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SCIENCE DIRECT®
International Dairy Journal 16 (2006) 945–960
INTERNATIONAL DAIRY JOURNAL

Review

Bioactive peptides: Production and functionality

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Abstract

Milk proteins exert a wide range of nutritional, functional and biological activities. Many milk proteins possess specific biological properties that make these components potential ingredients of health-promoting foods. Increasing attention is being focused on physiologically active peptides derived from milk proteins. These peptides are inactive within the sequence of the parent protein molecule and can be liberated by (1) gastrointestinal digestion of milk, (2) fermentation of milk with proteolytic starter cultures or (3) hydrolysis by proteolytic enzymes. Milk protein derived peptides have been shown in vivo to exert various activities affecting, e.g., the digestive, cardiovascular, immune and nervous systems. Studies have identified a great number of peptide sequences with specific bioactivities in the major milk proteins and also the conditions for their release have been determined. Industrial-scale technologies suitable for the commercial production of bioactive milk peptides have been developed and launched recently. These technologies are based on novel membrane separation and ion exchange chromatographic methods being employed by the emerging dairy ingredient industry. A variety of naturally formed bioactive peptides have been found in fermented dairy products, such as yoghurt, sour milk and cheese. The health benefits attributed to peptides in these traditional products have, so far, not been established, however. On the other hand, there are already a few commercial dairy products supplemented with milk protein-derived bioactive peptides whose health benefits have been documented in clinical human studies. It is envisaged that this trend will expand as more knowledge is gained about the multifunctional properties and physiological functions of milk peptides.

Keywords: Milk proteins; Bioactive peptides; Hydrolysis; Fermentation; Enrichment; Functionality; Health effects

Contents

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1. Introduction

The role of proteins as physiologically active components in the diet is being increasingly acknowledged. Many of the proteins that occur naturally in raw food materials exert their physiological action either directly or upon enzymatic hydrolysis in vitro or in vivo. In recent years it has been recognized that dietary proteins provide a rich source of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and can be released in three ways: (a) through hydrolysis by digestive enzymes, (b) through hydrolysis by proteolytic microorganisms and (c) through the action of proteolytic enzymes derived from microorganisms or plants. It is now well established that physiologically active peptides are produced from several food proteins during gastrointestinal digestion and fermentation of food materials with lactic acid bacteria. The production and properties of bioactive peptides have been reviewed in many recent articles (FitzGerald & Meisel, 2003; Korhonen & Pihlanto, 2003a; Li, Le, Shi, & Shrestha, 2004; Pihlanto & Korhonen, 2003). Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). Upon oral administration, bioactive peptides, may affect the major body systems—namely, the cardiovascular, digestive, immune and nervous systems (Fig. 1)—depending on their amino acid sequence. For this reason, the potential of distinct dietary peptide sequences to promote human health by reducing the risk of chronic diseases or boosting natural immune protection has aroused a lot of scientific interest over the past few years. These beneficial health effects may be attributed to numerous known peptide sequences exhibiting, e.g., antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory activities (FitzGerald & Meisel, 2003; Korhonen & Pihlanto, 2003a; Shimizu, 2004). 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 & FitzGerald, 2003). Today, milk proteins are considered the most important source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products, as reviewed in many recent articles (Clare & Swaisgood, 2000; FitzGerald, Murray, & Walsh, 2004; Kilara & Panyam, 2003; Korhonen & Pihlanto-Leppälä, 2001; Korhonen & Pihlanto, 2003b, c; Korhonen & Pihlanto-Leppälä, 2004; Matar, LeBlanc, Martin, & Perdigón, 2003; Meisel, 1998, 2001, 2004; Schanbacher, Talhouk, Murray, Gherman, & Willet, 1998; Silva & Malcata, 2005).

This paper reviews the current knowledge about bioactive peptides derived from milk proteins, with emphasis on their production, occurrence in fermented dairy products, physiological functionality and potential use for health promotion.

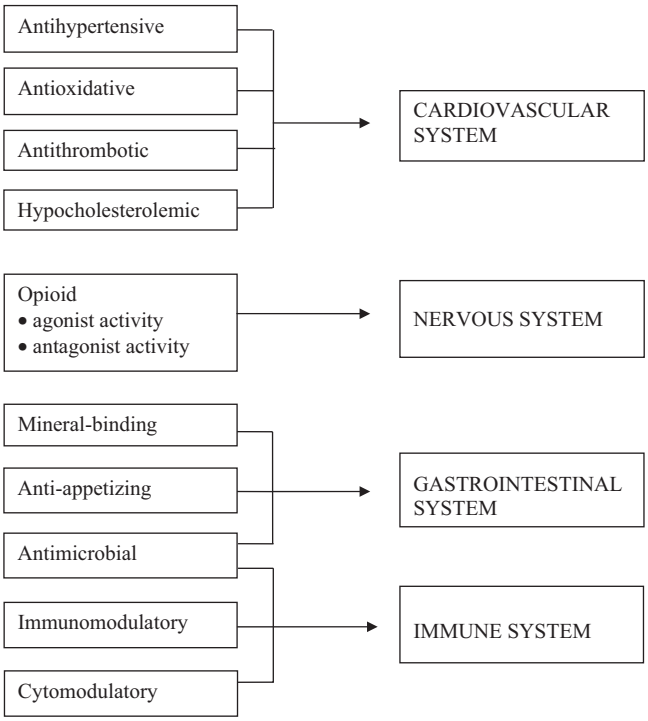

Fig. 1. Physiological functionality of milk-derived bioactive peptides.

2. Production of bioactive peptides

Basically, biologically active peptides can be produced from precursor milk proteins in the following ways: (a) enzymatic hydrolysis by digestive enzymes, (b) fermentation of milk with proteolytic starter cultures, (c) proteolysis by enzymes derived from microorganisms or plants. In many studies, combination of (a) and (b) or (a) and (c), has proven effective in generation of short functional peptides (Korhonen & Pihlanto, 2003b). Examples of bioactive peptides produced by the above treatments are given below.

2.1. Enzymatic hydrolysis

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. Many of the known bioactive peptides have been produced using gastrointestinal enzymes, usually pepsin and trypsin. Angiotensin-converting enzyme (ACE)-inhibitory peptides and calcium-binding phosphopeptides (CPPs), for example, are most commonly produced by trypsin (FitzGerald et al., 2004: Gobbetti, Minervini, & Rizzello, 2004; Meisel & FitzGerald, 2003; Vermeirssen, van Camp, & Verstraete, 2004; Yamamoto, Ejiri, & Mizuno, 2003). Moreover, ACE-inhibitory peptides have recently been identified in the tryptic hydrolysates of bovine α_{s2}-casein (Tauzin, Miclo, & Gaillard, 2002) and in bovine, ovine and caprine κ-casein macropeptides (Manso & López-Fandino, 2003).

Other digestive enzymes and different enzyme combinations of proteinases—including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources—have also been utilized to generate bioactive peptides from various proteins (Kilara & Panyam, 2003; Korhonen & Pihlanto, 2003a).

Apart from conventional production of peptides from natural protein sources by proteolytic enzymes, recombinant DNA techniques have been experimented for the production of specific peptides or their precursors in microorganisms. Kim, Yoon, Yu, Lönnerdal, and Chung (1999) succeeded in expressing recombinant human α_{s1}-casein in *Escherichia coli* and in purifying it. The trypsin digest of this protein was found to contain several ACE-inhibitory peptides.

2.2. Microbial fermentation

Many industrially utilized dairy starter cultures are highly proteolytic. Bioactive peptides can, thus, be generated by the starter and non-starter bacteria used in the manufacture of fermented dairy products. The proteolytic system of lactic acid bacteria (LAB), e.g. *Lactococcus lactis*, *Lactobacillus helveticus* and *Lb. delbrueckii* ssp. *bulgaricus*, is already well characterized. This system consists of a cell wall-bound proteinase and a number of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases (Christensen, Dudley, Pederson, & Steele, 1999). Rapid progress has been made in recent years to elucidate the biochemical and genetic characterization of these enzymes. The fact that the activities of peptidases are affected by growth conditions makes it possible to manipulate the formation of peptides to a certain extent (Williams, Noble, Tammam, Lloyd, & Banks, 2002). Table 1 gives a list of experimental studies on the release of bioactive peptides upon fermentation of milk using different live proteolytic microorganisms or proteolytic enzymes derived from such microorganisms.

Many recent articles and book chapters have reviewed the release of various bioactive peptides from milk proteins through microbial proteolysis (Gobbetti et al., 2004; Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002; Korhonen & Pihlanto-Leppälä, 2001, 2004; Matar et al., 2003). Most of these studies report the production of ACE-inhibitory or antihypertensive peptides, and immunomodulatory, antioxidative and antimicrobial peptides have also been identified. *Lb. helveticus* is widely used as a dairy starter in the manufacture of traditional fermented milk products, such as Emmental cheese and highly proteolytic *Lb. helveticus* strains capable of releasing ACE-inhibitory peptides, in particular, have been demonstrated in several studies. The best known ACE-inhibitory peptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), have been identified in milk fermented with *Lb. helveticus* strains (Nakamura, Yamamoto, Sakai, Okubo et al., 1995; Sipola, Finckenberg, Korpela, Vapaatalo, & Nurminen, 2002). Pihlanto-Leppälä, Rokka, and Korhonen (1998) studied the potential formation of ACE-inhibitory peptides from cheese whey and caseins during fermentation with various commercial dairy starters used in the manufacture of yoghurt, ropy milk and sour milk. No ACE-inhibitory activity was observed in these hydrolysates. Further digestion of the above samples with pepsin and trypsin resulted in the release of several strong ACE-inhibitory peptides derived primarily from α_{s1}-casein and β-casein. Gobbetti, Ferranti, Smacchi, Goffredi, and Addeo (2000)

Table 1
Examples of bioactive peptides released from milk proteins by various microorganisms and microbial enzymes

Micro-organisms used	Precursor protein[a]	Peptide sequence	Bioactivity	References
Lactobacillus helveticus, *Saccharomyces cerevisiae*	β-cn, κ-cn	Val-Pro-Pro, Ile-Pro-Pro	ACE inhibitory, antihypertensive	Nakamura et al. (1995); Nakamura, Yamamoto, Sakai, and Takano (1995)
Lactobacillus GG enzymes + pepsin & trypsin	β-cn, α_{s1}-cn	Tyr-Pro-Phe-Pro, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Thr-Met-Pro-Leu-Trp	Opioid, ACE inhibitory, immunostimulatory	Rokka et al. (1997)
Lb. helveticus CP90 proteinase	β-cn	Lys-Val-Leu-Pro-Val-Pro-(Glu)	ACE inhibitory	Maeno et al. (1996)
Lb. helveticus CPN 4	Whey proteins	Tyr-Pro	ACE inhibitory	Yamamoto et al. (1999)
Lb. delbrueckii subsp. *bulgaricus* SS1 *Lactococcus lactis* subsp. *cremoris* FT4	β-cn, κ-cn	Many fragments	ACE inhibitory	Gobbetti et al. (2000)
Lb. delbrueckii subsp. *bulgaricus* IFO13953	κ-cn	Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met	Antioxidative	Kudoh et al. (2001)
Lb. rhamnosus +digestion with pepsin and Corolase PP	β-cn	Asp-Lys-Ile-His-Pro-Phe, Tyr-Gln-Glu-Pro-Val-Leu, Val-Lys-Glu-Ala-Met-Ala-Pro-Lys	ACE inhibitory Antioxidative	Hernández-Ledesma et al. (2004a)
Lb. delbrueckii subsp. *bulgaricus*	β-cn	Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly Pro-Ile	ACE inhibitory	Ashar and Chand (2004)
Streptococcus thermophilus+*Lc. lactis* subsp. *lactis* biovar. *diacetylactis*	β-cn	Ser-Lys-Val-Tyr-Pro	ACE inhibitory	Ashar and Chand (2004)
Lb. helveticus ICM 1004 cell-free extract	Skim milk hydrolysate	Val-Pro-Pro, Ile-Pro-Pro	ACE inhibitory	Pan et al. (2004)

[a]Abbreviations: cn = casein, ACE = angiotensin I-converting enzyme.

demonstrated the formation of ACE-inhibitory peptides with two dairy strains, *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*, after fermentation of milk separately with each strain for 72 h. The most inhibitory fractions of the fermented milk mainly contained β-casein-derived peptides with inhibitory concentration (IC_{50}) values ranging from 8.0 to 11.2 $\mu g\,mL^{-1}$. Yamamoto, Maeno, and Takano (1999) identified an ACE-inhibitory dipeptide (Tyr-Pro) from a yoghurt-like product fermented with *Lb. helveticus* CPN4 strain. This peptide sequence is present in all major casein fractions, and its concentration was found to increase during fermentation, reaching a maximum concentration of 8.1 $\mu g\,mL^{-1}$ in the product. Fuglsang, Rattray, Nilsson, and Nyborg (2003) tested a total of 26 strains of wild-type LAB, mainly belonging to *Lc. lactis* and *Lb. helveticus*, for their ability to produce a milk fermentate with ACE inhibitory activity. All test strains produced ACE inhibitory substances in varying amounts, and two of the strains exhibited high ACE inhibition and a high OPA index, which correlates well with peptide formation. The inhibitory effect of active fermentates on in vivo ACE activity was demonstrated in normotensive rats. More recently, Ashar and Chand (2004) identified an ACE-inhibitory peptide from milk fermented with *Lb. delbrueckii* ssp. *bulgaricus*. The peptide showed the sequence Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly Pro-Ile from β-casein with an IC_{50} value of 1.7 $mg\,mL^{-1}$. In combination with *Streptococcus thermophilus* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*, a peptide structure with a sequence of Ser-Lys-Val-Tyr-Pro was obtained from β-casein with an IC_{50} value of 1.4 $mg\,mL^{-1}$. Both peptides were markedly stable to digestive enzymes, acidic and alkaline pH, as well as during storage at 5 and 10 °C for 4 days.

A number of studies have demonstrated that hydrolysis of milk proteins by digestive and/or microbial enzymes may produce peptides with immunomodulatory activities (Gill, Doull, Rutherfurd, & Cross, 2000). Sütas, Hurme, and Isolauri (1996) demonstrated that digestion of casein fractions with both pepsin and trypsin produced peptides that provoked immunomodulatory effects on human blood lymphocytes in vitro. Peptides derived from total casein and α_{s1}-casein mainly suppressed the proliferation of lymphocytes, while those derived from β- and κ-casein primarily stimulated the proliferation rate. When the caseins were hydrolysed by enzymes isolated from a probiotic strain of *Lactobacillus GG* var. *casei* prior to pepsin–trypsin treatment, all hydrolysate fractions were immunosuppressive and the highest activity was again found in α_{s1}-casein. The same hydrolysates also down-regulated in vitro the generation of interleukin-4 by lymphocytes (Sütas et al., 1996). Matar, Valdez, Medina, Rachid, and Perdigón (2001) fed milk fermented with a *Lb. helveticus* strain to mice for 3 days and detected significantly higher numbers of IgA secreting cells in their intestinal mucosa, compared with control mice fed with similar milk incubated with a non-proteolytic variant of the same strain. The immunostimulatory effect of fermented milk was attributed to peptides released from the casein fraction. These results suggest that LAB may modulate the immunological properties of milk proteins prior to or after oral ingestion of the product. Such modulation may be beneficial, e.g. in the down-regulation of hypersensitivity reactions to ingested proteins in patients with food protein allergies.

In addition to live microorganisms, proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins. Yamamoto, Akino, and Takano (1994) reported that casein hydrolysed by the cell wall-associated proteinase from *Lb. helveticus* CP790 showed antihypertensive activity in spontaneously hypertensive rats (SHR). Several ACE-inhibitory peptides and one antihypertensive peptide were isolated from the hydrolysate. Maeno, Yamamoto, and Takano (1996) hydrolysed casein using the same proteinase and identified a β-casein-derived antihypertensive peptide Lys-Val-Leu-Pro-Val-Pro-Gln, which was shown to be dose-dependent in a rat model at a dosage level from 0.2 to 2 mg of peptide per kg body weight. In a recent study, Mizuno, Nishimura, Matsuura, Gotou, and Yamamoto (2004) measured the ACE-inhibitory activity of casein hydrolysates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease isolated from *Aspergillus oryzae* showed the highest ACE-inhibitory activity in vitro per peptide. The *A. oryzae* peptide had the dose-dependent antihypertensive effect in a rat model with spontaneously hypertensive rats (SHR).

Most of the documented ACE-inhibitory peptides are usually short peptides with a proline residue at the carboxyl terminal end. Also, proline is known to be resistant to degradation by digestive enzymes and may pass from the small intestines into the blood circulation in the sequence of short peptides (Yamamoto et al., 2003). This hypothesis is supported by a recent study by Pan, Luo, and Tanokura (2004) who hydrolysed skimmed milk with a cell-free extract of *Lb. helveticus* JCM1004 and purified the antihypertensive tripeptides VPP and IPP from the hydrolysate with three runs of HPLC. The IC_{50} values of the peptides were 9.13 ± 0.21 and 5.15 ± 0.17 μM, respectively. A significant ($p < 0.01$) decrease in systolic blood pressure in SHR was measured after a single gastric intubation of VPP or IPP at 8 or 4 h, respectively. Furthermore, Ueno, Mizuno, and Yamamoto (2004) purified and characterized an endopeptidase from *Lb. helveticus* CM4 and demonstrated that this peptidase can generate the above two antihypertensive peptides using synthetic pro-peptides as a substrate.

2.3. Fractionation and enrichment of bioactive peptides

Commercial production of bioactive peptides from milk proteins has been limited by a lack of suitable large-scale technologies. Until now, membrane separation techniques have provided the best technology available for the

enrichment of peptides with a specific molecular weight range (Korhonen & Pihlanto, 2003b). Ultrafiltration is routinely employed to enrich bioactive peptides from protein hydrolysates. Enzymatic hydrolysis can be performed through conventional batch hydrolysis or continuous hydrolysis using ultrafiltration membranes. The traditional batch method has several disadvantages, such as the relatively high cost of the enzymes and their inefficiency as compared with a continuous process.

Use of enzymatic membrane reactors for continuous production of specific peptide sequences was introduced during the 1990s. It has already been widely applied for total conversion of food proteins of various origins to hydrolysates with improved nutritional and/or functional properties (Martin-Orue, Henry, & Bouhallab, 1999; Perea & Ugalde, 1996). Ultrafiltration membrane reactors have been shown to improve the efficiency of enzyme-catalysed bioconversion and to increase product yields, and they can be easily scaled up. Furthermore, ultrafiltration membrane reactors yield a consistently uniform product with desired molecular mass characteristics. Continuous extraction of bioactive peptides in membrane reactors has been mainly applied to milk proteins. Bouhallab and Touzé (1995) employed this technique for the recovery of antithrombotic peptides derived from hydrolyzed caseinomacropeptide (CMP). Gauthier and Pouliot (1996) combined enzymatic hydrolysis and ultrafiltration in order to produce emulsifying peptides from β-lactoglobulin (β-lg). Bordenave, Sannier, Ricart, and Piot (1999) demonstrated that α-lactorphin could be successfully generated with continuous hydrolysis of goat whey in an ultrafiltration reactor. On the other hand, they noted severe fouling problems with peptide-membrane interactions, especially in ultrafiltration of casein hydrolysates. Righetti, Nembri, Bossi, and Mortarino (1997) proposed a multicompartment enzyme reactor operating under an electric field for the continuous hydrolysis of milk proteins. This technique allowed for the continuous harvesting of some biologically active peptides, such as phosphopeptides and precursors of casomorphins from the tryptic digest of β-casein.

Stepwise ultrafiltration using cut-off membranes of low molecular mass have been found useful for separating out small peptides from high molecular mass residues and remaining enzymes. Turgeon and Gauthier (1990) used a two-step ultrafiltration process and were able to produce a mixture of polypeptides and a fraction rich in small peptides with a molecular mass below 2000 Da. Pihlanto-Leppälä, Koskinen, Paakkari, Tupasela, and Korhonen (1996) applied selective ultrafiltration membranes (30 and 1 kDa) for the enrichment of opioid peptides (α- and β-lactorphin) from pepsin-hydrolysed α-lactalbumin (α-la) and from pepsin- and trypsin-hydrolysed β-lg, respectively. The same technique has been successfully used to enrich ACE-inhibitory peptides from purified α-la and β-lg (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000).

Several ion exchange chromatographic methods have been developed for the enrichment of CPPs from casein hydrolysates, but the production costs of this technique were prohibitive for large-scale operation. Ellegård, Gammelgård-Larsen, Sörensen, and Fedosov (1999) developed a process-scale method for the isolation of high-purity CPPs using acid precipitation, diafiltration and anion-exchange chromatography. Recently, ion exchange membrane chromatography has emerged as a promising technique for the enrichment of peptide fractions from protein hydrolysates. Recio and Visser (1999) described a method where the protein of interest was concentrated within a chromatographic medium and hydrolysed in situ by an appropriate enzyme. The resulting active peptides were retained on the ion exchanger, while the other peptides were washed out. Finally, the fraction containing the active peptides was eluted from the chromatographic medium. With this method it was possible to isolate and enrich cationic antibacterial peptides from lactoferrin and α_{s2}-casein, as well as negatively charged phosphopeptides from β-casein. The advantages of this process are that isolation of the precursor protein is unnecessary and the enzyme used in this process can be recovered. This technique provides new possibilities for enriching peptides with a low molecular mass, and can be easily up-scaled to yield gram or even kilogram quantities (Recio, Floris, & Visser, 2000).

3. Occurrence of bioactive peptides in dairy products

It is now well documented that bioactive peptides can be generated during milk fermentation with the starter cultures traditionally employed by the dairy industry. As a result, peptides with various bioactivities can be found in the end-products, such as various cheese varieties and fermented milks (for reviews see Gobbetti et al., 2002; Korhonen & Pihlanto-Leppälä, 2001; Korhonen & Pihlanto, 2003b, c; Korhonen & Pihlanto-Leppälä, 2004; Matar et al., 2003; Meisel & Bockelmann, 1999). These traditional dairy products may under certain conditions carry specific health effects when ingested as part of the daily diet. Table 2 lists a number of studies which have established the occurrence of peptides in various fermented milk products. Some of these studies are described in more detail hereunder.

A great variety of peptides are formed during cheese ripening, many of which have been shown to exert biological activities. CPPs have been found as natural constituents in Comté and Cheddar cheese (Roudot-Algaron, LeBars, Kerhoas, Einhorn, & Gripon, 1994; Singh, Fox, & Healey, 1997). Furthermore, secondary proteolysis during cheese ripening may lead to the formation of other bioactive peptides, and the occurrence of bioactivity appears to be dependent on the ripening stage of the cheese. Meisel, Goepfert, and Günther (1997) detected higher ACE-inhibitory activities in middle-aged Gouda cheese than in short-termed or long-termed ripened cheese. These results suggest that the concentration of active peptides in cheese increases with cheese maturation,

Table 2
Bioactive peptides identified in fermented milk products

Product	Examples of identified bioactive peptides[a]	Bioactivity	References
Cheese type			
Parmigiano–Reggiano	β-cn *f*(8–16), *f*(58–77), α_{s2}-cn *f*(83–33)	Phosphopeptides, precursor of β-casomorphin	Addeo et al. (1992)
Cheddar	α_{s1}- and β-casein fragments	Several phosphopeptides	Singh et al. (1997)
Italian varieties: Mozzarella, Crescenza, Italico, Gorgonzola	β-cn *f*(58–72)	ACE inhibitory	Smacchi and Gobbetti (1998)
Gouda	α_{s1}-cn *f*(1–9), β-cn *f*(60–68)	ACE inhibitory	Saito et al. (2000)
Festivo	α_{s1}-cn *f*(1–9), *f*(1–7), *f*(1–6)	ACE inhibitory	Ryhänen et al. (2001)
Emmental	α_{s1}- and β-casein fragments	Immunostimulatory, several phosphopeptides, antimicrobial	Gagnaire et al. (2001)
Manchego	Ovine α_{s1}-, α_{s2}- and β-casein fragments	ACE inhibitory	Gomez-Ruiz et al. (2002)
Emmental	Active peptides not identified	ACE inhibitory	Parrot et al. (2003)
Fermented milks			
Sour milk	β-cn *f*(74–76, *f*(84–86), κ-cn *f*(108–111)	Antihypertensive	Nakamura et al. (1995 a)
Yoghurt	Active peptides not identified	Weak ACE-inhibitory	Meisel et al. (1997)
Dahi	Ser-Lys-Val-Tyr-Pro	ACE inhibitory	Ashar and Chand (2004)

[a]Abbreviations: α_{s1}-cn = α_{s1}-casein, β-cn = β-casein, κ-cn = κ-casein.

but starts to decline when proteolysis exceeds a certain level. Accordingly, ACE-inhibitory activity was low in products having a low degree of proteolysis, such as yoghurt, fresh cheese and quark. The above findings are consistent with the results obtained by Ryhänen, Pihlanto-Leppälä, and Pahkala (2001), who observed that ACE-inhibitory peptides developed gradually during cheese ripening and their concentration was highest in a Gouda-type cheese at the age of 13 weeks, declining slowly thereafter. Saito, Nakamura, Kitazawa, Kawai, and Itoh (2000) detected ACE-inhibitory activity in several cheese varieties and measured the highest activity in Gouda cheese aged 2 years. In feeding experiments on SHR, the decrease in systolic blood pressure was statistically significant with four cheese varieties (Gouda, Blue, Edam and Havarti) at 6 h after gastric intubation. Several peptides were isolated and identified from 8-month-old Gouda cheese, and two peptides derived from α_{s1}-casein *f*(1–9) and β-casein *f*(60–68), respectively, showed potent ACE-inhibitory activity. For Manchego cheese, which is prepared from ovine milk, only cheese that was at least 15 days old showed ACE-inhibitory activity that was comparatively low (Gomez-Ruiz, Ramos, & Recio, 2002). Furthermore, this inhibitory activity decreased during the first 4 months, increased when proteolysis advanced, and decreased again in 12-month-old cheese. Altogether, 22 peptide fragments were identified in the chromatographic fractions, corresponding to the sequences of ovine α_{s1}-, α_{s2}- and β-casein. In another study, Gagnaire, Molle, Herrouin, and Leonil (2001) identified a total of 91 peptides in Emmental cheese, 28 of which showed various bioactivities in vitro, e.g. mineral-carrying, antimicrobial, antihypertensive and immunostimulatory activities. Besides the well-known action of plasmin on β- and α_{s2}-caseins, two other proteinases seem to be involved in the hydrolysis of α_{s1}-casein in Emmental cheese: cathepsin D originating from milk and cell-envelope proteinase from thermophilic starters. Moreover, peptidases released from both starter and non-starter LAB seem to contribute to the formation of bioactive peptides throughout the ripening period. Perhaps due to degradation during the ripening process, active opioid peptides have not been detected in mature Cheddar cheese (Muehlenkamp & Warthesen, 1996). On the other hand, Sabikhi and Mathur (2001) found small quantities of β-casomorphin-3 in Edam cheese during ripening, whereas longer casomorphins were not detectable. These studies suggest that the presence of the bioactive peptides which are naturally formed in cheese depends on the equilibrium between their formation and the degradation exerted by the proteolytic systems involved in the ripening process. Interestingly, peptides isolated from several Italian cheese varieties have been shown to inhibit the activity of the proteolytic enzymes of certain strains of LAB and the activity of ACE (Smacchi & Gobbetti, 1998). Furthermore, the same peptides were found to be inhibitory to microbial enzymes, such as thermostable proteinases from the psychrotrophic *Pseudomonas fluorescens*, which causes bitterness in ultra-high temperature (UHT) treated milk, contributes to the age gelation of UHT milk, and reduces the shelf-life of dairy products.

Apart from generation during the ripening process, more bioactive peptides are likely to be formed in the gastrointestinal tract upon ingestion of a piece of cheese. This was demonstrated under in vitro conditions by Parrot, Degraeve, Curia, and Martial-Gros (2003) who showed that consecutive digestion of the water-soluble extract (WSE) of

Emmental cheese with pepsin and trypsin, respectively, induced an increase in ACE inhibition as compared with undigested WSE. On the other hand, a 10 kDa ultrafiltered WSE lost a part of its ACE-inhibitory activity after the above digestion process. These results suggest that the generation of ACE-inhibitory peptides during digestion depends on the molecular weight of the precursor peptides present in the cheese. Hernández-Ledesma, Amigo, Ramos, and Recio (2004a) evaluated the ACE-inhibitory activity of several commercial fermented milks and fresh cheeses and found that most of these products showed moderate inhibitory activity. The ACE-inhibitory activity of these commercial products remained stable or increased after simulated gastrointestinal digestion with pepsin and Corolase PP (from pig pancreas, showing mainly trypsin and chymotrypsin activities). Similar results were obtained in further studies by the same researchers (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004b) when a number of infant formulas were tested for potential ACE-inhibitory activity. Most of these products showed moderate inhibitory activity which increased when subjected to simulated digestion, except for two extensively hydrolysed milk protein-based formulas (one whey and one casein formula). These studies support the view that physiological digestion may promote the formation of bioactive peptides from the proteins and oligopeptides present in dairy products and that at least part of the active peptides survive the digestion process.

The occurrence of various bioactive peptides in fermented milks, e.g. yoghurt, sour milk and "Dahi", has been reported in many studies, as shown in Table 2. ACE-inhibitory, immunomodulatory and opioid peptides, e.g., have been found in yoghurt and in milk fermented with a probiotic *Lb. casei* ssp. *rhamnosus* strain (Rokka, Syväoja, Tuominen, & Korhonen, 1997). Most studies have employed strongly proteolytic *Lb. helveticus* strains for the production of antihypertensive peptides in fermented milk products (Gobbetti et al., 2004; FitzGerald et al., 2004). At present, at least two fermented sour-milk products containing the ACE-inhibitory tripeptides VPP and IPP have been launched commercially in Japan and Finland, respectively. The Japanese product "Calpis" is fermented with a culture containing *Lb. helveticus* and *S. cerevisiae* (Takano, 1998) and the Finnish product "Evolus" contains the same tripeptides produced by *Lb. helveticus* LBK-16H strain (Seppo, Kerojoki, Suomalainen, & Korpela, 2002). In animal model studies, single oral administration of these products has been shown to have an antihypertensive effect in SHR (Nakamura, Yamamoto, Sakai, & Takano, 1995; Sipola et al., 2002), and "Evolus" has also been demonstrated to prevent the development of hypertension in SHR (Sipola et al., 2001). As described in the following chapter, both of these fermented drinks have proven effective in the reduction of blood pressure in mildly hypertensive human subjects.

An increasing number of ingredients containing specific bioactive peptides based on casein or whey protein hydrolysates have been launched on the market within the past few years or are currently under development by international food companies. Such peptides possess, e.g., anticariogenic, antihypertensive, mineral-binding and stress-relieving properties. A few examples of these commercial ingredients and their applications are listed in Table 3.

4. Functionality of bioactive peptides

Dietary proteins are traditionally known to provide a source of energy and the amino acids essential for growth and maintenance of various body functions. In addition, they contribute to the physicochemical and sensory properties of protein-rich foods. In recent years, food proteins have gained increasing value due to the rapidly expanding knowledge about physiologically active peptides. Milk proteins provide a rich source of peptides which are latent until released and activated, e.g. during gastrointestinal digestion or milk fermentation. Once activated, these peptides are potential modulators of many regulatory processes in living systems. The primary and secondary structures of major human and bovine milk proteins are well characterized and the potential bioactivities of peptides released from these proteins are currently a subject of intensive research worldwide. There is now a considerable amount of scientific data to demonstrate that a wide range of milk peptides can regulate specific physiological functions in experimental animals and humans. These functions relate to general health conditions or a reduced risk of certain chronic diseases. Examples of milk peptides which have exerted physiological effects in animal model or human intervention studies are given in the following.

4.1. Regulation of the gastrointestinal system

Food-derived proteins and peptides may play important functions in the intestinal tract before hydrolysis to amino acids and subsequent absorption. These include regulation of digestive enzymes and modulation of nutrient absorption in the intestinal tract (Shimizu, 2004). In the case of the latter functions, the role of bioactive peptides has been disputed for a long time. The first reference to bioactive peptides in the scientific literature was made by Mellander in 1950, who suggested that casein-derived phosphorylated peptides, caseinophosphopeptides (CPPs), enhanced vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). Bovine α_{s1}-, α_{s2}- and β-casein contain phosphorylated regions which can be released by digestive enzymes. Specific CPPs can form soluble organophosphate salts and lead to enhanced calcium absorption by limiting the precipitation of calcium in the distal ileum. Published data on the effect of CPPs on mineral solubility and absorption are inconsistent, however, partly due to the diversity of the experimental approaches used (FitzGerald, 1998; Meisel & FitzGerald, 2003; Scholz-Ahrens &

Table 3
Commercial dairy products and ingredients with health or function claims based on bioactive peptides

Brand name	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-GMP	Whey protein isolate	κ-casein *f*(106–169) (Glycomacropeptide)	Prevention of dental caries, influence the clotting of blood, protection against viruses and bacteria	Davisco, USA
PRODIET F200/Lactium	Flavoured milk drink, confectionery, capsules	α_{s1}-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	α_{s1}-casein f (1–9), α_{s1}-casein f (1–7), α_{s1}-casein f (1–6)	No health claim as yet	MTT Agrifood Research Finland
Cysteine Peptide	Ingredient/hydrolysate	Milk protein derived peptide	Aids to raise energy level and sleep	DMV International, the Netherlands
C12	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

Schrezenmeir, 2000). Many animal and human studies have reported the presence of CPPs in vivo following ingestion of milk, fermented dairy products, casein and crude CPP preparations (Meisel & FitzGerald, 2003). The stomach and intestinal contents of adult humans fed milk or yoghurt have been found to contain CPPs (Chabance et al., 1998). Recently, CPPs were detected in the distal small ileum (ileum) of humans administered milk or crude CPP preparations orally (Meisel et al., 2003).

Since CPPs can bind and solubilize minerals, they have been considered physiologically beneficial in the prevention of osteoporosis, dental caries, hypertension and anemia. Conflicting results have been obtained in animal studies which have assessed the potential of CPPs to enhance mineral (primarily calcium) bioavailability. In general, animal studies using tracers have revealed a positive effect of CPPs on calcium absorption, whereas most of the balance studies have failed to find any effect of CPP addition (FitzGerald, 1998). In human studies, increased calcium and zinc absorption has been demonstrated in adults administered a rice-based infant gruel. This beneficial effect was abolished when the volunteers were fed cereal-based meals containing phytate (Hansen, Sandström, & Lönnerdal, 1996). More recently, Narva, Halleen, Väänänen, and Korpela (2004) showed that *Lb. helveticus*-fermented whey and the tripeptides VPP and IPP stimulated the proliferation of osteoblasts in vitro, whereas sour-milk whey and calcium had no effect. The fermented whey contained 26 mg L^{-1} of these peptides. No significant effects on osteoclast formation were observed in vitro with any of the studied products. Bouhallab et al. (2002) reported that a purified phosphopeptide (β-(f1–25)) exhibited a positive effect on iron bioavailability in vivo in a rat model and a mechanism of absorption of the caseinophosphopeptide bound iron was suggested by Pérès et al. (1999). More cell culture and human studies are, however, necessary to demonstrate the potential of CPPs and other peptides to enhance dietary mineral bioavailability and to modulate bone formation (Bouhallab & Bouglé, 2004). Another interesting property associated with CPPs is their potential to enhance mucosal immunity. This idea is supported by a study (Otani, Kihara, & Park, 2000) which showed that oral administration of a commercial CPP preparation enhanced the intestinal IgA levels of piglets. The anticariogenic effect of CPPs has been well documented in both human intervention and animal model studies (Meisel, 2001). CPPs can have an anticariogenic effect by promoting recalcification of tooth enamel, whereas glycomacropeptide (GMP) derived from κ-casein seems to contribute to the anticaries effect by

inhibiting the adhesion and growth of plaque-forming bacteria on oral mucosa (Brody, 2000; Malkoski et al., 2001). Various dental care products containing CPPs and/or GMP have been launched on the market in some countries.

Antimicrobial peptides have been identified from many milk protein hydrolysates (for reviews see Clare, Catignani, & Swaisgood, 2003; Floris, Recio, Berkhout, & Visser, 2003; Gobbetti et al. 2004; Pellegrini, 2003). The most studied are the lactoferricins, derived from bovine and human lactoferrin (Kitts & Weiler, 2003; Wakabayashi, Takase, & Tomita, 2003). Also, a few antibacterial peptides have been identified from α_{s1}-casein (Lahov & Regelson, 1996) and α_{s2}-casein (McCann et al., 2005). These peptides exhibit antimicrobial activity against various Gram-positive and -negative bacteria, e.g. *Escherichia, Helicobacter, Listeria, Salmonella* and *Staphylococcus,* yeasts and filamentous fungi. The disruption of normal membrane permeability is at least partly responsible for the antibacterial mechanism of lactoferricins. The physiological importance of antimicrobial milk peptides remains to be established, although it has been suggested that they may modulate the intestinal microflora when formed during milk digestion in vivo (Shimizu, 2004). On the other hand, these peptides may find interesting applications in the field of food safety and as pharmaceuticals.

Many studies over the last 10 years have attempted to establish the potential role of GMP and its non-glycosylated form, CMP, in regulation of intestinal functions (for reviews see Brody, 2000; Pihlanto & Korhonen, 2003; Manso & López-Fandino, 2004). CMP has been reported to inhibit gastric secretions and slows down stomach contractions. Further, it has been suggested that CMP stimulates the release of cholecystokinin (CKK), the satiety hormone involved in controlling food intake and digestion in the duodenum of animals and humans (Yvon, Beucher, Guilloteau, Le Huerou-Luron, & Corring, 1994). There is evidence that CMP triggers stimuli from intestinal receptors without being absorbed. Intact CMP has been detected in the stomach during digestion (Fosset et al., 2002) but, on the other hand, it has also been observed that GMP can be absorbed as intact and partially digested into the blood circulation of adult humans after milk or yoghurt ingestion (Chabance et al., 1998). Based on above studies, commercial products containing GMP have been launched on the market for the purpose of appetite control and weight management. However, the clinical efficacy of such products remains to be established. A study conducted with human adults for a short time period revealed that CMP had no effect on food energy intake or on subjective indicators of satiety (Gustafson, McMahon, Morrey, & Nan, 2001). On the other hand, GMP may have a beneficial role in modulating the gut microflora, as this macropeptide is known to promote the growth of bifidobacteria due to its carbohydrate (mainly sialic acid) content (Manso & López-Fandino, 2004).

4.2. Regulation of the nervous system

Peptides with opioid activity have been identified in various casein fractions hydrolyzed by digestive enzymes (Brantl, Teschemacher, Henschen, & Lottspeich, 1979; Pihlanto-Leppälä, Antila, Mäntsälä, & Hellman, 1994; Teschemacher, 2003). These opioid peptides are opioid receptor ligands with agonistic or antagonistic activities. Opioid receptors 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 and with exogenous opioids and opioid antagonists. Thus, orally administered opioid peptides may modulate absorption processes in the gut and influence the gastrointestinal function in two ways: first, by affecting smooth muscles, which reduces the transit time, and second, by affecting the intestinal transport of electrolytes, which explains their anti-secretory properties. The actual physiological effects of milk-derived opioid peptides remain, however, to be confirmed. β-Casein-derived opioid peptides (β-casomorphins) or their precursors 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 & FitzGerald, 2000, FitzGerald & Meisel, 2003). Opioid casein fragments have not been detected in the plasma of adult mammals and, therefore, it is suggested that only the neonatal intestine is permeable to casomorphins. Interestingly, a α_{s1}-casein-derived peptide f(91–100) has been demonstrated to possess anxiolytic-like stress-relieving properties in animal model and human studies (Lefranc, 2001). This peptide has been employed commercially as an ingredient, e.g. for confectionery and soft drinks.

4.3. Regulation of the cardiovascular system

The angiotensin I-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) has been associated with the renin-angiotensin system, which regulates peripheral blood pressure. Inhibition of this enzyme can exert an antihypertensive effect. A great number of ACE-inhibitory peptides have been isolated from the enzymatic digest of various milk proteins and they are, at present, the most studied group of bioactive peptides. Apart from ACE inhibition, milk peptides may exert antihypertensive effects also through other mechanisms, such as inhibition of the release of endothelin-1 by endothelial cells (Maes et al., 2004), stimulation of bradykinin activity (Perpetuo, Juliano, & Lebrun, 2003), enhancement of endothelium-derived nitric oxide production (Sipola et al., 2002) and enhancement of the vasodilatory action of binding to opiate receptors (Nurminen et al., 2000). This view is supported by the study of Fuglsang, Nilsson, and Nyborg (2003) who screened several milk-protein derived short peptides for their potential ACE inhibition and observed that in vitro 8 of 9 dipeptides were competitive inhibitors of ACE. Using three different in vivo models in rats for ACE inhibition, a

very moderate effect was observed for three only of the above active peptides. The authors suggested that there are probably also other mechanisms of action for the known milk derived inhibitors which are reported to be hypotensive. To exert an antihypertensive effect after oral ingestion, active peptides must be absorbed in an intact form from the intestine and further be resistant to degradation by plasma peptidases in order to reach the target sites. In fact, it has been demonstrated using monolayer-cultured human intestinal Caco-2 cells that the ACE-inhibitory tripeptide VPP can be transported intact through the cell layer via paracellular and transcellular routes, although a significant amount of the peptide is hydrolysed to amino acids by intracellular peptidases (Satake et al., 2002). It is also known that proline-containing peptides are generally resistant to degradation by digestive enzymes. Masuda, Nakamura, and Takano (1996) detected two ACE-inhibitory tripeptides (VPP and IPP) in the abdominal aorta of SHR after oral administration of sour milk containing these tripeptides. Furthermore, a dose-dependent antihypertensive effect has been established in animal model studies with SHR after single oral administration of small di- and tripeptides (Li et al., 2004). On the other hand, recent in vitro results by Walsh et al. (2004) indicated that β-lg f(142–148), the tryptic peptide Ala-Leu-Pro-Met-His-Ile-Arg which is known to be a potent inhibitor of ACE activity in vitro (Mullally, Meisel, & FitzGerald, 1997) is probably not sufficiently stable to gastrointestinal and serum proteinases and peptidases to act as an hypotensive agent in humans following oral ingestion. This conclusion was supported by the results showing that synthetic β-lg f(142–148) was rapidly degraded upon incubation with human serum. Furthermore, this peptide could not be detected by an enzyme immunoassay in the sera of two human volunteers following its oral ingestion or in sera from these volunteers subsequently spiked with β-lg f (142–148). Interestingly, it was shown in a previous study (Vermeirssen et al., 2002) that the same peptide was resistant to degradation during gastrointestinal passage, but was only transported at very low levels across Caco-2b monolayers. Furthermore, α-la f(50–53), i.e., the tetrapeptide, α-lactorphin (Tyr-Gly-Leu-Phe) which is known to produce an antihypertensive effect in vivo when administered subcutaneously to normotensive Wistar Kyoto and SHR rats (Nurminen et al., 2000) elicited no effect typical of active opioids in behavioural tests in mice after intraperitoneal administration (Ijäs et al., 2004). These studies would suggest that not all potent peptide inhibitors of ACE in vitro, may necessarily be antihypertensive in vivo.

Only a few among the great number of milk peptides identified as antihypertensive under in vitro conditions have so far proven clinically effective in animal and human studies (for reviews see FitzGerald et al., 2004; Gobbetti et al., 2004; Li et al., 2004; Takano, 2002; Vermeirssen et al., 2004; Yamamoto & Takano 1999; Yamamoto et al., 2003). In most of these studies moderate or significant reduction of blood pressure was observed after consumption of specific milk protein hydrolysates or fermented dairy products. Table 4 lists human clinical studies carried out using these products. In a placebo-controlled trial with mildly hypertensive subjects a significant reduction in blood pressure was recorded after daily ingestion for 4 weeks of 95 mL of ''Calpis'' sour milk containing the potent ACE-inhibitory peptides Vpp and IPP. It is noteworthy that the ingested dose of these peptides was small, only about 2.6 mg per day. Blood pressure was reverted gradually to pre-trial level after the intervention period ceased. No major changes in blood pressure were observed in the placebo group (Hata et al., 1996; Yamamoto et al., 2003). These results were supported by a recent double-blind randomized controlled study by Mizushima et al. (2004) in which the effect of ''Calpis'' was assessed in borderline hypertensive men upon oral administration of 160 g of the product for 4 weeks. Systolic blood pressure in the test group decreased significantly after 2 and 4 weeks of ingestion of ''Calpis''. No significant change in blood pressure was observed in the placebo group, which was administered unfermented acidified milk. Similar results were obtained with the ''Evolus'' product in two double-blind, placebo-controlled studies with mildly hypertensive subjects who ingested 150 mL of the product daily. ''Evolus'' was found to decrease both systolic and diastolic blood pressure during the 8-week and 21-week treatment periods, respectively. No such influence was reported in subjects with normal blood pressure

Table 4
In vivo human studies on bioactive peptides derived from milk proteins

Product administered	Peptide precursor/peptides identified[a]	Effect observed	References
Rice-based cereal gruel + Caseinophosphopeptides	Caseinophosphopeptides (CPP)	Improvement of calcium and zinc absorption	Hansen et al. (1996)
Tryptic casein hydrolysate	α_{s1}-casein	Reduction of blood pressure	Sekiya et al. (1992)
Sour milk	β-casein, κ-casein, Val-Pro-Pro/Ile-Pro-Pro	Reduction of blood pressure	Hata et al. (1996)
Sour milk	Val-Pro-Pro/Ile-Pro-Pro	Reduction of blood pressure	Seppo et al. (2003)
Sour milk	Val-Pro-Pro, Ile-Pro-Pro	Reduction of blood pressure	Mizushima et al. (2004)
Sour milk (Dahi)	β-casein, Ser-Lys-Val-Tyr-Pro	Reduction of blood pressure	Ashar and Chand (2004)

[a]Abbreviations: α_{s1}-cn = α_{s1}-casein, β-cn = β-casein, κ-cn = κ-casein.

(Seppo et al., 2002; Seppo, Jauhiainen, Poussa, & Korpela, 2003). Another recent placebo-controlled study (Ashar & Chand, 2004) tested the effect of "Dahi" fermented milk containing the ACE-inhibitory peptide Ser-Lys-Val-Tyr-Pro (SKVYP) on hypertensive subjects. The product was produced by fermentation of milk with *Lb. delbrueckii* subsp. *bulgaricus*, *Str. thermophilus* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*. The subjects received either 100 mL of the test product or the placebo product for 4 weeks. In the test group, a significant decline in systolic blood pressure was recorded after 2 and 4 weeks from the start-up of the trial. No significant change in blood pressure was noticed in the placebo group during the intervention period. The placebo product was prepared using the same starters as the test product, but these strains did not produce the above ACE-inhibitory peptide. Further studies are still required for a better understanding of the blood pressure reducing mechanisms of milk peptides and well controlled clinical human studies are needed to demonstrate the long-term physiological effects delivered by consuming such peptides.

Peptide sequences which inhibit the aggregation of blood platelets and the binding of the human fibrinogen γ–chain to platelet surface fibrinogen receptors have been identified in CMP, which is split from κ-casein in milk coagulation with rennin (Fiat et al., 1993). The potential physiological effects of these antithrombotic peptides have not been established, but such peptides have been detected in the plasma of newborn children after breastfeeding or ingestion of cow milk-based infant formulae (Chabance et al., 1995).

Nagaoka et al. (2001) identified a hypocholesterolemic peptide (Ile-Ile-Ala-Glu-Lys) from the tryptic hydrolysate of β-lg. This peptide suppressed cholesterol absorption by Caco-2 cells in vitro and elicited hypocholesterolemic activity in vivo in rats upon oral administration of the peptide solution. The mechanism of the hypocholesterolemic effect remains to be clarified.

4.4. Regulation of the immune system

Milk protein hydrolysates and peptides derived from caseins and major whey proteins can enhance immune cell functions, measured as lymphocyte proliferation, antibody synthesis and cytokine regulation (Gill et al., 2000). Of special interest are peptides released during milk fermentation with lactic acid bacteria, as these peptides have been found to modulate the proliferation of human lymphocytes, to down-regulate the production of certain cytokines and to stimulate the phagocytic activities of macrophages (for reviews see Matar et al., 2003; Meisel & FitzGerald, 2003). The protective effect of a casein-derived immunopeptide on resistance to microbial infection by *Klebsiella pneumoniae* has been demonstrated in mice (Migliore-Samour, Floc'h, & Jollés, 1989). Also, it has been suggested that immunomodulatory milk peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract (Korhonen & Pihlanto, 2003a) In this way immunomodulatory peptides may regulate the development of the immune system in newborn infants. Recently it was demonstrated that commercial whey protein isolates contain immunomodulating peptides which can be released by enzymatic digestion (Mercier, Gauthier, & Fliss, 2004). This information is of high relevance when developing infant formulas with optimized immunomodulatory properties.

Furthermore, immunopeptides formed during milk fermentation have been shown to contribute to the antitumor effects observed in many studies with fermented milks (Matar et al., 2003). The fact that CPPs have been shown to exert cytomodulatory effects is of particular interest in this context. Cytomodulatory peptides derived from casein fractions inhibit cancer cell growth or stimulate the activity of immunocompetent cells and neonatal intestinal cells (Meisel & FitzGerald, 2003). In addition, GMP and its derivatives have been shown to exhibit a range of immunomodulatory functions, such as immunosuppressive effects on the production of IgG antibodies (Monnai, Horimoto, & Otani, 1998; Manso & López-Fandino, 2004) and immunoenhancing effects on proliferation and phagocytic activities of human macrophagelike cells U937 (Li & Mine, 2004). On the basis of results obtained in mouse model studies, Matar et al. (2003) concluded that peptides released by bacterial proteolysis might have important implications in modulation of the host's immune response and have an impact on inhibition of tumor development. Further fundamental research is needed in this field in order to establish the physiological importance of multifunctional immunopeptides.

4.5. Other functionalities

Recent studies have shown that antioxidative peptides can be released from caseins in hydrolysis by digestive enzymes and in fermentation of milk with proteolytic LAB strains (Korhonen & Pihlanto, 2003a). Most of the identified peptides are derived from α_s-casein and have been shown to possess free radical-scavenging activities and to inhibit enzymatic and non-enzymatic lipid peroxidation (Rival, Boeriu, & Wichers, 2001; Rival, Fornaroli, Boeriu, & Wichers, 2001; Suetsuna, Ukeda, & Ochi, 2000). In the future, antioxidative peptides may find applications as ingredients in different fields, e.g. in the prevention of oxidation in fat-containing foodstuffs, cosmetics and pharmaceuticals. More studies are needed to demonstrate the potential health benefits of the antioxidative peptides formed during milk fermentation.

Many milk protein-derived peptides have more than one functional role; e.g., peptides from the sequence 60–70 of β-casein show immunostimulatory, opioid and ACE-inhibitory activities. This sequence has been defined as a strategic zone (Migliore-Samour & Jollés, 1988; Meisel, 1998). The sequence is protected from proteolysis because of its high hydrophobicity and the presence of proline

residues. Other examples of the multifunctionality of milk-derived peptides include the α_{s1}-casein fraction 194–199 showing immunomodulatory and ACE-inhibitory activity, the opioid peptides α- and β-lactorphin also exhibiting ACE-inhibitory activity and the CPPs, which possess immunomodulatory properties (Korhonen & Pihlanto, 2003a).

5. Future outlook

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. So far, antihypertensive, mineral-binding and anti-cariogenic peptides have been most studied for their physiological effects. A few commercial developments have been launched on the market and this trend is likely to continue alongside with increasing knowledge about the functionalities of the peptides. The optimal exploitation of bioactive peptides for human nutrition and health possesses an exciting scientific and technological challenge, while at the same time offering potential for commercially successful applications. 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. This approach has been taken initially at target group level but will ultimately address individuals. Bioactive peptides offer an excellent basis for the novel concept of "personalized nutrition". Many scientific, technological and regulatory issues must, however, be resolved before these substances can be optimally harnessed to this end.

Firstly, there is a need to develop novel technologies, such as chromatographic and membrane separation techniques, to enrich active peptide fractions from the hydrolysates of various food proteins (Korhonen, 2002; Mehra & Kelly, 2004; Tolkach & Kulozik, 2004; DeSilva, Stockmann, & Smithers, 2003). In addition to enzymatic hydrolysis, microbial fermentation provides a natural technology applicable for the production of bioactive peptides either from animal or plant proteins. The potential of this approach is already well demonstrated by the presence of bioactive peptides in fermented dairy products. Production of bioactive peptides from protein-rich raw materials may be scaled up to industrial level using controlled fermentation in bioreactors with known LAB. In the future, the commercial production of specific peptide sequences is likely to employ recombined enzyme technology and specific production strains or alternatively make use of peptidases isolated from suitable microorganisms.

Secondly, it is important to study the technological properties of active peptide fractions and to develop model foods that contain these peptides and retain their activity for a guaranteed period. It is recognized that, due to their lower molecular weight, peptides can be more reactive than proteins, and the peptides present in the food matrix may react with other food components. The interaction of peptides with carbohydrates and lipids, as well as the influence of the processing conditions (particularly heating) on peptide activity and bioavailability, should also be addressed (Korhonen, Pihlanto-Leppälä, Rantamäki, & Tupasela, 1998). In this respect, the possible formation of toxic, allergenic or carcinogenic substances warrants intensive research. Modern analytical methods need to be developed to study the safety of foodstuffs containing biologically active peptides.

Thirdly, molecular studies are needed to assess the mechanisms by which bioactive peptides exert their activities. For this approach, it is necessary to employ proteomics and associated technologies (O'Donnell, Holland, Deeth, & Alewood, 2004). By developing such novel facilities it will be possible to study the impact of proteins and peptides on the expression of genes and hence optimize the nutritional and health effects of these compounds. This research area is currently considered highly challenging and will revolutionize the protein research in the near future. The majority of the known bioactive peptides are not absorbed from the gastrointestinal tract into the blood circulation and their effect is, therefore, probably mediated directly in the gut lumen or through receptors on the intestinal cell wall. In this respect, the target function of the concerned peptide is of utmost importance. It is anticipated that such targets will be related to various lifestyle-related disease groups, such as cardiovascular diseases, cancers, diabetes, osteoporosis, stress and obesity. Bioactive peptides derived from milk proteins offer a promising approach for the promotion of health by means of a tailored diet and provide interesting opportunities to the dairy industry for expansion of its field of operation.

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