. Several mechanisms have been proposed for cationic antimicrobial peptides. Hydrophobicity, cationicity and secondary structure have been implicated in the antimicrobial effect. The mode of action of cationic peptides against Gram-negative bacteria has been unraveled. The outer membrane (OM) of Gram-negative bacteria constitutes a semi-permeable barrier against a variety of substances. The enterobacterial OM bilayer consists of an inner monolayer containing phospholipids and an outer monolayer that is mainly lipopolysaccharide (LPS). The susceptibility of Gram negative bacteria to cationic peptides has been proposed to be associated with factors that facilitate the transport of the peptides across the outer membrane. It has been suggested that the disruption of the outer membrane structure is most likely not the primary factor leading to cell death. The peptides interact with and cross both the outer and cytoplasmic membranes, and cause cell death by a multihit mechanism that involves action on more than one ionic target [5].

2-1-2 Lactoferricins

The fragments from human and bovine lactoferrins were characterized and named human (H) and bovine (B) lactoferricins. Both peptides are derived from the N-terminal region of the N-lobe and have greater antibacterial activity than their parent proteins. Lactoferricin-H corresponds to amino acid residues 1-47 from the N-terminal region of the protein and includes an 18-residue loop formed by an internal disulphide bridge. Residues 1-11 constitute a separate fragment, which remains bound to the main loop by a disulfide bridge [5]. Antibacterial activity of lactoferricins towards enterotoxigenic *Escherichia coli* and *Listeria monocytogenes* is reported [15]. The antimicrobial properties of lactoferricins can be related to tryptophan/arginine-rich proportion of the peptide [11].

However, there remains a potential pharmacological interest in the therapeutic use of lactoferricin produced on an industrial scale [1].

2-1-3 Lactoferrampin

An antimicrobial cationic domain identified in the N1-domain of lactoferrin in close proximity to lactoferricin was designated lactoferrampin. It includes amino acids 268-284 of bovine lactoferrin. This novel peptide exhibited candidacidal activity, which was substantially higher than the activity of lactoferrin. Furthermore, lactoferrampin was active against *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, but not against the fermenting bacteria, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Streptococcus mutans* and *Streptococcus sanguis*. Lactoferrampin plays a crucial role in membrane-mediated activities of lactoferrin [25].

2-2 α – lactalbumin-derived antibacterial peptides

Pellegrini *et al.* [16] showed that α – lactalbumin yielded bactericidal peptides after digestion with trypsin and chymotrypsin, but not with pepsin. The bactericidal activity was restricted to Gram-positive bacteria.

2-3 β – lactoglobulin- derived antibacterial peptides

The first time, Pellegrini *et al.* [17] showed that bactericidal domains (f15-20, f25-40, f78-83, f92-100) are present in the β – lactoglobulin molecule and suggested a possible biological function of this protein after its partial digestion by endopeptidases of the pancreas. The bactericidal activity of the β – lactoglobulin fragments was restricted to Gram-positive bacteria. In fact, most of the bactericidal peptides derived from this protein were negatively charged. This character explains why they were only weakly effective against Gram negative bacteria whose membranes contain lipopolysaccharide, a negatively charged molecule. However, the cationic peptide β – CN (f78-83) was only effective against Gram-positive bacteria, and also displayed an hydrophobic character.

2-4 Proteoseptone- 3 (PP3)- derived antibacterial peptides

PP3 is a component present in the whey fraction and has an unknown function. It is a 135 amino-acid glycoprotein and does not derived from the enzymatic hydrolysis of casein. The sequence of PP3 clearly indicates that the protein

contains at least two distinct domains . The first one covers the N-terminal part (residues 1-97) and is largely negatively charged. The second domain from residue 98 to the C-terminal residue 135 is positively charged , displayed an amphipathic character adopting an α – helical structure and exhibited the ability to permeabilize planar lipid bilayers . This domain , called lactophorin , was chemically synthesized and displayed bacteriostatic activity against Gram-positive and Gram-negative bacteria, except for *Streptococcus thermophilus* for which it demonstrated bactericidal activity [11]. This peptide displayed a moderate inhibitory-growth activity but the low minimal inhibitory concentrations (MIC $10\ \mu\text{M}$) and the minimal lethal concentrations (MLC $20\ \mu\text{M}$) observed in *Streptococcus thermophilus* strain look promising for further trials against untested pathogens[24]. Lactophorin would be noncytotoxic when used in concentrations between 2 to $200\ \mu\text{M}$ [2].

3- Caseins –derived antibacterial peptides

3-1 κ -casein –derived antibacterial peptides

3-1-1 Kappacin

Kappacin is an antimicrobial peptide derived from κ -casein. Kappacin corresponds to nonglycosylated and phosphorylated form of caseinomacropeptide. The release of kappacin in the stomach could be a mechanism to limit gastrointestinal tract infection in the developing neonate, by increasing the sensitivity of bacteria to gastric acid by collapsing essential transmembrane cation gradients [11].

3-1-2 Caseinomacropeptide

Many pathogens and enterotoxins adhere to cells by recognizing carbohydrate receptors. Caseinomacropeptide (CMP) interacts with toxins, viruses and bacteria, exerting health promoting activities that are strongly mediated by the carbohydrate fraction. Glycosylated CMP inhibits the binding of cholera toxins to their oligosaccharide receptors on cell walls and protects cells from infection by influenza virus . CMP also inhibits the adhesion of cariogenic bacteria such as *Streptococcus mutans*, *S.sanguis* and *S.sobrinus* to the oral cavity and modulates the composition of the dental plaque microbiota . This could help to control acid formation in the dental plaque , in turn reducing hydroxyapatite dissolution from tooth enamel and promoting remineralisation. For this, it has applied for oral care products to prevent dental caries [23].

The human glycomacropeptide was also known to exhibit antimicrobial effects by its sugar composition . It inhibits *Helicobacter pylori* infection by interfering the adhesion of these bacteria to their target cells. In addition , adhesion inhibitory properties of the glycomacropeptide towards *Haemophilus influenzae* and *Streptococcus pneumoniae* were reported [10].

CMP inhibits the growth of the oral pathogens *Streptococcus mutans* and *Porphyromonas gingivalis* as well as *Escherichia coli* , and the active form identified was the nonglycosylated Ser (P)¹⁴⁹ κ -casein (f106-169) , designated as kappacin . These findings could help to protect against dental caries [24].

3-1-2-1 Caseinomacropeptide (CMP)- derived peptides

The monophosphorylated sequence ser (P)₁₄₉ κ -casein – A, f(138-158) , has antibacterial properties against *S.mutans* , *Porphyromonas gingivalis* and *E.coli*. It has generated by digestion of CMP [23].

3-1-3 κ -casecidin

The antimicrobial role of κ -casein also involves κ -casecidin, f17-21. This peptide was prepared using trypsin digestion of bovine κ -casein. It has antibacterial activity against *Listeria innocua* , *Salmonella carnosus* . However , κ -casecidin was found to display cytotoxic activity towards some mammalian cells , including human leukemic cells lines, probably due to apoptosis [24].

3-1-4 Other κ -casein-derived antibacterial peptides

Other antimicrobial peptides derived from κ -casein are f(18-24) , f(139-146) and f(30-32) . These showed antibacterial activity against *Listeria innocua* , *Sal.carnosus* and *E.coli* [11].

The human casein- κ (63-117) is a proline –rich cationic peptide , and likely it belongs to the large group of polycationic antimicrobial peptides. The spectrum of chemically synthesized f(63-117) includes inhibition of the growth of several Gram-positive and Gram-negative bacteria and yeasts[10].

3-2 α_{s1} – casein –derived antibacterial peptides

3-2-1 Caseicin

A study showed that *Lactobacillus acidophilus* DPC6026 offers interesting perspectives for the generation of multiple antimicrobial peptides (Caseicin A, B and C) from α_{s1} – casein , against pathogen or undesirable bacteria. These peptides have common features with other reported antibacterial peptides, given by a high degree of homology with isracidin for instance . Caseicin A and B were able to inhibit *Escherichia coli* O157:H7 and *Enterobacter sakasaki*, while Caseicin C displayed only minor activity against *Listeria innocua* [24].

3-2-2 Caseicidin

Caseicidin was obtained by chymosin –mediated digestion of casein α_{s1} – at neutral pH. It was among the first defense peptides actually purified. This peptide exhibited antibacterial activity against *Staphylococcus* spp., *Sarcina* spp., *Bacillus subtilis* , *Diplococcus pneumoniae* and *Streptococcus pyogenes* [3,9,22]. Inhibition occurred at high concentrations , in vitro , compared with commercial antibiotics [9].

3-2-3 A novel fragment from α_{s1} – casein

McCann *et al.* [13] isolated an antibacterial cationic peptide , which corresponded to residues 99-109 of bovine α_{s1} –casein. It derived from a pepsin digest of bovine sodium caseinate. This peptide exhibited minimum inhibitory concentration (MIC) of 125 $\mu\text{g ml}^{-1}$ against the Gram-positive bacteria (*Bacillus subtilis*, *Listeria innocua*, *Listeria monocytogenes* FSAW 2310, *Listeria monocytogenes* NCTC 11994) and MIC ranging between 125 and > 1000 $\mu\text{g ml}^{-1}$ against the Gram negative bacteria (*Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium*).

3-2-4 Isracidin

The N-terminal segment of bovine α_{s1} -casein , viz. f1-23, was known as isracidin. It has demonstrated antibiotic type activity in vivo versus *Staphylococcus aureus* and *Candida albicans*. This peptide can protect sheep and cows against mastitis [3,9,22].

3-3 casein- α_{s2} – derived antibacterial peptides

3-3-1 Casocidin-I

Zucht *et al.* [26] reported the isolation and characterization of an antibacterial peptide, known as casocidin-I, from bovine milk. It was inhibited the growth of *Escherichia coli* , and *Staphylococcus carnosus* . The peptide includes amino acids 165-203 of bovine casein- α_{s2} . Both terminal amino acids of the peptide represent potential tryptic cleavage sites. Casein - α_{s2} is not present in human milk and so they proposed that casocidin – I or related peptides of bovine milk influence the human intestinal flora , particularly of the suckling .

3-3-2 other peptides

Ricio and Visser [21] isolated two cationic domains f(183-207) and f(164-179) with antibacterial activity from a peptic hydrolysate of bovine α_{s2} -casein . Both fragments showed antibacterial activity against Gram-positive (*L. innocua* , *B. cereus* P7 , *M. flavus* DSM 1790 , and *St. thermophilus* Rs) and Gram-negative (*E. coli* ATCC 25922 and *E. coli* MC 1061) bacteria.

New antibacterial peptides have identified from a chymosin digest of bovine sodium caseinate , all of them originated from the C-terminal of bovine α_{s2} -casein . Four antibacterial peptides corresponding to amino acid residues 180-207 , 175-207 , 164-207 and 172-207 of bovine α_{s2} -casein were isolated. The minimal inhibitory concentration of peptides f(175-207) and f(172-207) was determined against a range of Gram-positive and Gram-negative bacteria and showed similar activities to that of the bacteriocin nisin and the antibacterial peptide lactoferricin B against certain Gram-positive bacteria [11].

Lopez-Exposito and Ricio [11] demonstrated the presence of four cationic antibacterial peptides derived from the ovine α_{s2} - casein molecule f(165-170), f(165-181), f(184-208), and f(203-208). Gram-positive microorganisms were more susceptible to the action of these peptides than Gram-negative bacteria.

McCann *et al.* (2005) isolated five antibacterial peptides Cr1, Cr3, Cr4, Cr5 and Cr7, using chymosin digest of sodium caseinate. These peptides corresponded to amino acid residues 181-207, 180-207, 175-207, 164-207 and 172-207 of bovine α_{s2} -casein, respectively. It is shown that Cr1, Cr4 and Cr5 have similar activities to those of nisin and lactoferricin B against certain Gram-positive bacteria.

McCann *et al.* [13] isolated an antibacterial cationic peptide, which corresponded to residues 183-207 of bovine α_{s2} -casein. It derived from a pepsin digest of bovine sodium caseinate. This peptide was generally far more potent against all Gram-positive bacteria tested, exhibiting a MIC of $21 \mu\text{g ml}^{-1}$, compared to MIC ranging from 332 to $> 664 \mu\text{g ml}^{-1}$ against most of the Gram-negative bacteria tested.

3-4 β – Casein – derived antibacterial peptides

Lopez-Exposito & Recio [11] reported in their review article that rabbit β – casein f(64-77) with anionic character has antibacterial activity against Gram-positive bacteria. Also, human β – casein f(184-210) showed a large inhibition spectrum against Gram-positive and Gram-negative bacteria. A peptide derived from ovine β – casein showed inhibition of bioluminescent production by *E. coli* JM103.

Fragments of human β – casein have also a protective effect against *Klebsiella pneumoniae* in mice. The immunomodulatory peptide derived from bovine β – casein, viz. f193-209, was shown to enhance the antimicrobial activity of mouse macrophages [22].

4- Lysozyme-derived antibacterial peptides

It is demonstrated milk lysozyme has antibacterial activity against Gram-positive and some Gram-negative bacteria that are completely resistant to egg white lysozyme. Amino acid residues 87-114 of chicken lysozyme and 87-115 of human-milk lysozyme were potentially bactericidal against Gram-positive and Gram-negative bacteria. Both lysozyme fragments can be used for the design of novel antimicrobial peptides [11].

5- Future perspectives for antibacterial peptides

With the great concerns about antibiotic – resistant bacterial threatening on both animal and human health, an increasing need to develop effective but human and environment compatible antibiotic alternatives for feed and medical industries has been arisen. More attentions have been paid to the research of antimicrobial peptides derived from animals and plants due to their advantage of possessing more effective activity as well as exhibiting unique mechanism of killing bacteria compared with current antibiotics [6].

Antimicrobial peptides generated from food proteins present the great advantage to be derived from harmless substances, therefore one can expect their safety for use in medicine and food industry [18].

The successful strategy, by enzymatic cleavage, may initiate future studies on clarifying a possible common antibacterial mechanism of food proteins that are frequently found *in vivo* in a proteinase-rich environment. Antibacterial domains of such proteins may have a potential use in therapy, functional foods, or infant formulas [20].

So, bioactive peptide preparations have the potential to be used in the formulation of functional foods, cosmetics and as potent drugs having well defined pharmacological effects. With the rise of consumer concerns about the deleterious effects of chemical preservatives and the increasing preference for natural components, milk derived bioactive substances may have value in food preservation and nutraceuticals

[7].

Application of enrichment protocols such as membrane processing and chromatographic isolation may also be an area of future interest in the extraction of potent biofunctional peptides from fermented dairy products and their subsequent utilization as functional food ingredients [7,14]. Hence, these peptides are claimed to be health enhancing nutraceuticals for food and pharmaceutical preparations [14].

The role of lactoferrampin in the activity of bovine lactoferrin can be expected throughout the body, with the only exception of sites where pepsin activity may have destroyed the specific domain. For oral or local application, bovine lactoferrin or lactoferrampin could well be used as a therapeutic agent. When desired, cleavage in the stomach could be avoided by using a delivery system in which the peptide is protected in a capsule [25].

The applications of peptides derived from lactoferrin or lactoferrin hydrolysates include clinical areas because of their immunomodulatory effect or for the chemoprevention of carcinogenesis. The use of lactoferrin derivatives in oral care products has also been proposed and several studies have examined the effect of lactoferricin on food preservation. However, it is not very effective as food preservative although some reports have described selective reduction of pathogenic bacteria [11].

Rennet digest of lactoferrin was shown to have potential for use as a natural and antimicrobial food preservative against both Gram-negative and Gram-positive bacteria. Natural preservatives, like nisin and lysozyme, are known to be active against Gram-positive bacteria only. Also, rennet lactoferrin hydrolysate would be applicable as a substitute for porcine pepsin digest, especially in Islamic communities to fulfill the specific consumer's demand [4].

Lactoferricin B is one of the most potential substitutes for antibiotics. Feng et al. [6] demonstrated successful expression of Lactoferricin B in *E. coli* provides a possible method to produce lactoferricin B in large amounts. Recombinant Lfcin B has been obtained and proved to be functional in that study. Since lactoferricin B possesses the advantage of a broad antibacterial spectrum without inducing the resistance against antibiotics, recombinant lactoferricin is promising for being used as an alternative of widely used antibiotics currently [6].

The modification performed on the bactericidal domain of β -lactoglobulin (f92-100) suggested a potential therapeutical use of the modified β -lactoglobulin as functional food or infant formula. In this case, the modified β -lactoglobulin molecule would not only deliver nutrition to the organism but it would also provide, after its gastrointestinal digestion, protection against microbial infection. Several drugs cause problems when taken orally. A protein molecule such as the HBSO₅₅₋₆₁ – modified β -lactoglobulin, which could carry the microbicidal peptide through the gastrointestinal tract and release it after natural digestion of the carrier protein in blood, would represent a great advantage [17].

Although potent antibiotics are available for the treatment of microbial infections, antimicrobial peptides derived from casein show advantages of being able to kill target cells rapidly, while having a broad spectrum of activity. In addition, as the rate of killing by peptides is higher than the rate of bacterial multiplication, the problem of drug resistance may be overcome [19].

Some researchers have demonstrated bactericidal activity of cationic peptides in minimal media, but have observed a substantial loss of activity when the peptides were added to foods including milk and carrot juice. Further studies identified the presence of metal cations as the major reason for the reduced activity of cationic antibacterial peptides in certain foods. It has been proposed that the metal cations stabilize negatively charged target sites such as the lipopolysaccharides of the outer membrane of Gram-negative bacteria thereby reducing the affinity of the peptides for these sites. The successful application of cationic antibacterial peptides as antimicrobials in biological systems where metal cations are likely to interfere will require the development of delivery strategies and approaches to minimize the adverse effects of metal cations on their biological activity [12].

The potential health benefits of milk protein –derived peptides have been a subject of growing commercial interest in the context of health – promoting functional foods. Bioactive peptides can be incorporated in the form of ingredients in functional and novel foods, dietary supplements and even pharmaceuticals with the purpose of delivering specific health benefits. Such tailored dietary formulations are currently being developed worldwide to optimize health through nutrition [8].

The current research into delivery systems and the developments in nanoencapsulation and nanoemulsion technology may also ultimately lead to application of specific formulations for dairy bioactive peptides that have now been identified [13].

CONCLUSION

Milk proteins are not only essential to supply nutrition and specific immunological protection to the neonate but that they also provide bioactive peptides which defended the organism against infectious agents. These antibacterial peptides have been derived from lactoferrin, α_{s1} –, α_{s2} –, β –, and κ -casein, α -Lactalbumin, β -lactoglobulin, proteoseptone-3 and lysozyme. Antibacterial domains of milk proteins may have a potential use in therapy, functional foods, or infant formulas.

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