Review article

Bioactive peptides in dairy products

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ABSTRACT

Bioactive peptides are specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health. Most of the biological activities are encrypted within the primary sequence of the native protein and can be released by enzymatic hydrolysis and proteolysis or by food processing. Milk is a rich source of bioactive peptides which may contribute to regulate the nervous, gastrointestinal and cardiovascular systems as well as the immune system, confirming the added value of dairy products that, in certain cases, can be considered functional foods. The main biological activities of these peptides and their bioavailability in dairy products are reviewed. The natural concentration of these biomolecules is quite low and, to date one of the main goals has been to realize products enriched with bioactive peptides that have beneficial effects on human health and proven safety. Even though several health-enhancing products have already been launched and their integration in the diet could help in the prevention of chronic diseases such as hypertension, cancer and osteoporosis, more clinical trials are required in order to develop a deeper understanding of the activity of biopeptides on the human physiological mechanisms and also to assess the efficacy of their effects in a long term view. New scientific data are also needed to support their commercialisation in compliance with current regulations.

Key words: Bioactive Peptides, Dairy products, Health-promoting food, Regulatory status.

RIASSUNTO

I PEPTIDI BIOATTIVI NEI PRODOTTI LATTIERO-CASEARI

I peptidi biologicamente attivi sono specifici frammenti proteici che hanno un impatto positivo sulle funzioni e sulle condizioni dell'organismo, e possono sostanzialmente influire sulla salute. Molte delle attività biologiche sono contenute, in forma inattiva, all'interno della sequenza primaria della proteina e possono essere liberate mediante idrolisi e/o proteolisi enzimatica e/o durante la trasformazione degli alimenti. Il latte è una ricca sorgente di peptidi bioattivi che possono contribuire a regolare il sistema nervoso, ga-

strointestinale, cardiovascolare e immunitario, confermando il valore aggiunto del latte e dei suoi derivati, talvolta considerati cibi funzionali. Questa review raccoglie le conoscenze sulle principali attività dei peptidi bioattivi identificati nei prodotti lattiero-caseari. La concentrazione naturale di queste biomolecole è relativamente bassa e quindi uno dei principali obiettivi è realizzare prodotti arricchiti con peptidi bioattivi aventi effetti benefici sulla salute umana e di comprovata sicurezza. Anche se diversi prodotti salutistici sono stati già commercializzati ed il loro consumo potrebbe essere utile nella prevenzione di malattie croniche quali l'ipertensione, alcune forme di cancro e l'osteoporosi, tuttavia un maggior numero di studi clinici sono necessari per comprendere appieno l'attività dei biopeptidi sulla fisiologia umana e verificare l'efficacia dei trattamenti a lungo termine. Infine altri dati sperimentali sono necessari per supportare la commercializzazione di tali prodotti in conformità con le norme vigenti in materia.

Parole chiave: *Peptidi bioattivi, Prodotti lattiero-caseari, Alimenti salutistici, Stato legislativo.*

Introduction

Many food proteins can exert a physiological action, either directly or, after their degradation, in the form of fragments. Peptides represent a quite heterogeneous class of compounds and their characteristics deeply depend on the amino acidic composition and on the length of the chain. The acid-basic behaviour is determined by the free terminal residues and by the ionic lateral group of the residues in the chain; the reactivity of the terminal groups is also useful for their detection and quantification. Protein physic-chemical properties remarkably change after degradation and, consequently, some oligopeptides may play an important role in determining the rheological characteristics of a food. In fact they have been successfully used as additives as long as they are more soluble, less viscous and with greater emulsifying and foaming properties than the native proteins.

Peptides are generally tasteless or bitter except for dipeptides containing Glutamic or Aspartic acid which, typically sweet, are widely used in food industry since they do not cause dental caries and do not contribute to obesity or pathologies such as diabetes mellite. Bitter peptides, such as dichetopiperazins and cyclic dipeptides, have been found in caseinic hydrolysates (Minagawa, 1989), and in cheeses (Lee, 1995). The bitter taste is mainly correlated to the hydrophobic amino acid content (Cliffe, 1990) and to the increasing length of the chain, though over a certain length, it is no more perceptible. Nevertheless, an extended hydrolysis of the proteins may produce peptides with a very intense, bitter taste (Vegarud and Langsrud, 1989).

In food matrices containing sugars, some peptides can undergo the Maillard reaction by heating process, thus modifying the appearance of the product; in milk, lactose and lysine residues in proteins (mainly in caseins) can give rise to a series of undesirable brown pigments and aromatic compounds which weigh upon the colour and the flavour of heated milk (Martins *et al.*, 2000).

From a nutritional point of view, peptides represent a more bio available form of essential amino acids than proteins, even compared to free amino acids, both in terms of increasing assimilation rate at the brush border membrane of the human intestine and of reducing osmotic pressure (Adibi, 1997). In this context, protein hydrolysates and peptides with definite characteristics are being used for clinical applications: for instance, peptide preparations, rich in branched-chain amino acids and poor in aromatic amino acids, have been related to improved conditions in patients affected by liver encephalopathy and they could be addressed to parenteral nutrition and to people suffering from hepatic pathologies (Adachi, 1991).

Low molecular weight peptides are also less allergenic than native proteins; therefore, milk protein hydrolysates are commonly utilized to formulate hypoallergenic food for infants (Host and Halken, 2004).

The physiological activity of some peptides, able to positively affect human health, have attracted the interest of researchers and the food industries.

Bio-active peptides

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health (Kitts and Weiler, 2003). Most of these bioactivities are encrypted within the primary sequence of the native protein and peptides require to be released through one of the following ways:

- hydrolysis by digestive enzymes
- enzymatic cleavage by proteases derived from microrganisms or plants
- food processing or manufacturing (acids, alkali, heating etc.).

Sometimes, these processes may overlap since the protelytic action can start in food and continue in the organism.

Apart from these conventional sources, recombinant DNA techniques have been experimented for the production of specific peptides or their precursors in microrganisms. Kim *et al*. (1999), succeeded in expressing recombinant human α_{s1}-casein in *Escherichia coli* and in purifying it: the tryptic digest of the protein was found to contain several bio-active peptides.

Whatever the origin, once released, bioactive peptides must reach the target receptors in the intestinal lumen or in other peripheral organs, passing via the systemic circulation.

The activity is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to reveal multifunctional properties (Meisel and FitzGerald, 2003). In fact, some regions contain overlapping peptide sequences that exert different activities; these regions have been considered as "strategic zones" that are partially protected from further proteolytic breakdown. A strategic zone, for instance, is located in the sequence 60-70 of cow and human β-casein (Fiat *et al*., 1993). The sequence is protected from proteolysis because of its high hydrophobicity and the presence of Proline residues. Proline, in fact, has an exceptional conformational rigidity compared to other amino acids; hence it loses less conformational entropy upon folding.

Other examples of the multifunctionality of milk-derived peptides include the αs_1-casein fraction (f194-199) showing immunomodulatory and antihypertensive activity, the opioid peptides α- and β-lactorphin exhibiting also antihypertensive activity and the Caseinophosphopeptides, which possess mineral-carrier and immunomodulatory properties (Korhonen and Pihlanto, 2003).

Biologically active peptides are produced from several dietary proteins during gastrointestinal digestion and fermentation, but milk is considered the main source of biopeptides, with specific nutritional, sensorial and functional properties.

Biopeptides in dairy products

Milk naturally contains an array of bioactivity due to lysozyme, lactoferrin, growth factors, and hormones, which are secreted in their active form by the mammary gland. Colostrum is especially rich in nutrients and provides protection against pathogens thanks to its high concentration of antimicrobial proteins and, in particular, immunoglobulins (Pakkanen and Aalto, 1997).

In addition, the evidence that milk proteins are the main source of many biopeptides, with different important physiological functions, proves that their role is not only to feed the neonate but also to regulate the complete growth of the body (Zabielski, 2007).

Bioactive peptides can be generated by digestive enzymes and during milk fermentation with the starter cultures traditionally employed by the dairy industry (Korhonen and Pihlanto, 2006). Peptides with various bioactivities have been identified in several dairy-products, such as milk protein hydrolysates, fermented milks and many cheese varieties (Gobbetti *et al*., 2002; Korhonen and Pihlanto-Leppälä, 2004).

Some products and ingredients, which have already been launched on the market and their applications are listed in Table 1.

At least two fermented sour-milk products with antihypertensive activity have been launched in Japan and Finland, respectively. The Japanese product "Calpis" is a soft drink, made from skim milk inoculated with starter cultures containing *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae* (Takano, 1998), where the peptides Val-Val-Pro and Ile-Pro-Pro have been isolated and identified. In animal model studies, single oral administration of these tripeptides has been shown to have an antihypertensive effect in Spontaneously Hypertensive Rat (SHR). "Calpis" has also been demonstrated to prevent the development of hypertension in mildly hypertensive human subjects (Haque and Rattan, 2006).

The Finnish milk product "Evolus" contains the tripeptide Ile-Pro-Pro and exerts a similar antihypertensive effect but it is produced by *Lactobacillus helveticus* LBK-16H strain as starter (Seppo *et al.*, 2002).

PeptoPro® is a sport drink obtained by the cleavage of caseins, by means of a patented technology; it results rich in di- and tri-peptides that are stable, no longer bitter nor allergenic and supply energy and fast muscle refuelling by stimulating the production of insulin (Dutch State Mines, 2004).

BioPure-Alphalactalbumin (Davisco©, 2007) is a Davisco product with a minimum of 90% purified alpha-lactalbumin on a protein basis, containing the highest level of tryptophan naturally available from a protein source (4.4g tryptophan per 100g powder). Tryptophan is the precursor of serotonin in the brain and is associated with many health benefits, including improved sleep, memory, mood, *etc*. (Markus *et al*, 2005).

The presence of some of these fractions in food and beverages has given rise to the term "functional food" which is so called if it is satisfactorily demonstrated to beneficially affect one or more target functions in the body through active compounds, beyond adequate nutritional effects. According to this definition, functional food must remain food and cannot be made of pills or capsules. Another category of foodstuffs is labelled as "nutraceuticals" which, in proper cases, contain physiologically active components at a concentration significantly higher than the one naturally occurring in the original product (Childs, 1999).

A nutraceutical is any substance that provides health or medical benefits, including the improved state of well-being and a reduction of risks related to certain diseases (DeFelice, 1995). However, it is advisable to make a clear distinction between health enhancing nutraceuticals, which are good adjuvant for prevention, and drugs for treatment of diseases when pharmacologically active compounds are needed.

Health-promoting food products are specifically aimed for weight management (prevention of obesity), natural defence (boosting of immunity), bone calcification (prevention of osteoporosis), digestion (prevention of intestinal disorders), cardiovascular health

Table 1. Commercial dairy products and ingredients with health or function claims based on bioactive peptides (Korhonen and Pihlanto, 2006).

Type of product	Claimed functional bioactive peptides	Health/function claims	Manufacturers
"Calpis" Sour milk	Val-Pro-Pro, Ile-Pro-Pro, derived from β-casein and κ–casein	Reduction of blood pressure	Calpis Co., Japan
"Evolus" Calcium enriched fermented milk drink	Val-Pro-Pro, Ile-Pro-Pro, derived from β-casein and κ–casein	Reduction of blood pressure	Valio Oy, Finland
"BioZate" Hydrolysed whey protein isolate	β-lactoglobulin fragments	Reduction of blood pressure	Davisco, USA
"BioPure-Alphalactalbumin" Whey protein isolate	α-lactalbumin	Helps sleep and memory	Davisco, USA
"BioPure-GMP" Whey protein isolate	κ-casein f (106–169) (Glycomacropeptide)	Prevention of dental caries, influence the clotting of blood, protection against microrganisms	Davisco, USA
"Prodilet F/200 Lactium" Flavoured milk drink, confectionery, capsules	αs1-casein f (91–100) (Tyr-Leu-Gly Tyr-Leu-Glu-Gln-Leu-Leu-Arg)	Reduction of stress effects	Ingredia, France
"Festivo" Fermented low-fat hard cheese	αs1-casein f (1–9), αs1-casein f (1–7), αs1-casein f (1–6)	No health claim as yet	MTT Agrifood Research Finland
"Cystein Pepetide" Ingredient/hydrolysate	Milk protein derived peptide	Aids to raise energy level and sleep	DMV International, The Netherlands
"C 12" Ingredient/hydrolysate	Casein derived peptide	Reduction of blood pressure	DMV International, The Netherlands
"Capolac" Ingredient	Caseinophosphopeptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
"PeptoPro" Ingredient/hydrolysate	Casein derived peptide	Improves athletic performance and muscle recovery	DSM Food Specialties, The Netherlands
"Vivinal Alpha" Ingredient/hydrolysate	Whey derived peptide	Aids relaxation and sleep	Borculo Domo Ingredients (BDI), The Netherlands

(prevention of heart diseases by lowering the cholesterol level or blood pressure).

Recently casein hydrolysates, mainly derived from α_s-caseins, obtained by proteolytic enzymes from *Lactobacillus* strains have shown antioxidant properties (Korhonen and Pihlanto, 2003), free radical-scavenging activities and the ability to inhibit enzymatic and non-enzymatic lipid peroxidation (Suetsuna *et al.*, 2000; Rival *et al.*, 2001). However the potential health benefits in the human diet of these antioxidative peptides need to be thoroughly investigated.

The bioactivities highlighted by isolated peptides from fermented milks, yoghurt and from a great variety of cheeses have been reported in many studies and they take part in the regulation of the nervous, gastrointestinal, immune and cardiovascular systems (Clare *et al*., 2003; Florisa *et al.*, 2003; Kitts and Weiler, 2003; Bouhallab, and Bouglè, 2004; Janecka *et al*., 2004; Rizzello *et al*., 2005; Korhonen and Pihlanto, 2006).

Effects on the nervous system

Recent studies have shown that the consumption of dairy products causes interactions with the nervous system through the action of opioid peptides; basically they are receptor ligands with agonistic or antagonistic activities which are located in the nervous, endocrine and immune systems as well as in the gastrointestinal tract of mammals and can interact with their endogenous ligands (normally synthesized by the organism) or exogenous ligands (introduced by food). There are at least three types of opioid receptors: μ-type regulating the emotional behaviour and the intestinal mobility, δ-type involving the emotional behaviour and the κ-type regulating calmness and appetite. They show different affinity, even though all of them present cross-interactions.

The common structural feature of opioid peptides (except for α-casein opioids) is the presence of a Tyr residue at the N-terminal, coupled with the presence of another aromatic residue, such as Phe or Tyr, in the third or fourth position. This is an important factor that ensures fitting into the binding site of the receptors; furthermore, the negative potential, localized around the phenolic hydroxyl group of Tyrosine, seems to be essential for opioid activity (Silva and Malcata, 2005).

The major and the first discovered opioid peptides, deriving from milk, are the so called β-casomorphins (Teschemacher, 2003) which are fragments of β-casein between the 60th and the 70th residues, mainly f60-63, f60-64, f60-65, f60-66 and f60-70, classifiable as μ-type ligands (Smacchi and Gobetti, 2000).

The most potent seems to be the pentapeptide f60-64 (Fiat *et al.,* 1993) whose sequence appears similar in β-casein from sheep (Richardson and Mercier, 1979) and from water buffalo (Petrilli *et al.,* 1983) along with the fragment f60-63 of bovine β-casein called Morphiceptin (Chang *et al.,* 1981; Mierke *et al*., 1990). The fragment f51-54 of human β-casein is also supposed to exert an agonistic opioid activity (Fiat *et al.*, 1993).

β-casomorphins have been detected in the duodenal chyme of minipigs, in the plasma of newborn calves and in the human small intestine, upon oral administration of casein or milk (Meisel, 1998; Meisel and FitzGerald, 2000; Meisel and FitzGerald, 2003) whereas their absorption in the gut or plasma of adult mammals has not been reported to date.

Gastric and pancreatic digestion are thought to originate those active sequences although their absorption through the intestinal epithelium has not been proven (Silva and Malcata, 2005). During diges-

tion, caseins, because of the acidity of the stomach, spontaneously precipitate; slowly, they empty the gut in the form of degraded products, including putative bioactive peptides like β-casomorphins. Therefore the opioid activity is performed only at a peripheral level, whereas β-casomorphins may modulate the absorption of amino acids and the transport of electrolytes by decelerating the intestinal transit time (Meisel and Schlimme, 1994). As soon as peptides enter the blood stream, they are quickly hydrolysed (Meisel, 1997b).

On the other hand, β-casein derived peptides may pass through the intestinal mucosa in neonates via passive transport, so babies, thanks to greater intestinal permeability, may become calm and sleepy after milk consumption (Sturner and Chang, 1998).

Peptides influencing the nervous system are also the Exorphins (called formons or food hormones), fragments of α_{s1}-casein mainly, which exhibit pharmacological properties similar to opium (morphine) and exert naloxone-inhibitory activities (Meisel and Schlimme, 1990); they also induce apnea and irregular breathing, modulate sleep patterns, stimulate pancreatic insulin and gastrointestinal somatostatin release, modulate animal behaviour and food intake by modifying the endocrine activity of the pancreas, hence causing an increase in insulin output (Xu, 1998). A large body of evidence has shown that opioid peptides reduce feeding in many species including humans and may implicate potential treatment of obesity (Clapham *et al*., 2001).

After enzymatic proteolisys of α-Lactoalbumin, α-Lactorphin which binds to opioid receptors and possesses opioid-like activities, can be released. It is a tetrapeptide (Tyr-Gly-Leu-Phe) and, probably due to its conformation, it does not easily cross the blood-brain barrier; consequently it would not induce nervous centrally mediated effects. Contrariwise, α-Lactorphin has improved *in vitro* endothelium-dependent vasorelaxation in rat mesenteric arteries via nitric oxide-mediated mechanism (Ijas *et al*., 2004), whereas β-Lactorphin (Tyr-Leu-Leu-Phe), the analogous peptide originated from β-Lactoglobulin, induced endothelium-independent relaxation (Sipola *et al*., 2002). In addition, both had positive effects on the cardiovascular system (Nurminen *et al*., 2000).

Another agonistic opioid peptide has been detected in the whey, namely Serorphine which derives from fragment f399-404 of the Bovine serum albumin (Meisel and FitzGerald, 2000).

Opioid antagonists are those peptides that suppress the action of endogenous and exogenous agonistics: known as casoxins, they have been found in both bovine and human κ-casein, as well as in α_{s1}-casein (Chiba *et al.*, 1989). Some agonistic and antagonistic opioid peptides isolated from milk proteins are listed in Table 2.

Casoxins A and B are opioid receptor ligands of the μ-type, even though they may also bind κ-type receptors; their antagonistic potency appears relatively low as compared with naloxone, a common analgesic drug. Casoxin C is an opioid antagonist obtained from tryptic digests of bovine κ-casein and possesses the highest biological potency among the casoxins, showing a 50% inhibitory concentration (IC_{50}) of 50.0 μmol/L (Xu, 1998). Casoxin D (Yoshikawa *et al.,* 1994; Clare and Swaisgood, 2000), composed of seven residues, was generated from α_{s1}-casein and was also efficiently produced by using a plasmid hosted by *Bacillus brevis* (Kato *et al.,* 1995).

After methoxylation some casoxins show a greater biological activity than the correspondent non-methoxylated ones (Meisel, 1997a).

Effects on the gastrointestinal system

During gastroenteric digestion, whole proteins and peptides reach the intestinal tract where they are involved in the regulation of digestive enzymes and modulation of nutrient absorption. Here the formation of biopeptides can take place and further proteolysis by the peptidases of the apical microvilli may complete or ruin all their biological effects.

Generally the bioactivity of short-chain peptides may be better preserved rather than longer molecules, but *in vitro* digestion experiments demonstrated that the hydrolysis degree of known peptides can vary depending on peptide chain length, nature of the peptide and presence of other peptides in the medium (Roufik *et al.*, 2006); performing *in vivo* studies is important to confirm the true potential role of peptides for nutraceutical applications.

Casein-derived phosphorylated peptides, caseinophosphopeptides (CPPs), that enhance vitamin D-independent bone calcification in rachitic infants, were the first bioactive peptides reported in literature (Mellander, 1950).

CPPs have been found after *in vitro* and/or *in vivo* digestion of α_{s1} ,α_{s2} or β-casein and recently, they have been detected in the distal small ileum of humans administered with milk or crude CPP preparations, confirming the ability of such peptides to survive gastrointestinal passage (Meisel *et al.*, 2003).

Most CPPs share a common feature: they consist of a sequence of three phosfoseryl residues, followed by two Glutamic acid residues, i.e. SerP-SerP-SerP-Glu-Glu (Meisel *et al.*, 1997). The high concentration of negative charges of phosphate peptides makes them resistant to further proteolysis (FitzGerald, 1998; Clare and Swaisgood,

Table 2. Opioid milk peptides. Modified from Clare and Swaisgood (2000).

Protein substrate	Bio-peptide	Amino acid segment	Reference
Bovine αs1-CN	Exorphin	f90-95, f90-96, f91-96	Loukas *et al.*, 1983
Human β-CN	β-Casomorphin (4,5)	f51-54, f51-55	Brantl, 1984
Bovine & Human α-LA	α-Lactorphin	f50-53	Chiba and Yoshikawa, 1986; Fiat and Jolles, 1989
Bovine β-Lg	β-Lactorphin	f102-105	Fiat et al.,1993; Yoshikawa *et al.*, 1986
Bovine β-CN	Mofphicetin	f60-63	Chang *et al.*, 1981; Mierke *et al.*, 1990
Bovine & Human k-CN	Casoxin A, B, C	f25-34, f35-41, f57-60	Yoshikawa *et al.*, 1986; Chiba *et al.*,1989
Lactotransferrin	Lactoferroxin A, B, C	f318-323, f536-540, f673-679	Tani *et al.*, 1990
Human αs1-CN	Casoxin D	f158-164	Yoshikawa *et al.*, 1994
Bovine serum albumin	Serorphin	f399-404	Tani *et al.*, 1994

2000) and, at the same time, represents the binding sites for macroelements such as Ca, Mg, and Fe as well as for oligoelements as Zn, Cr, Ni, Co and Se (Meisel, 1998).

The ability of CPPs to retain minerals allows the prevention of different diseases caused by their deficiency such as osteoporosis, dental caries, hypertension and anaemia.

It is well known that dairy products are a rich source of Ca^{2+} that can form with CPPs soluble complexes, enhancing calcium absorption and avoiding the precipitation of insoluble phosphates (Berrocal *et al.*, 1989). Following the same mechanism, Fe^{2+} can be retained to restore Fe tissues as demonstrated by *in vivo* studies with rats showing that low molecular-weight casein phosphopeptides, namely β-casein f1-25, can more efficiently bind to Fe compared to whole casein and inorganic salts (Ait-Oukhartar *et al*., 1999). That peptide can also improve Zinc absorption without interfering with other nutrients like calcium or iron (Meisel and Bockelmann, 1999; Bouhallab and Bouglè, 2004); an increase in both Ca and Zn was noticed when infant food enriched with CCP was given to twenty-two volunteers, (20-30 years of age), during a feeding trial (Hansen *et al.*, 1997).

The net increased absorption of calcium in the gut was not confirmed when large doses of CPPs through CPP-enriched preparations were administered to 15 adults; during the 5 experimental days, each volunteer completed 3 absorption tests consuming, in random order, a control drink and two drinks with additional 69 or 138 mg of Calcium with 1 or 2 g of CPP added, respectively. The observed differences were not effective to assess any evident calcium-enhancement absorption (Lopez-Huertas *et al*., 2006; Teucher *et al*, 2006). In light of these results, the need to perform more in depth studies on humans seems compelling, especially when fortified products are thought to ensure consumers some benefits. However, some current studies are devoted to the explanation of the real role of CPP in the promotion of Calcium uptake (Gravaghi *et al*., 2007).

An interesting aspect associated with CPPs is their anticariogenic activity, promoting the recalcification of the dental enamel with a caries-protective effect; hence, their application as additives to toothpaste and to sugar-free chewing gums have been proposed (Morgan *et al*., 2008; Reynolds *et al*., 2008).

On the other hand, heating processes affect the bioavailability of CCPs; sterilization of milk can induce dephosphorylation of phosphoseryl residues that occur mainly as monoesters of Ser in clusters, and the formation of dehydroalanine from residues occupying isolated positions in the peptide chain (Meisel *et al*., 1991).

Glycomacropeptide (GMP) and its non-glycosylated form Caseinmacropeptide (CMP) are released after specific cleavage of κ-casein by chymosin (Farrell *et al*., 2004). Their potential role in the regulation of intestinal functions has been widely investigated (Brody, 2000; Pihlanto and Korhonen, 2003; Manso and López-Fandino, 2004).

GMP has a unique amino-acid composition as it lacks aromatic residues and is rich in branched chain ones; thus it might be useful for diets aimed at controlling several liver diseases, in cases where branched chain amino acids appear to be used as a carbon source (El Salam *et al.,* 1996).

Glycomacropeptides also have a positive effect in selecting the intestinal microflora determining a prevalent growth of bifidobacteria (Manso and López-Fandino, 2004).

CMP, the casein derived whey peptide, seems to inhibit gastric secretions, slow down stomach contractions and stimulate the release of cholecystokinin (CKK), the satiety hormone involved in controlling food

intake and digestion (Yvon *et al*., 1994). This peptide could be directed to products destined to weight programmes and appetite control, although a study conducted on volunteers fed with CMP revealed no effects on food energy intake (Gustafson *et al*., 2001).

Some opioid peptides belonging to casomorphins can also influence the gastrointestinal system by interacting with opiate receptors in the serosal side of the intestinal epithelium, leading to a subsequent modification of the electrolyte transport (Tome and Debbai, 1998).

Furthermore the opioid antagonist lactoferroxins have been found in human lactoferrin and they presumably inhibit the gut motility induced by casomorphins (Yoshikawa *et al*., 1988).

Effects on the immune system

The immune system is made up of specialized cells, antibodies and a lymphatic circulatory system since it protects the organism. Milk protein hydrolysates and peptides derived from caseins and the major whey proteins can enhance immune cell functions, measured as lymphocyte proliferation, antibody synthesis and cytokine regulation (Gill *et al*., 2000).

The physiological properties attributed to these diet related peptides have a common mechanism based on the inhibition of target enzymes which are somehow involved in essential processes like blood coagulation, phagocytosis and pathological infections.

It is used to distinguish two main activities: the immunomodulatory and the antimicrobial one.

Immunomodulating peptides

Breast feeding, especially at the beginning of lactation (colostrum), is the best way to provide the neonate with all the nutrients and, in particular, an adequate resistance against bacterial and viral infections. When gastrointestinal digestion occurs, many peptides with immunomodulating capacity are released from both the whey proteins and caseins.

There are various hypotheses about the physiological action of such peptides: they might stimulate the proliferation and maturation of T-cells and natural killer cells for the defence of the newborn against different bacteria, especially enteric bacteria. Cow and goat milk proteins were investigated by Eriksen and Vegarud (2007) by using human gastric and duodenal juice in comparison to commercial pig derived enzymes to simulate *in vivo* digestion; the hydrolysates obtained, in particular those from whey proteins, revealed a dose-dependant inhibition of human peripheral blood mononuclear cells (PBMC).

The first isolated and sequenced peptide was the tryptic hydrolisate of human β-casein, Val-Glu-Pro-Ile-Pro-Tyr (corresponding to f54-59), that revealed immunostimulating activity (Jollès, 1981; Parker *et al.,* 1984). Later on several other peptides were identified, namely f63-68 and f191-193 from bovine β-casein and f194-199 from bovine α_{s1}-casein (Migliore-Samour and Jollès, 1988) which stimulate phagocytosis in mice and humans *in vitro* and protect against *Klebsiella pneumoniae* infection in mice *in vivo* (Migliore-Samour *et al.*, 1989).

Kayser and Meisel (1996) reported that di- and tri-peptides like Tyr-Gly and Tyr-Gly-Gly (partial sequences in the primary structure of bovine k-casein and α-lactalbumin respectively), significantly increased the proliferation of human peripheral blood lymphocytes *in vivo*. Recently these peptides were used for immunotherapy of human immunodeficiency virus infections, for example, to inhibit the development of infections in patients with pre-AIDS (Hadden, 1991). Trials conducted in 93 patients with

AIDS-related syndrome showed encouraging results after a bi-weekly treatment, reducing the tendency to progress to a critical endpoint or to AIDS (Gobbetti *et al.*, 2002).

Moreover, β-casokinin-10 and β-casomorphin-7, (different fragments of β-casein), also induce a proliferative response in rat lymphocytes, showing a suppression as well as a stimulation, depending on peptide concentration (Meisel and Bockelmann, 1999).

Immunoenhancing effects were also pointed out by the bovine Glycomacropeptide and its derivatives on the cell proliferative response of human macrophage-like cell, U937; Li and Mine, (2004) experienced the dose response of GMP on cell proliferation of U937 finding an enhanced activity at the dose of 1 to a maximum of 10 µg/mL, whereas the activity rapidly decreased at 100 µg/mL, suggesting that GMP acts as an immuno-enhancer at low concentrations *in vitro*. Pepsin-digested fragments of GMP enhanced cell proliferation about three times more than GMP itself while the trypsin-digest did not affect it. In the same study, GMP derivatives were tested on phagocytic activities of U937 and the sialic acid, terminal sugar unit of GMP, showed greater activity but *in vivo* trials on humans are in progress to establish whether these effects are maintained when GMP is included in the diet.

Furthermore, immunopeptides formed during milk fermentation have been shown to contribute to the antitumoural effects observed in many studies with fermented milks. Bioactive peptides present in yoghurt actually decreased tumour cell proliferation which may explain, at least partially, why consumption of yoghurt has been associated with a reduced incidence of colon cancer (Ganjam *et al.,* 1997).

A commercially available caseinophosphopeptide preparation CPP-III, consisting mainly of f1-32 of bovine α_{s2}-casein and f1-28 of β-casein, enhances the proliferative response and immuglobulin production in mouse spleen cell cultures; this immunostimulating activity was attributed to the o-phospho-l-serine residue, hence suggesting that such bioactivity is relatively stable to proteinase action in the intestinal tract (Hata *et al.,* 1999). This information is of high relevance when developing infant formulas with optimized immunomodulatory properties.

Antimicrobial peptides

Additively and synergistically to peptide hydrolysates, some intact milk proteins can participate in the host defence and interesting antiviral effects have been reported *in vivo* in mouse and rat models (Pan *et al.*, 2006). Lysozyme, whose content is particularly rich in the milk of humans and equids, works by peptidoglycan hydrolysis causing the lysis of the bacterial cell wall, although an increasing body of evidence supports the existence of a non-enzymatic and/or non-lytic mode of action (Masschalck and Michiels, 2003).

Lactoperoxidase catalyses the peroxidation of thiocyanate and some halides (I^-, Br^- but not Cl^-) to generate products which are harmful for mammalian cells but kill or inhibit the growth of many species of microorganism (Boots and Floris, 2006). Lactoferrin, an iron-binding whey glycoprotein, shows indeed the most important antimicrobial activities (Chierici, 2001). However, this review deals chiefly with the antimicrobial peptides derived from milk proteins; several have been detected and some of them are listed in Table 3.

The sequence fragment f17-41, having one intramolecular disulfide bond, is generated *in vitro* upon enzymatic cleavage of lactoferrin with pepsin in a region distinct from its iron-binding sites. The released peptide, named lactoferricin (Wakabayashi *et al*.,

Table 3. Antimicrobial milk peptides.

Milk peptide fragment	Release protease	Gram (+) activity	Gram (-) activity	Yeast and fungi*
Casecidin κ-CN (f 17-21) αs1-CN	Chymosin and Tripsin	*Staphylococcus aureus Sarcina Bacillus subtilis Diplococcus pneumoniae Streptococcus pyogenes*		
Casocidin-I αs2-CN (f 165-203)	Synthetic peptide	*Staphylococcus carnosus*	*E. coli*	
Isracidin αs1-CN (f 1-23)	Chymosin and Tripsin	*Staphylococcus aureus*		*Candida albicans*
Caseicin αs1-CN A (f 21-29) B (f 30-38) C (f 195-208)	Synthetic peptide Synthetic peptide Synthetic peptide	 *Listeria innocua*	*E. coli, E. sakazakii* *E. coli, E. sakazakii*	
Kappacin k-CN (f106-169)	Chymosin	*Streptococcus mutans*	*E. coli*	
Lactoferricin B Lactoferrin (f17-41)	Pepsin	*Bacillus Listeria Streptococci Staphylococci*	*E. coli 0111 E. coli 0157H:7 Klebsiella Proteus Pseudomonas Salmonella*	*Candida albicans Dermatophytes: *Cryptococcus unigulattulus *Penicillum pinophilum *Trichophyton mentagrophytes*
Lactoferrampin Lactoferrin (f265-284)	Pepsin	*Streptococcus mutans*	*E. coli*	*Candida albicans*

2003), has bactericidal properties more potent than undigested lactoferrin, suggesting that its much smaller size may facilitate access to target sites on the microbial surface (Meisel, 1998). The antimicrobial activity of lactoferricin seems correlated to its net positive charge, which kills sensitive microorganisms by increasing cell membrane permeability (Bellamy *et al*., 1993). Besides, it is reckoned that lactoferricin may hit additional intracellular targets since it is able to translocate across the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria (López-Espósito and Recio, 2006), inhibiting bacterial protein synthesis, although the exact mechanism of this inhibition is not known (Ulvatne *et al*, 2004).

In the sequence of bovine lactoferrin a

new antimicrobial peptide has been identified, Lactoferrampin (f265-284), which has shown broad-spectrum activity against the yeast *Candida albicans* and many gram positive and negative bacteria (van der Kraan *et al*., 2005).

The whey fraction of fermented skim milk may also include component-3 of proteose peptone (PP3), a minor phosphoglycoprotein (135 residues). A synthetic peptide of 23 residues corresponding to the cationic domain f113-135 of PP3, subsequently named lactophoricin, is endowed with the pore-forming ability to interact with natural lipidic bilayers, such as bacterial membranes (Campagna *et al.*, 2001). This peptide displayed a moderate inhibitory-growth activity but the low minimal inhibitory concentrations (MIC 10 µM) and the minimal lethal concentrations (MLC 20 µM) observed in *Streptococcus thermophilus* strain look promising for further trials against untested pathogens (Campagna *et al.*, 2004).

Among the caseins, by digestion of α_{s1}-casein, the first defence peptide actually purified is Caseicidin which exhibits activity against *Staphylococcus spp., Sarcina spp., Bacillus subtilis, Diplococcus pneumoniae* and *Streptococcus pyogenes* (Lahov and Regelson, 1996).

A peptide derived from the fragment f1-23 of α_{s1}-casein, called isracidin, has demonstrated antibiotic-type activity *in vivo* versus *Staphylococcus aureus* and *Candida albicans*; isracidin may be useful to protect the udder of sheep and cow from mastitis (Sayer *et al*., 1996).

Hayes *et al.* (2005) studied the production of three peptides generated by *Lactobacillus acidophilus* DPC6026 fermentation of α_{s1}-casein (Caseicin A, B and C) which have common features with other reported antibacterial peptides, given by a high degree of homology with isracidin for istance. Caseicin A and B were able to inhibit *Escherichia coli* O157:H7 and *Enterobacter sakazakii*, while Caseicin C displayed only minor activity against *Listeria innocua*.

This study showed that *Lactobacillus acidophilus* DPC6026 offers interesting perspectives for the generation of multiple antimicrobial peptides from casein, against pathogen or undesirable bacteria.

From the bovine α_{s1}-casein a novel fragment (f99–109) has been isolated and identified; this peptide, positively charged and obtained by hydrolysis with pepsin, presented activity against *Salmonella typhimurium* (MIC 125 µg/ml), *Escherichia coli* (MIC 250 µg/ml), *Salmonella enteritidis* (MIC 125 µg/ml) and *Citrobacter freundii* (MIC 500 µg/ml). With respect to Gram-positive bacteria *Bacillus subtilis* and *Listeria innocua,* f99–109 has an MIC of 125 µg/ml (McCann *et al*., 2006).

In the sequence of α_{s2}-casein, the cationic fragment f165-203, known as casocidin-I, can inhibit growth of *Escherichia coli* and *Staphylococcus carnosus* (Zucht *et al*., 1995). The search for antibacterial activity from α_{s2}-casein has been extended to milk from other species. Recently, four antibacterial peptides have been identified from a pepsin hydrolysate of ovine α_{s2}-casein (López-Expósito *et al.*, 2006). The peptides correspond to sequences α_{s2}-casein (f165–170), (f203–208), (f165–181), and (f184–208), taking into account that the last two fragments were homologous to those previously identified in the bovine protein. In this study, the ovine α_{s2}-casein peptides (f165–181) showed the highest antibacterial activity against all bacteria tested while the fragment (f203–208) revealed itself a good example of a multifunctional peptide because it exhibited not only antimicrobial activity, but also, potent antihypertensive and antioxidant activity previously studied by Recio *et al*., (2005).

Even caseinomacropeptide (CMP) and glycomacropeptide (GMP) derived from

k-casein have demonstrated antimicrobial activity; CMP inhibits the growth of the oral pathogens *Streptococcus mutans* and *Porphiromonas gingivalis* as well as *Escherichia coli,* and the active form identified was the nonglycosylated Ser(*P*)[149] k-casein (f106-169), designated as kappacin (Malkoski *et al*., 2001). These findings could help in protecting against dental caries.

Numerous physiological functions can be attributed to GMP; among those are highlighted the ability to inhibit bacterial and viral adhesion and to bind *Cholera* and *Escerichia coli* enterotoxins (Kawasaki *et al*., 1992; Brody, 2000).

The antimicrobial role of k-casein also involves a pentapeptide f17-21, k-casecidin, identified from a trypsin digest of bovine κ-casein by Matin *et al.* (2000) and other six peptides with antibacterial activity against *Listeria innocua*, *Salmonella carnosus* reviewed by López-Expósito and Recio *et al.* (2006). However, k-casecidin was found to display cytotoxic activity towards some mammalian cells, including human leukemic cells lines, probably due to apoptosis (Matin and Otani, 2002).

Few studies have considered cheeses as a potential source of antimicrobial peptides (Smacchi and Gobetti, 2000). Antibacterial peptides were isolated and determined from water-soluble extracts of nine Italian cheese varieties characterized by the use of different types of milk, starter and especially by a different time of ripening, demonstrating how different conditions in cheese making might affect the synthesis of bioactive peptides. Parmigiano Reggiano, Fossa, and Gorgonzola water-soluble extracts did not show the presence of antibacterial peptides probably because they are subjected to a very intense proteolysis during ripening. On the contrary, Pecorino Romano, Canestrato Pugliese, Crescenza and Caprino del Piemonte contained peptides with inhibitory activity towards many potentially pathogenic bacteria, including *Staphylococcus aureus* and *Listeria innocua*. Instead, in Mozzarella cheese and Caciocavallo, two pasta filata cheeses, have been identified fragments of isracidin, i.e. cow α_{S1}-casein (f10–14 and f1–23) (Rizzello *et al.*, 2005).

Effects on the cardiovascular system

Bioactive peptides derived from milk or dairy products, mainly from caseins, have shown effects on the cardiovascular system, generally via antithrombotic and antihypertensive peptides.

Antithrombotic peptides

The similarity between the clotting process of milk and the clotting of blood is well known since the undecapeptide (f106-116) from cow's k-casein involved in the coagulating mechanism presents a high structural homology with the human fibrinogen γ-chain (f400-411) (Jollès *et al*., 1978).

Casoplatelins, which are casein-derived peptides, behave like inhibitors of both the aggregation of ADP-activated platelets and the bound of human fibrinogen γ-chain to a specific receptor on the platelet surface (Fiat and Jollès, 1989). A blood anti-clotting effect is also displayed by the k-casein fragment f103-111 which can avoid the platelet aggregation, although is not able to affect fibrinogen binding to ADP-treated platelets (Fiat *et al.,* 1993).

Furthermore k-caseinoglycopeptide, fragment f106-171 of sheep's k-casein, was shown to decrease thrombin- and collagen-induced platelet aggregation in a dose-dependent manner (Qian *et al*., 1995).

Milk might also provide bioactive peptides with cholesterol-lowering effects. Nagaoka *et al.* (2001) isolated from milk β-lactoglobulin tryptic hydrolysate, a hypocholesterolemic peptide, which was identified to be the amino acid sequence Ile-Ile-Ala-Glu-Lys.

Antihypertensive peptides

The angiotensin I-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) is involved in the renin-angiotensin system, which partially regulates peripheral blood pressure. This enzyme is responsible for the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor, and for the degradation of bradykinin, a vasodilatatory peptide.

Inhibition of ACE can therefore exert a hypotensive effect and may also influence different regulatory systems involved in immunodefence and nervous system activity (Meisel, 1997).

Food derived ACE inhibitors peptides are of great interest since they are natural preventive measures for the control of hypertension and could lead to a decrease in the requirement of medicines which are known to exert strong side effects.

Biopeptides may also exert the antihypertensive function by means of:

- interaction with opioid receptors having vasodilatatory effects (Nurminen *et al*., 2000; Fuglsang, 2003)
- inhibition of the release of endothelin-1, a 21 residues long peptide with vasoconstrictor properties (Maes *et al.*, 2004)
- mineral carrier peptides that increase Calcium bioavailability (Seppo *et al*., 2003).

Active peptides must be absorbed in an intact way from the intestine and, in addition, be resistant to degradation by plasma peptidases to obtain the physiological effect. In fact, using monolayer-cultured human intestinal Caco-2 cells, it has been demonstrated, that the ACE-inhibitory tripeptide Val-Pro-Pro can be transported intact through the intestinal wall into the blood via paracellular and transcellular routes, although a significant amount of the peptide is degraded to amino acids by intracellular peptidases (Vermeirssen *et al.,* 2004).

To date, Walsh *et al*. (2004) indicated that β-lactoglobulin fragment f142-148, known as a potent inhibitor of ACE activity *in vitro* (Mullally *et al.,* 1997), is degraded when it is incubated with gastrointestinal and serum proteinases and peptidases, simulating human digestion. This is of practical importance because not all potent peptide inhibitors of ACE that might be produced *in vitro* (such as the f142-148 of β-lg), may necessarily act as a hypotensive agent in humans i*n vivo*.

Peptides with ACE-inhibition action can derive both from caseins, named casokinins (Meisel and Schlimme, 1994), and whey proteins, named lactokinins (FitzGerald and Meisel, 2000). Highly active casokinins are present in the bovine α_{s1}-casein sequence 23-27 and in the β-casein sequence 177-183.

Microbial proteases are capable of producing several ACE-inhibitors during fermentation (Yamamoto *et al.*, 1999; Gobbetti *et al.,* 2002; Ashar and Chand, 2004) and cheese-making (Addeo *et al.*, 1992; Stepaniak *et al.*, 2001) and the type of starter culture used is one of the main factors influencing their synthesis in dairy products. Potent ACE-inhibitory peptides VPP and IPP were purified from the fermented sour-milk "Calpis" (Nakamura *et al. ,*1995) and a significant reduction in blood pressure was recorded in mildly hypertensive patients after daily ingestion of 95 ml of "Calpis" for an eight-week period; although the ingested dose of peptides was small, only about 2.6 mg per day, the hypotensive effect remained even four weeks after the end of treatment (Yamamoto *et al.*, 2003).

As stated previously, another fermented milk, "Evolus", proved effective in Spontaneously Hypertensive Rats; the treatment lasted 14 weeks and the calculated intake of IPP was 0.4 mg/d and 0.2 mg/d in the groups receiving fermented milk (A and B), respectively, whereas the corresponding amounts for VPP were 0.6 mg/d and 0.3 mg/d. At the end of the experiment, lower blood pressure

was detected in the two groups (group A with greater effect than B), while the control group, fed simply skim milk, did not show any considerable change in blood pressure (Sipola *et al*., 2002). "Evolus" was also tested in two double-blind, placebo-controlled studies with mildly hypertensive subjects who ingested 150 ml of the product daily. It was found to decrease both systolic and diastolic pressure during the 8-week and 21-week treatment periods, respectively. No such influence was reported in subjects with normal blood pressure (Seppo *et al*., 2002; Seppo *et al*., 2003).

Minervini *et al*. (2003) prepared sodium caseinates from bovine, sheep, goat, pig, buffalo and human hydrolysates by a partially purified proteinase of *Lactobacillus helveticus* PR4. Peptides in each hydrolysate were fractioned by reverse-phase fast-protein liquid chromatography (RP-FPLC). The fractions that showed the highest ACE-Inhibitory activity were sequenced by mass spectrometry and Edman degradation analysis and the peptide profiles obtained differed according to the species. Bovine sodium caseinate hydrolysates fractions showed the highest activity as the IC_{50} (peptide Concentration Inhibiting the activity of ACE by 50%) settled from 16.2 to 57.2 µg/ml; this was slightly lower than sheep sodium caseinate hydrolysate which had an IC_{50} of 120.2 µg/ml; goat and buffalo fractions IC_{50} ranged from 112.6 to 210.5 µg/ml and human sodium caseinate also showed a considerable ACE-inhibitory activity (IC_{50}, 228.1 µg/ml). This study gave evidence that milks of different species all have the potential to yield hypotensive peptides after enzymatic hydrolysis and the different bioactive peptides generated are related to the level of sequence identity and native conformation of the protein.

Moreover these caseinate hydrolisates and related fermented milks may be considered as suitable functional foods, as the IC_{50} values of the tested peptides are consistent with the IC_{50} (in the order of 14 µM) artificially synthesized by Maruyama *et al.* (1989), and compatible with the amount of bioactive peptides (10 to 60 mg) potentially produced during proteolysis of 1 g of caseins (Meisel, 1998), which are necessary to exert their action.

Besides the functional properties, the release of peptides in yoghurt fermentation is also interesting. It has been claimed that the traditional yoghurt starters *Lactobacillus bulgaricus* and *Streptococcus thermophilus* act synergistically, and symbiosis is promoted by the release of free amino acids and peptides (Bracquart and Lorient, 1979). Thus, some of the peptides also act as growth promoters or stimulatory peptides in the mixed starter culture (Van Boven *et al*., 1986).

In several ripened cheeses Meisel *et al*. (1997) have found the presence of low molecular mass peptides with ACE-inhibitory activity which increases as proteolysis develops, whereas the ACE inhibition index decreases when cheese ripening exceeds a certain level (e.g. the ACE-inhibitory activity detected in medium-aged Gouda was about double compared to the long-ripened Gouda cheese).

Many studies compare the ACE-inhibitory activity of a specific cheese with different ripening degrees but only a few have tackled the search in Protected Denomination of Origin (PDO) cheeses elaborated with different technologies (mould-ripened, smoked, hard), starters and milk from different species. Recently, in six Spanish cheeses (Cabrales, Idiazàbal, Roncal, Manchego, Mahòn and a goat's milk cheese) - five of them with PDO - a total of 41 ACE-inhibitory peptides mainly derived from β- and α_{s1}-casin were identified. Although there is not a clear relationship between proteolysis and ACE-inhibitory activity, Cabrales cheese that had the highest proteolysis index also showed the highest

inhibitory activity while Mahòn showed the lowest (Gòmez-Ruiz *et al*., 2006).

Peptides bearing ACE-inhibitory and antioxidant activity have been found in raw and sterilized ovine and caprine cheeses, manufactured with enzymes from Cinara Cardunculus as the coagulant agent. The milk clotting activity is caused by two aspartic proteases, cardonsis A and B, which resemble chymosin and pepsin, respectively, in activity and specificity (Verissimo *et al*., 1995).

These products contain a complex mixture of peptides that may also have opioid binding capabilities.

Health-promoting foods and their legal compliance

The great success in the retail of functional foods and concerns about health that have emerged in recent years have led food industries to new marketing strategies that embrace consumer expectations with health-promoting foodstuffs while acquiescing to the requirements of legislation.

As for EU legislation, regulation 258/97/CE concerned the placing on the market of "Novel foods" which are foods and food ingredients that were not used for human consumption to a significant degree within the Community before 15 May 1997 (EU Regulation 258, 1997). By May 2004, 14 novel foods were approved to be marketed in the EU.

The European Parliament and the Council of the European Union are currently working to issue new directives regarding the definitions of "Enriched foods" in order to regulate the production and the market of nutritionally adequate products. Regulation 1924/2006/CE, already in force in Member States, harmonises the provisions laid down by law which relate to nutrition and health claims in order to ensure the effective functioning of the internal market while providing a high level of consumer protection. The European Commission has the task to state, by 19 January 2009, the nutritional profiles that have been approved by the scientific evaluation of European Food Safety Authority (EFSA), as criteria that food products must satisfy in order to receive any type of health rating. The mentioned regulation makes it possible to indicate the product's role in the reduction of risk of certain diseases; in addition, any misleading label or promotion is forbidden and it is necessary to specify that the mentioned disease is caused by multiple risk factors and intervening on one of them may not have any beneficial effect (EU Regulation 1924, 2006).

In Canada and Japan, food with health promoting effects must follow a scientific protocol that proves the claimed properties before they are labelled as functional and nutraceuticals. Japan has the world's first policy of legally permitting the commercialization of numerous functional foods and in 1991 the Ministry of Health and Welfare approved the term FOSHU (Foods for Specific Health Use) which is officially used to designate foods that have enough scientific evidence to support health claims (Hartman and Meisel, 2007). Japanese food industries have a prominent as well as innovative role in the functional food sector; until 2007 FOSHU approved products numbered 755. The gastrointestinal health claims category represented the majority (51%) of FOSHU sales (Japan Health Food and Nutrition Food Association, 2007).

In the USA, where the largest market of this sector is recorded, the term nutraceuticals has been coined with similar attributes as those of functional foods, with no legal difference. The pertinent American authority in this field is the Food and Drug Administration (FDA), which decides whether a health claim meets the significant scientific agreement standards with "reasonable certainty of no harm"(US CFR 190.6, 2008).

In 2006 a non profit organization separate from government, the US Pharmacopeial (USP) incorporated the Food Chemicals Codex (FCC) by means of elected volunteer experts and set reliable quality standards (including analytical tests, procedures and acceptance criteria) which manufacturers and regulators globally rely on. Although these public standards are not always recognized in Food and Drug Administration regulations, there is a proactive help to ensure the highest quality of functional foods in public commerce (Griffiths *et al.,* 2009).

Conclusions

This paper reviews the main studies performed on biopeptides in dairy products. As pointed out by the extensive experimental data, regular consumption of foods containing such peptides may bring health benefits or contribute to the treatment of some form of diseases. To date, the most investigated peptides appear to be those with hypotensive activity, probably because they are aimed at the numerous consumers affected by hypertension, a pathology of high social relevance in developed countries.

The occurrence of biological active peptides in dairy products is now well established, but numerous scientific and technological issues have to be resolved before biopeptides can be optimally exploited for human nutrition and health.

Most of the claimed physiological actions of milk biopeptides have been carried out *in vitro* or in animal model systems; indeed, some of these hypothesized functions remain to be proven in human studies as well as in human cell culture models in order to give evidence of their health enhancing effect. A novel approach appears to be the investigation of milk and dairy products directly subjected to human gastric and duodenal juices to mimic a normal human digestion.

Additional, in-depth molecular studies may likely help in explaining the complex mechanisms by which biopeptides exert their activity and, in particular, proteomics offers a cutting edge approach in studying the impact of proteins and peptides on the gene expression, providing a spin-off for nutrition.

At the same time, we deem necessary further clinical studies able to assess the effect of bioactive peptides on human physiology in a long term view.

An important challenging task is still the identification of novel bioactive sequences and a desirable perspective may be to orient research towards non-bovine milks as precious sources of biopeptides. The minor species, such as small ruminants, water buffalo, camelids and equids are also worthy of investigation. In order to obtain a preliminary screening in finding a definite biopeptide within major proteins, an interesting tool could be the use of softwares and databases of bioactive peptide sequences (Zamyatnin *et al*., 2006; Shtatland *et al.,* 2007). These applications, such as BIOPEP (Minkiewicz *et al*., 2008), enable the building of profiles of potential precursors of bioactive peptides inside known protein sequences and make it possible to predict the bonds susceptible to hydrolysis by endopeptidases in a protein chain.

Once identified, the development of suitable techniques for the separation, characterization and quantification of those compounds is thus made necessary in order to transfer their biological effects and functional properties into food applications. Until now, membrane separation techniques (such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis) have provided the best technology available for the enrichment of peptides with a specific molecular weight range or charge. Different techniques can also be used in combination (i.e. peptide fractions obtained from membrane filtration can be further purified using HPLC to achieve higher separations).

However, commercial production of bioactive peptides from milk proteins has been limited by the low concentration found in dairy products and by the lack of large-scale technologies (Korhonen and Pihlanto, 2007).

The main technological problems to overcome are linked to the development of enriched products and of methods to retain and store the biopeptides in a suitable matrix, in the form of ingredients or nutraceuticals, guaranteeing their activity for a certain period; micro encapsulation with edible coatings is a potential solution (Korhonen, 2002).

On the other hand, it is assumed that peptides can be more reactive than proteins due to their lower molecular weight. Consequently, although the possibilities for designing new dietary products look promising, testing their safety is mandatory and should include the absence of toxicity, cytotoxicity and allergenicity (Korhonen and Pihlanto, 2006). Strict and unambiguous legislation is therefore necessary to ensure the protection of consumers from potentially harmful or misleading products.

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