Animal Science Papers and Reports vol. 29 (2011) no. 4, 269-280
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Bovine milk proteins as the source of bioactive peptides influencing the consumers' immune system – a review

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(Received January 21, 2011; accepted September1, 2011)

The present review describes selected peptides derived from bovine milk proteins and demonstrates their effect on the human immune system. Apart from their obvious nutritive value milk proteins and products of their degradation (peptides) have multiple biological functions. Bovine milk, fermented milk drinks and cheeses are the most abundant sources of biologically active peptides. One of the primary functions of milk is to protect the health of a newborn organism by the virtue of the fact that milk contains many proteins, which exhibit bacteriostatic and bactericidal properties in their intact form. Ingestion of bovine milk by humans causes that bioactive peptides are evoked from delivered proteins during the course of digestion. They possess not only immunomodulatory, but also antibacterial, antiviral and antifungal properties.

KEY WORDS: antimicrobial activity / cattle / immunomodulation / milk / peptides / protein

Proteins are essential and integral components of food as a source of energy and exogenous amino acids necessary for growth of a living organism. Bovine milk is an excellent source of proteins of high biological value. It is characterized by well-balanced amino acid profile and exceptionally high digestibility. Apart from their obvious nutritive value, milk proteins and products of their degradation (peptides)

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exert a wide range of biological functions [Barłowska and Litwińczuk 2008, Król *et al.* 2008, Litwińczuk *et al.* 2011, Król *et al*. 2011].

Biologically active peptides derived from milk proteins are defined as specific protein fragments (3-20 amino acid residues), which have a positive impact on the physiological functions of the body ultimately affecting the health of living organism [Kitts and Weiler 2003, Möller *et al.* 2008]. Functional properties of such peptides are revealed only after degradation of the native protein structure. This degradation may be a consequence of enzymatic hydrolysis, fermentation of milk conducted by the starter cultures of proteolytic bacteria and other processes used in dairy production [Michalidou 2008]. Native sequences of milk proteins may contain fragments that exert various activities, such as antihypertensive, antithrombotic, opioid, opioid antagonist, immunomodulatory, antibacterial, antifungal, antiviral, antioxidant, binding and transporting metals, preventing amnesia and causing smooth muscle contractions [Seppo *et al.* 2003, Haug *et al.* 2007a, Ebringer *et al.* 2008, Atanasova and Ivanova 2010]. In addition, such peptides show a much lower allergenicity than the protein from which they were formed. It is related to their lower molecular weight [Host and Halken 2004].

The aim of the present paper was to present selected peptides derived from bovine milk proteins and their influence on the human immune system.

Obtaining bioactive peptides from bovine milk proteins

General scheme of obtaining bioactive peptides from bovine milk proteins is shown on Figure 1. Enzymatic hydrolysis is one of the most common processes, which result in formation of bioactive peptides [Korhonen and Pihlanto 2006]. The release of bioactive peptides from milk proteins in the gastrointestinal tract results from the action of digestive enzymes such as pepsin and pancreatic enzymes (trypsin, chymotrypsin, carboxy- and aminopeptidases). The efficiency of physiological activity of biopeptides depends on their ability to maintain integral state during transport to the various functional systems of the body [Vermeirssen *et al.* 2004, Picariello *et al.* 2010]. Many well-known bioactive peptides have been generated *in vitro* by the action of digestive enzymes, mostly pepsin and chymotrypsin. Other digestive enzymes and combinations of proteinases (alcalase, chymotrypsin, pancreatin, pepsin, thermolysin) as well as enzymes derived from bacteria and fungi are also used for the production of bioactive peptides from various sources.

The process of fermentation carried out by microorganisms has been known and used in dairy production for many thousands of years in order to extend the consuming period of milk [Hayes *et al.* 2007]. Achievements of food science and technology of food production have allowed exploring the broad spectrum of opportunities created by the lactic fermentation, *i.e.* formation of bioactive peptides. Numerous bacterial strains used in dairy production exert proteolytic activity, which eventually leads to the release of bioactive fragments from the native protein structures. Peptides with

different chain lengths are released during the action of bacterial proteases and peptidases (secondary proteolysis) – Sieber *et al.* [2010]. Type of bacterial culture used is the most important factor determining the nature of the synthesized bioactive peptides. For example, bacterial strain *Lactobacillus helveticus* was the subject of many promising experiments in anti-cancer research. Some authors suggest that milk fermented by these bacteria exerts numerous actions causing the growth reduction of tumor cells. Moreover, peptides produced by *Lactobacillus helveticus* fermentation support the production of antibodies also against the O157:H7 *Escherichia coli* strain [LeBlanc *et al.* 2002, 2004, Rachid *et al.* 2006]. Another example is fermentation with the use of *Lactobacillus lacti*s, which leads to the release of oligopeptides containing the amino acid sequences characteristic of various biologically active peptides such as casomorphines, lactorphines, casokinines and immunopeptides [Ebringer *et al.* 2008].

Peptides with diverse biological activities have been identified in many dairy products, not only in milk protein hydrolysates and fermented milk, but also in cheeses [Gobetti *et al.* 2000, Walther *et al.* 2008] that are among the most important dairy products in the world. Variety of enzymatic processes occurs during cheese ripening.

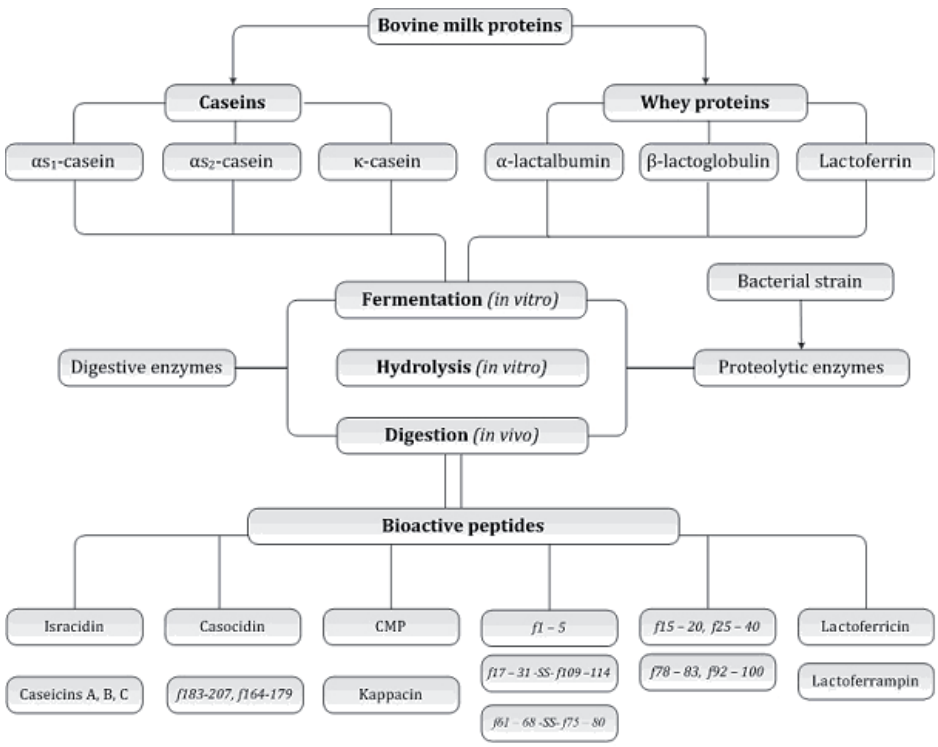

Fig. 1. Scheme of obtaining bioactive peptides from bovine milk proteins. Source: own elaboration based on Short and O'Brien, 2004.

They involve action of chymosin and participation of specialized bacterial strains which contribute not only to the formation of the characteristic sensoric properties of cheese. They also induce the release of numerous antibacterial peptides derived mainly from caseins [Rizello *et al*. 2005, Losito *et al*. 2006, Pritchard *et al*. 2010]. For example, the Emmentaler cheese is abundant in antibacterial and immunomodulatory peptides derived from α- and β-casein [Gagnaire *et al*. 2001] – Figure 1.

The influence of peptides released from bovine milk proteins on the immune system

One of the primary functions of milk is to protect the health of a newborn mammal. Milk contains many peptides and proteins, which exhibit bacteriostatic and bactericidal properties in their intact form [Bagnicka *et al*. 2010]. These properties of proteins [Litwińczuk *et al*. 2011] are primarily associated with whey proteins such as immunoglobulins, lactoferrin, lactoperoxidase and lysozyme [Atanasova and Ivanova 2010]. Ingestion of bovine milk by humans causes that bioactive peptides are evoked from consumed proteins throughout the course of digestion. They possess not only immunomodulatory, but also antibacterial, antiviral and antifungal properties. The disintegration of bacterial cells by the antibacterial peptides involves the destruction of the cell membrane or mitochondrial membrane. The mechanism of action is related to the binding affinity depending on the electrostatic interaction of peptide with the surface of the cell membrane [Brodgen 2005]. Bechinger [1997] and Hancock and Lehrer [1998] claim that antibacterial peptides derived from various sources are able even to destroy antibiotic-resistant bacteria developing in hospitals. It is related to the fact that the rate of destroying these microorganisms is greater than the rate of their reproduction. The immunomodulatory action of biopeptides is related to the stimulation of proliferation of human lymphocytes and macrophages phagocytic activity [Clare *et al.* 2003]. Furthermore, many cytochemical studies indicate that peptides can induce apoptosis of cancer cells [López-Expósito and Recio 2008].

Numerous studies focus on multipotential activity of lactoferricin (Lfcin) that is a product of hydrolytic degradation of lactoferrin (LF). Lactoferrin itself exhibits a strong bactericidal activity through its ability to bind iron. Lactoferricin consists of 25 amino acid residues from the N-terminal region of lactoferrin and is released by pepsin digestion of LF under acidic pH [Haug *et al.* 2007b, Oo *et al.* 2010]. This peptide shows a considerably higher antimicrobial activity than the native protein. Antibacterial spectrum of Lfcin is broad, while its minimum concentration necessary to inhibit growth of microbes is low (0.5 mg/ml). Moreover, Lfcin is heat-resistant and exerts its activity over a wide range of pH. It is well established that the capacity of the peptide to bind to the surface of Gram-negative bacteria results in the release of lipopolysaccharide (LPS) from the bacterial cell wall, which causes damage to cell walls and other morphological changes [Bellamy *et al.* 1992, Appelmelk *et al.* 1994; Nibbering *et al.* 2001, Tomita *et al.* 2001, Małaczewska and Rotkiewicz 2007].

Furthermore, Lfcin has been found effective in the treatment of some cancer varieties, such as leukemia and neuroblastoma [Gifford *et al.* 2005].

Lactoferrampin (Lfampin) is another peptide derived from lactoferrin. It has a wide spectrum of antifungal and antibacterial properties. The peptide exerted antifungal (against *Candida*) activity higher than LF and was also active against *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* [van der Kraan *et al.* 2004, 2005]. Lactoferrin and its derivatives show the antibacterial activity *in vitro* against various pathogens, e.g. *Clostridium perfringens, Candida albicans, Haemophilus influenzae, Helicobacter pylori, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella enteriditis, Staphylococcus aureus, Streptoccccus mutans, Vibrio cholerae* as well as antiviral activity against hepatitis C, G, and B virus; HIV-1; cytomegalovirus; poliovirus; rotavirus; and herpes simplex virus [Farnaud and Evans 2003, Pan *et al*. 2007]. It has been suggested that lactoferrin and its derivatives affect the production of cytokines involved in immune reactions of the organism [Möller *et al.* 2008].

LF-derived peptides are proposed for clinical applications due to their chemopreventive and immunomodulatory properties. Moreover, it is also alluded to use them as food preservatives [Haque and Chand 2008, Król *et al*. 2011].

The whey protein fraction of bovine milk consists mainly of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). Biologically active antibacterial peptides are released during the digestion of β-lactoglobulin with trypsin. Such peptides demonstrate activity against food pathogens (*Staphylococcus aureus, Listeria monocytogenes, Salmonella spp. and Escherichia coli* O157) – Pellegrini *et al.* [2003]. Earlier Pellegrini *et al.* [2001] explained that action of peptides depends on their electrostatic charge. For example, some peptides derived from β-LG (*f15 – 20, f25 – 40, f78 – 83* and *f92 – 100)* have the negative electrostatic charge, and their activity is restricted mainly to the Gram-positive bacteria. Native α-lactalbumin has immunomodulatory properties, but does not affect microorganisms, whereas products of its degradation by trypsin and chemotrypsin (*f1 – 5, f17 – 31*-SS-*f109 –114* and *f61 – 68*-SS-*f75 – 80*) or pepsin exhibit both immnunomodulatory and antimicrobial properties against bacteria, viruses and fungi [Fiat *et al.* 1993, Pellegrini *et al.* 1999, Kamau *et al.* 2010]. Biological properties of peptides encoded in the structure of β-LG have been a subject of studies aiming at inhibition of the human immunodeficiency virus type 1 by use of chemically modified β-LG (3-hydroxyphthaloyl-β–LG) – Neurath *et al*. [1997a,b], Oevermann *et al*. [2003], Taha *et al.* [2010]. Acidic peptides derived from β-LG under the action of peptidases of *Lactobacillus paracasei* decreased the stimulation of lymphocytes and regulated production of IL-10, IFN-γ and IL-4.This indicates an existing prospect of eliminating the cow milk allergy reaction [Prioult *et al.* 2004].

Numerous studies are dedicated to the casein fraction, which accounts for 80% of milk total protein and is a rich source of bioactive peptides that stimulate and aid the immune system. Hydrolysis of αs_2-casein (by chymosin acting at neutral pH) results in releasing casocidin. This peptide shows antibacterial properties against

Staphylococcus spp, Sarcina spp, Bacillus subtilis, Diplococcus pneumoniae, and *Streptococcus pyogenes* [Lahov and Regelson 1996, Clare and Swaisgood 2000, Silva and Malcata 2005]. It was also reported that antibacterial peptides obtained from αs_2-casein (*i.e*. f183-207 and f164-179) inhibit growth of both Gram-positive and Gram-negative bacteria when used in small (i.e. from 8 to 95 μmol/l) concentration [Recio and Visser 1999].

Another casein-derived peptide – isracidin – is encoded in the native sequence of αs_1-casein (N-terminal fragment *f1-23*) and is also released by of chymosin. It shoved *in vivo* antibiotic properties against *Staphylococcus aureus* and *Candida albicans*. Intramammary injection of *isracidin* protects cows and sheep from *mastitis* [Hayes *et al.* 2005, Sayer *et al.* 1996]. Haque and Chand [2008] reported that *isracidin* achieves 80% efficiency against chronic udder infection with *Staphylococcus* in cows. Moreover, the peptide exerts therapeutical and prophylactic properties, which provide a long-term increase of immune resistance [Lahov and Regelson 1996].

Caseicins A, B and C are released from αs_1-casein during fermentation of milk by *Lactobacillus acidophilus*. Their properties are similar to those previously mentioned of the other antimicrobial peptides. Characteristic of caseicins A (α_{s1}-CN *f21-29*) and B (α_{s1}-CN *f30-37*) is their particularly high activity against *Escherichia coli* O157:H7 and *Enterobacter sakazakii* [Hayes *et al.* 2005]. Presence of the latter bacterium was reported numerously in powdered infant formulas [FAO/WHO 2008, Oonaka *et al.* 2010]. The *Enterobacter sakazakii* may lead to severe neurological complications in infants, such as hydrocephalus and delayed development of the nervous system with a related mortality rate from 40 to 80% [Korpysa-Dzirba *et al.* 2007]. Furthermore, caseicins have been found active against various Gram-negative pathogens (*Cronobacter sakazakii, Cronobacter muytjensii, Salmonella enterica* subspecies 1 serovar Typhimurium, *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas fluorescens*) as well as the Gram-positive *Staphylococcus aureus* [Norberg *et al.* 2011]. It is also noteworthy that the peptides derived from α-casein promote the growth of probiotic *Lactobacillus acidophilus* while inhibit that of pathogenic bacteria [Srinivas and Prakash 2010] indicating their positive effect on the gastrointestinal tract and suitability in nutraceuticals production.

During the chymosin-induced hydrolysis of κ-casein, the peptide bound fractures between Phenylalanine (Phe^{105}) and Methionine (Met^{106}). Two polypeptides are released – a hydrophobic N-terminal para-κ-casein polypeptide (1-105 amino acid residues) and a hydrophilic C-terminal phosphorylated and glycosylated fragment known as caseinomacropeptide (CMP) – Malkoski *et al.* [2001] – or glycomacropeptide (GMP) – Brody [2000]. At least six genetic CMP variants were identified in bovine milk, A and B being most common. Fractionation of heterogenous CMP (*i.e*. by hydrolytic action of endoproteinase Glu-C) results in the formation of kappacine – the non-glycosylated form of CMP. The peptide has membranolytic properties, which determine its bactericidal properties [Malkoski *et al.* 2001, Dashper *et al.* 2005]. CMP and its derivatives possess immunomodulatory properties, they also inhibit

hemagglutination caused by the influenza virus, binding the toxins of *Vibrio cholerae* bacterium. In addition, these peptides inhibit the adherence of *Streptococcus mutans,* the Gram-positive bacterium responsible for caries development and growth of Gram-negative bacteria, such as *Porphyromonas gingivals* and *Escherichia coli* [Brody 2000, Malkoski *et al.* 2001, Haque and Chand 2008]. The action of selected biopeptides on the immune system is presented on Figure 2.

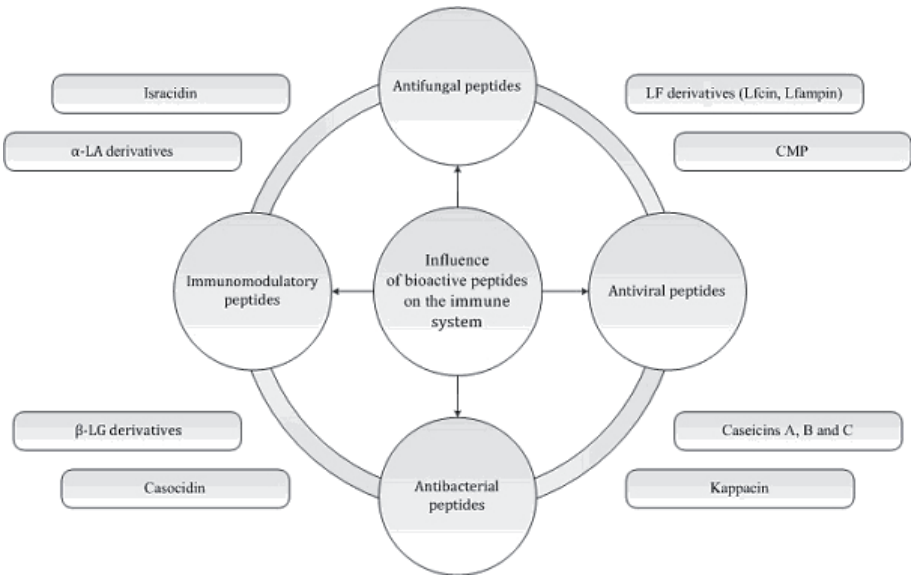

Fig. 2. Scheme of the influence of selected peptides released from bovine milk proteins on the immune system. Source: own elaboration.

Conclusion

Despite the current opinions that every protein can serve as the precursor of biologically active peptides; the most abundant source of bioactive peptides is bovine milk, as well as fermented and other milk products. Industrial and semi-industrial techniques of isolation and fractionation of milk proteins are already commonly used by dairy industry. The described examples of formation and functioning of bioactive peptides encourage pursuing further studies, aiming at utilization of these substances for production of food. Health beneficial nutraceuticals with incorporated bioactive peptides support the immune system in a non-invasive manner. Biologically active peptides may also be used as potential farmaceuticals, characterized by well-defined effects. Studies concerning the possible physiological impact of bioactive peptides are continuously developing. This area of research requires further experiments and investigations in order to acquire knowledge of their mechanism of action.

Furthermore, it is crucial to extend the durability and stability of peptides during the digestion, which causes their inactivation.

It can be anticipated that many new products, which are targeted at specific age groups of consumers or at individuals with the compromised immune system, will soon emerge on the market. Particular attention should be given to the studies on the well-known and popular products, which also contain biologically active peptides, formed during the technological processes used in dairy production.

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