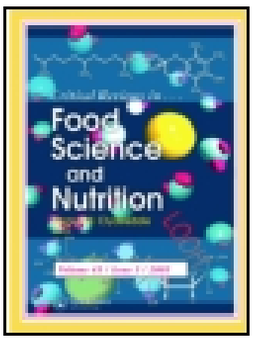

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Therapeutic potential of dairy bioactive peptides: A Contemporary Perspectives

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ABSTRACT

Dairy products are associated with numerous health benefits. They are good source of nutrients like carbohydrates, protein (bioactive peptides), lipids, minerals and vitamins which are essential for growth, development and maintenance of the human body. Accordingly, dairy bioactive peptides are one of targeted compounds present in different dairy products. Dairy bioactive compounds can be classified as anti-hypertensive, anti-oxidative, immmunomodulant, anti-mutagenic, anti-microbial,

opoid, anti-thrombotic, anti-obesity and mineral-binding agents depending upon biological functions. These bioactive peptides can easily be produced by enzymatic hydrolysis, during fermentation and gastrointestinal digestion. For the reason, fermented dairy products like yogurt, cheese and sour milk are gaining popularity worldwide and considered excellent sources of dairy peptides. Furthermore, fermented and non-fermented dairy products are associated with lower risks of hypertension, coagulopathy, stroke and cancer insurgences. The current review article is an attempt to disseminate general information about dairy peptides and their health claims to scientists, allied stakeholders and certainly readers.

Key words

Bioactive peptides, dairy peptides, dairy nutrition, food and health

BACKGROUND

Bioactive peptides are considered as a protein fragments that are produced via certain modification or cleavage from parent protein (Sharma et al., 2011). These bioactive peptides are known as active peptides or physiological active peptides, involves to carryout various body functions. Size of these bioactive peptides ranges from 2-20 amino acid residues depending upon their type, nature and composition (Korhonen and Pihlanto, 2006).

Many naturally occurring protein or protein fragment perform their biological function in their native form while some of the protein fractions or peptides may become active once they are released from precursor protein. There are three different possible ways involved in peptide activations: (i) by the action of proteolytic microorganisms, (Gobbetti et al., 2007) (ii) through breakdown by digestive enzymes (Korhonen and Pihlanto, 2006) and (iii) by hydrolysis with proteolytic enzymes from plants or microorganisms (Kilara and Panyam, 2003). It has been investigated that these functional peptides can also be produced during dairy industrial processing, fermentation with lactic acid bacteria (LAB) or during digestion in gastrointestinal tract (Li et al., 2004).

Bioactive peptides play different biological functions in human body including immune, nervous and digestive system however, their particular physiological function depends upon inherent amino acid composition and sequence (Meisel and FitzGerald,

2003). Health promoting perspectives accredited to different peptides are anti-thrombotic, anti-oxidative, anti-hypertensive, immunomodulatory and anti-microbial (Shimizu, 2004). Accordingly, various bioactive peptides have been identified in fermented and non-fermented dairy products. Milk protein hydrolysates are considered as a rich source of bioactive peptides (Silva and Malcata, 2005).

Fermented products as a potential source of bioactive peptides

The health promoting potential of cultured milk products can be attributed to generation of bacterial metabolites such as bacteriocins (Hernández et al., 2005), cell wall components (FitzGerald and Murray, 2006) and the hydrolysis by cell-free extracts having protein/peptide degrading enzymes (Pan et al., 2005). Proteolysis is a vital biochemical process that involves the production of various essential components. During fermentation process followed by storage, extracellular proteinases and some intracellular peptidases from bacterial cultures produce lactic acid, flavorful compounds along with broad range of oligopeptides (Donkor et al., 2007). Numerous proteins and peptide fractions with bioactive properties have been isolated from fermented dairy products (Chobert et al., 2005).

Yogurt is a coagulated product obtained via milk fermentation with *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. It is a traditional healthy food, its functionality can further be enhanced by lactic fermentation that involve in the production of various bioactive peptides (Shah, 2007). These peptides are encrypted in

the milk proteins and then released during fermentation (Roufik et al., 2006; Moller et al., 2008). Yoghurt peptides that are especially obtained from bovine and ovine milk have anti-hypertensive and anti-microbial properties (Lopez et al., 2006). Among hypotensive peptides, fragments like Val-Pro-Pro and Ile-Pro-Pro identified in sour milk fermented with *Lactobacillus helveticus*, potent anti-hypertensive fragment (Nakamura et al., 1995).

Cheese ripening involves bacterial proteolytic activities which result in formation of a wide variety of bioactive peptides. Calcium-binding phosphopeptides (CPPs) are natural components of Comté and Cheddar cheese (Hayes et al., 2007). Bioactivities of these peptides produced during secondary proteolysis depend upon the cheese ripening stages (Ardo et al., 2009). Hayes et al. (2007) investigated that higher concentration of hypotensive peptides were found in middle-aged Gouda cheese compared to short-termed or long termed stored ripened cheese. Furthermore, peptide concentration in cheese escalates with cheese maturation time, but it drops when proteolysis exceeds a certain point due to hydrolysis of active peptides. Saito et al. (2000) also investigated hypotensive potential of numerous cheese varieties and observed that maximum activity in Gouda cheese aged about two years. Moreover, they found that a number of peptides were derived from 8-month-old Gouda cheese, and two of them isolated from *a*S1-casein f(1--9) and *β*-casein f(60--68), respectively having strong ACE-inhibitory activity. Gagnaire et al. (2001) isolated 91 peptides fractions from

Emmental cheese, 28 of these fractions exhibited multiple bioactivities *in vitro*, *e.g.* anti-microbial, immunostimulatory, mineral-binding and Angiotensin converting enzyme (ACE) inhibitory activities. Additionally, proteinase and peptidases released from starter and non-starter LAB play a vital role in production of bioactive peptides during ripening time. Peptides identified in numerous Italian cheese varieties depicted inhibitory action towards proteolytic enzymes of LAB with specified strains (Hayes et al., 2007). Hernández-Ledesma et al. (2004) also found ACE-inhibitory potential in numerous fresh cheeses and commercial fermented milks. Moreover, anti-hypertensive activity of these products remained constant after gastrointestinal digestion with pepsin and Corolase PP (Hernández-Ledesma et al., 2004).

PRODUCTION OF HEALTH PROMOTING PEPTIDES

Figure 1 elaborates the production methodologies of functional peptides by three different ways: (i) hydrolysis by enzymes from plants or microorganisms (ii) fermentation with LAB (especially proteolytic cultures) (iii) breakdown by digestive enzymes. Many researchers have suggested that combined use of (i), (ii) and (iii) could be beneficial in production of physiological active peptides (Korhonen and Pihlanto, 2003). Highest production of bioactive peptides take place during hydrolysis with microbial enzymes include proteinases/peptidases from LAB or secondary starters, natural milk enzymes and coagulating or hydrolytic enzymes used in cheese production.

(1). Enzymatic hydrolysis

Protein breakdown via an enzymatic hydrolysis is most common method to generate functional peptides. Pepsin and trypsin, two well-known digestive enzymes have been used to produce many peptides with various bioactive properties like ACE-inhibitory activities (Ferreira et al., 2007). Furthermore, anti-hypertensive peptides have been generated with tryptic hydrolysis of bovine αS2-casein (Tauzin et al., 2002) and macropeptides from caprine and ovine (Manso and Lo´pez-Fandino, 2003). Pepsin, chymotrypsin, alcalase, thermolysin, pancreatin, multiple enzymes of different origin and their combinations can be used to produce these bioactive peptides (Kilara and Panyam, 2003).

(2). Microbial fermentation

Many starter cultures utilized in dairy industry at industrial scale are proteolytic in nature. Fermented dairy products produce various bioactive peptides using these cultures like *Lactococcus lactis*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus*. These cultures contain cell-wall bound proteinases and peptidease like dipeptidases, tripeptidases, aminopeptidases and endopeptidases (Griffiths and Tellez, 2013). It has been investigated that these starter cultures help to produce various peptides including ACE-inhibitory peptides anti-microbial, anti-oxidative and immunomodulatory peptides. *Lactobacillus helveticus* is one of those starter cultures that are commonly used in dairy products, being highly proteolytic in nature and is capable

of releasing anti-hypertensive peptides (Szwajkowska et al., 2011). Furthermore, two renowned anti-hypertensive peptides namely, Val-Pro-Pro and Ile-Pro-Pro have been isolated from fermented milk of *Lactobacillus helveticus* (FitzGerald et al., 2004).

Yamamoto et al., (1999) identified a hypotensive peptide (Tyr-Pro) from yoghurt by employing *Lactobacillus helveticus* CPN4 strain. This dipeptide fragment is a part of all casein fractions and its level increases during fermentation. Fuglsang et al. (2003) also investigated about more than 25 wild-type strains of LAB belonging to *Lactobacillus helveticus* and *Lactococcus lactis*, evaluated their capability to produce fermented milk with anti-hypertensive activity. Ashar and Chand (2004) also identified an anti-hypertensive peptide from fermented milk produced with *Lactobacillus delbrueckii* ssp. *bulgaricus*. The identified fragment had an amino acid sequence Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly Pro-Ile that showed it was a part of β-CN. Gill et al., (2000) reported immunomodulatory peptides produced by the breakdown of milk proteins using microbial enzymes. Casein hydrolysates produced by the action of microbial pepsin and trypsin triggered immunomodulatory effect on human blood lymphocytes *in vitro*. Furthermore, peptides produced by strains of *Lactobacillus* GG var. *casei* also showed immunosuppressive activity and maximum activity was demonstrated by αS1-casein (Bernard et al., 2000). It was also investigated that both bacterial cultures and their proteolytic enzymes have ability to produce biologically active peptides from dairy proteins. Mizuno et al., (2004) reported anti-hypertensive potential of casein fragments

produced by the action of nine different commercially available microbial proteolytic enzymes.

(3). Gastrointestinal digestion by the action of digestive/microbial enzymes

Dairy bioactive peptides can be produced during digestion of dairy-base products in gastrointestinal tract (GIT). These peptides are mostly released during casein breakdown due to action of protease enzymes like pepsin, trypsin or chymotrypsin (Gauthier et al., 2006, Ferreira et al., 2007). Bioactive peptides are produced throughout the GIT during digestion. Food protein after entering stomach from cardiac orifice are denatured and partially broken down by simultaneous action of HCl and pepsin. Microbial enzymes present in gut can only digest those milk proteins that move towards large intestine intact or partially digested (Moller et al., 2008). Microbial enzymes whether released by gut residing flora or used as starter for milk processing have different action sites as compared to gastrointestinal enzymes. Hence, bioactive peptides released by microbial enzymes are different from those liberated by digestive enzymes. However, it is still under observation whether peptides liberated by flora residing in large intestine or not. Moreover, bioactive peptides released by microbial enzymes could be attacked by other enzymes present in GIT and produce some other peptides (Shimizu, 2007; Moller et al., 2008). Although bioactive peptides released by the action of site specific protease like trypsin or chymotrypsin on milk protein are well

documented (Custódio et al., 2005) yet, there is limited research on milk protein digestion in human GIT (Miquel et al., 2005).

Dairy Processing and storage -- peptide production

Dairy processing is another way to produce bioactive peptides with specific physiological function. Proteases present in food (for example plasmin in milk) cause hydrolysis of proteins and produce peptides during processing and storage. On the other hand bacterial cultures used in dairy products contain numerous proteolytic enzymes which breakdown proteins into peptides and amino acids during processing. During fermentation process, several oligopeptides released by casein degradation, could act as precursors for bioactive peptides. In cultured dairy products, intracellular peptidases present in LAB would most likely to help in further hydrolysis (Kilpi et al., 2007). Phosphopeptides are natural component of cheese (Gagnaire et al., 2001) and further hydrolysis during processing result in production of other peptides with biological activity like anti-hypertensive peptides (Saito et al., 2000). Moreover, Parmesan cheese has been reported to contain precursors of β-casomorphins (Addeo et al., 1992).

Bioactive peptide used in dairy industries

Now a day's several dairy products are present in market which consists of different bioactive peptides with biological activities (Table 1). Calpis fermented milk cultured via *Saccharomyces cerevisiae* and *Lactobacillus helveticus*, poses two well-known ACE

inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro (Nakamura et al., 1995). In Japan, a product named "Food for special health use" consist of casein dodecapeptide, is available in market, reported to be beneficial food to avoid hypertension and cardiovascular diseases (Sugai, 1998). One of the fermented milk, cultured with *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains have different anti-hypertensive peptides derived from casein fraction (Gobbetti et al., 2000). Peptide FM is also one of the famous dairy commodity responsible for inhibition of dietary fat deposition in body and to modify fat metabolism; glutamine peptide, which help in sustaining the immune system, regulation of protein turnover, glycogen replenishment and caseinophosphopeptides- responsible for increasing Ca^{2+} and Fe^{2+} absorption and involve in prevention of dental caries (Jelen and Lutz, 1998).

BIOACTIVE PEPTIDES DIGESTION AND ABSORPTION

Gastrointestinal hydrolysis of milk proteins and peptides has been investigated in a number of *in vitro* trails. Protein digestion is achieved stepwise via several process like degradation with pepsin and pancreatic enzymes. It has been investigated from pervious researches that gastrointestinal degradation is a key element for determination of biological activities (Herna´ndez et al., 2004). The conditions of gastrointestinal hydrolysis *i.e.* pH, temperature, incubation time and enzyme preparation are critical factors for degree of hydrolysis and bioactivity of peptides (Vermeirssen et al., 2003).

Protein and peptide Digestion: overview of physiology

One of the most important sites for protein and peptide digestion is stomach and small intestine. There are number of finger like projections present on small intestine to increase the surface area for digestion and absorption. In particular, brush border membrane is the highly folded membrane that covers whole internal lumen and provides maximum surface area. This membrane is extremely advanced in terms of metabolic activities, including selection of enzymes, transporters and receptors (Woodley et al., 1994). Small intestine in the digestive system plays a key role in absorption of nutrients. In fact, it also acts as a barrier to foreign substances and digestive enzymes. The epithelial cells in small intestine are of diverse nature and include enterocytes or absorptive cells, endocrine cells, goblet cells (mucin secreting cells), M cells, Paneth cells and tuft and cup cells. Most common of the epithelial cells are enterocytes, accountable for majority of absorption of drugs and nutrients in small intestine.

Physiologically, function of GIT is to digest dietary proteins and peptides into smaller units, and make them small enough to be absorbed (Pauletti et al., 1996). Hydrolysis of protein and peptide is facilitated by multiple enzymes, known as peptidases, these peptidase have peptide bond specificity that help in the absorption of bioactive peptides (Woodley et al., 1994). On the basis of cleaving peptide bond, these peptidases can be classified into 2 groups: (1) exopeptidases, cleave peptide bond that link C-terminal

amino or N-terminal amino of peptide molecule and (2) endopeptidases responsible for cleavage of bonds present at interior of peptide chain (Pauletti et al., 1996).

Peptide hydrolysis is a function of small intestine while the initiation step of protein and peptide digestion takes place in stomach. Acid environment in stomach and presence of pepsin facilitate the initial step of protein breakdown. Pepsin, an endopeptidase and key enzyme in stomach hydrolyzes proteins and polypeptides to oligopeptides. These oligopeptides and few intact protein molecules move to intestinal lumen. After stomach hydrolysis, only few amino acids are released into blood stream while majority of polypeptides pass to duodenum (Harvey and Ferrier, 2011). During intraluminal digestion of polypeptides, oligopeptides with chain length of 2-8 amino acids and few free amino acids are released (Harvey and Ferrier, 2011). Luminal degradation depends on size and amino acid composition of peptide (Sienkiewicz et al., 2009).

Furthermore, brush border membrane is required for substantial degradation of peptides (Harvey and Ferrier, 2011). Carboxypeptidases and aminopeptidases present at brush border further hydrolyze these peptides into tri-, dipeptides and free amino acids (Woodley et al., 1994). There are some other enzymes endopeptidaes and dipeptidases also present at brush border membrane. For example, cleavage of dipeptides from C-terminus of oligopeptides with leucine, proline or phenylalanine at chain end takes place by the action of ACE or dipeptidyl carboxypeptidase. Similarly,

dipeptidyle aminopeptidase IV cleaves oligopeptides with proline or alanine at ultimate position resulting in production of dipeptides which are usually X-Pro type (Ganapathy and Leibach, 1999). Peptides containing proline or hydroxyproline are normally resistant to hydrolysis by digestive enzymes. Tri-peptides with pro-pro at Carboxyl end also shows resistant to enzymes having proline specificity (FitzGerald and Meisel, 2000).

Absorption of bioactive peptides

It has been investigated that physiologically active peptide derived from milk proteins can be absorbed without further degradation and enter direct into blood stream to produce specific effect. In addition, it is also investigated that parent protein with bioactive sequence may enter circulation system, provoke specific functionality after hydrolysis and release an active sequence on action site (Sienkiewicz et al., 2009).

Peptides could be absorbed either by paracellular diffusion or carrier-mediated transport, the two processes being referred as a key mechanism for peptide transport across cell monolayer (Shimizu, 2007). Specific transporters (PepT1) actively transport di- and tri- peptide, whilst oligopeptides are passively transported by paracellular diffusion (Satake et al., 2002). Paracellular route keep the peptide intact without degenerating it into subunits. Modulation of tight junction by other food substances facilitates paracellular transport (Tsukita et al., 2001). Furthermore, oligopeptides may also be transported by vesicle-mediated transcellular transport *i.e.* transcytosis (Shen et

al., 1992). Moreover, it has been concluded that ACE inhibitors (Zhu et al., 2008), opioid peptides (Sienkiewicz et al., 2009) and anti-hypertensive peptides (Sun et al., 2009) can cross epithelial cell monolayer. However, transport method not only depends upon size of peptide but also on molecular weight, hydrophobicity, charge and hydrogen bonding (Sienkiewicz et al., 2009).

BIOLOGICAL ACTIVITIES OF DAIRY PEPTIDES

Bioactive peptides have drug or hormone like properties. These peptides can alter biological functions via specific receptors binding on target cells and to initiate different biological responses. These bioactive peptides are hypocholesteremic (Kawase et al., 2000), anti-carcinogenic (LeBlanc et al., 2005), immunomodulation (Szwajkowska et al., 2011), mineral-binding (Lorenzen and Meisel 2005), bone formation (Narva et al., 2004), anti-microbial including bacteriocins (Hernández et al., 2005), peptidase inhibitory (Smacchi and Gobbetti, 1998) and opioid activities (Rokka et al., 1997). Table 2 highlights different bioactive peptides, their precursors and associated biological activities.

(1). Anti-hypertensive peptides

Hypertension is one of the prime causes of cardiovascular (CVD) diseases. The risk of CVD is positively correlated to blood pressure. Different interacting biochemical pathways control the risk of CVD especially blood pressure via renin-angiotensin system. Figure 2, demonstrates the mode of action of ACE-I, secreted by various tissues

and involve in blood pressure regulation. Angiotensinogen, inactive precursor and their hydrolysis via a renin, release peptides named angiotensin I (ACE-1). Accordingly, ACE hydrolyzes angiotensin-I by removing dipeptide His-Leu from C-terminus, consequently producing angiotensin-II (forceful vasoconstrictor). It also removes the C-terminus dipeptide from bradykinin that results in production of non-functional peptide fraction (Imig, 2004). Hence, anti-hypertensive effect is main consequence of ACE inhibition however, it may also involve in neural activity and allied immunodefence regulatory systems (Meisel, 1993).

There are several proteins or protein fragments that have ability to characterize as ACE inhibitory peptides. Anti-hypertensive peptides have been identified in various types of dairy products including processed milk, cheese and sour milk *etc.* Lactotripeptides peptides (Ile-Pro-Pro and Val-Pro-Pro) are found in various milk and cheese sources (Nakamura et al., 1995). Dipeptide (Tyr-Pro) was found in whey fraction of dairy products, have significant anti-hypertensive effect in spontaneously hypertensive rats (SHR). The strongest ACE-inhibitory peptides present in these hydrolysates are Arg-Tyr-Leu-Gly-Tyr, Ala-Tyr-Phe-Tyr-Pro-Glu-Lue, and Tyr-Gln-Lys-Phe-Pro-Gln-Tyr. These peptides also showed hypotensive effect when subjected to SHR rats, oral doses @ 5 mg/kg of body weight (Yamamoto et al., 1999). Maeno et al., (1996) also characterized a β-casein derived ACE inhibitory fraction from casein digest. The isolated peptide did not indicate a substantial ACE inhibitory potential but have an

analog similar to synthetic hexa-peptide deleted by Gln (Lys-Val-Leu-Pro-Val-Pro), it showed strong ACE inhibitory effects on SHR. Arg-Tyr-Lue-Gly-Tyr and Ala-Tyr-Phe-Tyr-Pro-Glu-Lue dairy peptides have also anti-hypertensive effect similar to Val-Pro-Pro (ACE inhibitory fragment), already formulated in different functional foods (Contreras et al., 2009).

Mizuno et al. (2004) investigated different level of casein fragments (Ile-Pro-Pro & Val-Pro-Pro) formed during fermentation (*Aspergillus oryzae)* and concluded that consumption of Ile-Pro-Pro reduces the blood pressure as compared to placebo. Murakami et al. (2004) isolated peptide from β-lactoglobulin f142-f145, termed "β-lactosin B" had ability to lower the blood pressure significantly in spontaneously hypertensive rats (oral dose). Some of the peptides have very strong *in vitro* ACE inhibitory effect while fail in *in vivo* trails. For example, αS1-casein f (23-27), has a potential ACE inhibitory effect *in vitro* while no hypotensive effect was found in animal models (Fitzgerald et al., 2004). Furthermore, by removing the C-terminal glutamine from β-casein f169-f175 increased the *in vitro* anti-hypertensive potential (Maeno et al., 1996). It is hard to estimate direct association between *in vitro* and *in vivo* activity because of the bioavailability, nature and mechanism of the ACE inhibitory peptides (Vermeirssen et al., 2004). It is also important for the peptides to remain active throughout digestion by human proteases. These peptides should also be transported through the intestinal wall into the blood stream. However, transcellular and

paracellular routes facilitate intact peptide absorption but bioavailability after absorption is inversely proportional to chain length (Shimizu, 2004).

Table 3, provides the information about various proteins and their ACE inhibitory characteristics. Casokinins are ACE inhibitors which are derived from milk proteins (FitzGerald et al., 2004). Commercial products like casein fractions (Casein DP, Kanebo, Ltd., Japan), C12 peptide (DMV, Netherlands), and a whey protein fragments (Biozate, Davisco, US), also have anti-hypertensive effect in humans (FitzGerald et al., 2004). Lactotripeptides is a valuable peptide alternative to existing pharmacological drugs that maintain blood pressure (Jakala and Vapaatalo, 2010). Evolus™ and Ameal S™ are two fermented dairy products with ACE inhibitory peptides (Val-Pro-Pro and Val-Pro-Pro), have been developed by Valio Company in Finland (Gobbetti et al., 2004; FitzGerald et al., 2004).

(2). Anti-oxidative potential of dairy peptide

Oxidative metabolism is one of the important functions for cell maintenance while malfunction in metabolism produce number of reactive oxygen species (ROS) and free radicals, causing oxidative stress. Free radicals like peroxide radical, hydroxyl radical, superoxide radical and hydrogen peroxide commonly known as ROS not only cause deterioration in food but also damage to biological systems. These free radical species have deleterious effect on endogenous defensive enzymes like peroxidase, catalase and superoxide dismutase (Yun-Zhong et al., 2002). These enzymes destroy and cause

mortal cellular effects (*e.g.* apoptosis) by oxidation of cellular proteins, lipids in membrane, enzymes and DNA (Harvey and Ferrier, 2011). These free radical species may also cause modifications at cellular level that lead to various human diseases like arthritis, hypertension, atherosclerosis and most importantly cancer (Frenkel, 1992).

Proteins and peptides have anti-oxidative potential due to their specific or non-specific biological mechanism, inactivation of ROS, by scavenging pro-oxidant metals, eradicate oxidants, decline hydroperoxide levels and change internal properties of the system (Elias et al., 2008). Dairy protein hydrolysates vary in their anti-oxidant potential depending on size and specific amino acid sequence, which in turn vary according to protein source and specific conditions of proteolytic process being employed (Pena and Xiong, 2002). Whey protein fractions having aromatic amino acids have strong anti-oxidant potential as compare to simple amino acids (Pena et al., 2004). Peptide fragment rich in histidine and hydrophobic amino acid residues showed high 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity because of hydrogen donating capacity of hydroxyl group on aromatic amino acids or phenolic anti-oxidants (Shahidi and Zhong, 2008).

Recently, Unal and Akalın (2012) investigated anti-oxidative and hypotensive activity of yoghurt fortified with whey proteins concentrates and sodium casinates. It has been showed that yoghurt fortified with 4% whey protein concentrates revealed highest DPPH scavenging activity. Moreover, the DPPH scavenging activity of fortified yoghurt

was dose dependent. High DPPH scavenging property of whey protein concentrate can be attributed to the fact that it contains lactoferrin, a potent anti-oxidative protein (Lindmark-Månson and Åkesson 2000), α lactalbumin (Sadat et al., 2011) and β-lactoglobulin (Del-Mar-Contreras et al., 2011). Pritchard et al. (2010) also studied anti-oxidative potential of peptides derived from cheddar cheese and confirmed their activity using DPPH system.

Few contemporary studies regarding anti-oxidative peptides showed that proteolysis of casein by gastrointestinal enzymes and milk fermentation with LAB results in production of peptides with potent anti-oxidative activity (Koh et al., 2003). Hydrolysates from αS--casein fractions have also showed anti-oxidative potential. Besides anti-oxidant activity, these peptides were also analyzed for free radical scavenging activities and enzymatic & non-enzymatic lipid peroxidation (Rival et al., 2001).

(3). Anti-thrombotic peptides

Anti-thrombotic peptides are protein fractions that can prevent thrombosis. High prevalence of thrombosis is accredited to up regulation of hemeostatic proteins like fibrinogen, hyper-reactive platelets, irregular fibrinolysis and elevated blood viscosity (Erdmann et al., 2008). Milk clotting proteins like chymosin and κ-casein share similar mechanism like blood clotting mechanism that involves thrombin and fibrinogen (Erdmann et al., 2008). Bovine κ-casein (f106-f116) has ability to repress platelet

aggregation via binding with platelets at receptor site and inhibit fibrinogen (Schlimme and Meisel, 1995). Jolles et al. (1992) also reported anti-thrombotic effect of peptides derived from bovine milk that glycomacropeptides exhibit anti-platelet effect better than ϒ chain of fibrinogen. Fragments (f106-116, f106-112 and f113-116) showed anti-thrombotic effect in dose dependent manner. *In vitro* study reported that sequence f106-116 showed strong competition against ADP induced platelet aggregation. However, other peptides 106-112, 113-116 and 103-116 revealed lower anti-thrombotic effect compared to aforementioned peptides. κ-casein fraction obtained by casein proteolysis by trypsin also demonstrated anti-thrombotic activity preventing fibrinogen binding platelet (Schlimme and Meisel, 1995). Such peptides are produced during gastrointestinal digestion and absorbed into blood stream without further hydrolysis, this mechanism supports the fact of *in vivo* anti-thrombotic activity.

Manso and Lopez (2003) identified anti-thrombotic peptide fragment from Yoghurt κ-casein with amino acid sequence Met-Ala-Ile-Pro-Pro-Lys (f113-f116). Dionysius et al., (2000) also characterized κ-casein from water soluble extract of Spanish fermented milks (Pro-Pro-Ly), very similar to aforementioned peptide. Both protein hydrolysates from κ-casein were found to be multifunctional properties like ACE inhibitory and anti-thrombotic (Rutherfurd and Gill, 2000). Rokka et al., (1997) also found peptide sequence from αS1-, β-casein and α-lactalbumin in UHT milk fermented with *Lactobacillus* GG followed by digestion with pepsin and trypsin with anti-thrombotic activity. Qian et al.

(1995a) studied the effect of sheep κ-casein fragments against platelet aggregation induced by collagen and thrombin. Various κ-casein fragments purified by RP-HPLC showed anti-thrombotic activity ranging from 0 to 100%. Simos et al. (2011) also revealed that goat milk has a higher ability to reduce platelet aggregation as compared to cow and donkey milk. Variation among breeds of different and same species can be attributed to genotype, rearing system and feeding diet.

(4). Anti-microbial peptides

Anti-microbial peptides have a potential to kill wide range of pathogenic organisms *e.g. Listeria*, *Salmonella, Escherichia*, *Staphylococcus* and *Helicobacter*, filamentous fungi and yeasts (Haque and Chand, 2008). Anti-microbial peptides are very essential for newborn babies because these are vulnerable to a broad spectrum of pathogenic microorganisms mainly present at mucosal surfaces like small intestine and lungs. It is reported that anti-microbial effect of milk is accredited by both immunoglobulins and non-immunoglobulin proteins like lysozymes, lactoferrin, lactoperoxidase system and peptides (Jabbari et al., 2012). These natural proteins and peptides are produced from latent proteins (Gobbetti et al., 2004). Dairy proteins or peptides are anti-bacterial peptide's precursors; these peptides have ability to boost natural defense of organisms against attacking pathogens (Pellegrini**,** 2003).

Peptides isolated from whey protein lactoferrin, called lactoferricin have very strong anti-microbial potential against various microorganisms (Bellamy et al., 1992).

Enzymatic hydrolysates of lactoferrin showed more anti-microbial activity compared to the parent protein (Tomita et al., 1991). Furthermore, anti-bacterial peptides from bovine f(17-41) and human lactoferrin f(1-47) were purified, these peptides have strong anti-bacterial potential against array of Gram +ve and Gram --ve bacteria (Wakabayashi et al., 2003). Goat milk lactoferrin showed less anti-bacterial activity as compared to the bovine fraction (Vorland et al., 1998). Anti-microbial peptides obtained by proteolysis of sheep and goat milk-lactoferrin, f(14-42) also reported in caprine. Furthermore, whey proteins like α-lactalbumin and β-lactoglobulin were also known for their anti-microbial potential on broad spectrum. It has also been investigated that few milk derived anti-microbial protein fragments can directly hit on intracellular targets (Recio and Visser 2000).

Milk casein is also a rich source of anti-bacterial peptides (Lopez et al., 2006). Casein fragments of ovine (β-casein fragments) obtained by enzymatic hydrolysis (with pepsine, trypsin and chymotrypsin) exhibited anti-bacterial activity against *Escherichia coli* JM103, while exact mechanism is still under research (Gomez et al., 2006). Anti-microbial peptides were also identified in αS1-, αS2- and κ-CN (Lahov and Regelson, 1996). Pepsin hydrolysis of ovine αS2-CN resulted in production of four anti-microbial peptide fragments like f165-170, f165-181, f184-208 and f203-208 (Lopez et al., 2006a). These fragments are potent anti-bacterial against Gram -ve bacteria. Furthermore, among all these fragment f165-181 showed highest anti-microbial activity while

fragment f203-208 also showed anti-oxidant and anti-hypertensive activity (Recio et al., 2005). Table 4, shows various anti-microbial peptides derived from milk proteins and their releasing mechanisms.

(5). Anti-cancer or anti-tumor peptides

Cancer is characterized by a group of diseases that involve uncontrolled or abnormal cell growths, potential to invade or spread to other parts of the body (Hoskin and Ramamoorthy, 2008). There are various factors involve in the occurrence of cancer disease while diet imparts a central role in the etiology of cancer. It has been observed that breast, prostate, colon and rectum cancer are common in developed countries because of poor dietary habits (Willett, 1995). However, about one-third of all cancers diseases can be reduced by modifying the diet plans (Kontou et al., 2011).

Dairy proteins and protein fractions have potential to cure various cancers. Likewise, intact whey proteins are less anti-mutagenic than derived peptides because whole whey protein molecules have comparably tight structure and unapproachable to the mutagen while caseins, on other hand, have a loose micellar structure, mutagen can access them easily (Bosselaers et al., 1994). Moreover, specific amino acid composition and the number of nucleophilic groups could also be responsible for the binding of mutagen within the protein. However, casein deprivation might raise the vulnerability of binding sites to mutagens. Several *in vitro* cell culture and animal experiments reported that whey protein fractions and their peptides can help to reduce tumor growth at various

sites mainly in breast and colon. Cancer inhibiting potential of these whey peptides are due to presence of ϒ- glutamylcystine and cysteine/cysteine dipeptides which are actually precursor for biological synthesis of glutathione (Parodi, 2007).

Fermented products have a strong potential to inhibit broad spectrum of mutagens because of the presence of various beneficial milk culture. Several studies about fermented milk and culture used reported the growth prevention of certain tumors and tumor cells (kumar et al., 2010). Epidemiological, biochemical and animal model also reported that LAB culture may inhibit tumors at various sites (Bahadoran et al., 2013). Guzel-Seydim et al., (2005), reported that milk cultured with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* has a strong anti-mutagenic potential against 4-nitroquinoline-N-oxide (4NQO). Likewise, Cassand et al., (1994) investigated that milk fermented by *Lactobacillus helveticus* or *Bifidobacterium* species has also anti-mutagenic properties induced by 4NQO, 2-nitrofluorene, quercetin, and benzopyrene.

Colon and mammary tumorigenesis studies reported that peptides derived from whey protein are more efficient in inhibiting tumor growth compared to other dietary proteins (Sasaki and Kume, 2007). Some other studies also reported that whey protein fractions, α- lactalbumin, β-lactoglobulin, and serum albumin are potent anti-cancer moieties because these fractions are helpful in reducing the toxic effect of cancer (Parodi, 2007). A smaller fraction of lactoferrin also showed significant results against intestinal tumors, it stimulates apoptosis, inhibits angiogenesis and modulates

carcinogen metabolizing enzymes and also acts as an iron scavenger (Wolf et al., 2007). Peptides which are derived from casein hydrolysis have also anti-mutagenic properties (Tellez et al., 2010). Van Boekel et al., (1993) also reported that casein has powerful inhibitory activity against mutagens. However, the mechanism involved is yet not confirmed. There is several assumptions involved, connection of mutagen with casein micelle or due to adsorption of mutagen on protein molecule to prevent the reaction on target cell. There are also chances of quenching reaction between proteolysis derived peptides and mutagen, or a chemical binding by a scavenging method (El-Aziem et al., 2007).

CONCLUSIONS

Dairy and dairy products are rich source of bioactive peptides which are essential for healthy life. These bioactive compounds show their activity only after being released from parent protein. It is very important to investigate whether and how these peptides can retain their sequence during gastrointestinal digestion, enter into blood stream and target on action site. In nutshell, these bioactive peptides could lead to market as dairy-based products or supplements to improve human health status.

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

Brand name	Bioactive peptide	Health claims	Country
Calpis	VPP, IPP from β-CN and k-CN in sour milk	Blood pressure reduction	Japan
Evolus	VPP, IPP from β-CN and k-CN in Calcium enriched fermented milk	Blood pressure reduction	Finland
BioZate	β -LG from Hydrolyzed whey protein	Blood pressure reduction	USA
BioPUREGMP	k-CN f(106--169)	Prevention of dental caries, influence the clotting of blood, protection against viruses and bacteria	USA
PRODIET F200/Lactium	αs1-CN (91--100) in Flavored milk and confectionery	Reduction of stress effects	France
Festivo	αs1-CN (1--9), αs1-CN (1--7), αs1-CN (1--6) from	No health claim as yet	Finland

	Fermented low-fat hard cheese		
Cysteine Peptide	Milk protein derived peptide	Aids to raise energy level and sleep	Netherlands
C12 peptide	Casein derived peptide	Reduction of blood pressure	Netherlands
Capolac	Casein derived peptide	Helps mineral absorption	Sweden
PeptoPro	Casein derived peptide	Improves athletic performance and muscle recovery	Netherlands
Vivinal Alpha	Whey derived peptide	Aids relaxation and sleep	Netherlands

Table 2: Bioactive peptides derived from milk proteins (Silva and Malcata 2005)

Bioactive	**Peptide Precursor**	**Protein Bioactivity**
Casomorphins	α CN, β CN	Opioid agonist
α -lactorphin	α -LA	Opioid agonist
β -lactorphin	β -LG	Opioid agonist
Lactoferroxins	LF	Opioid antagonist
Casoxins	k-CN	Opioid antagonist
Casokinins	α -CN, β -CN	ACE-inhibitory activity
Lactokinins	α -LA, β -LG	Hypotensive
Immunopeptides	α -CN, β -CN	Immunomodulatory
Lactoferricin	LF	Anti-microbial
Casoplatelins	k-CN,Transferrin	Anti-trombotic
Caseinophosphopeptides	α -CN, β -CN	Mineral binding
Met-His-Ile-Arg-Leu	α-LA, β-LG	Anti-oxidant
Ile-Ile-Ala-Glu-Lys	β-LG	Hypocholesterolemic

Table 3: ACE Inhibitory peptides produced from different parent proteins

Parent protein	**Peptide sequence**	**Reference**
ß- casein	Lys-Val-Leu-Pro-Val-Pro-(Glu)	Korhonen and Pihlanto (2006)
ß-casein, k-casein	Val-Pro-Pro, Ile-Pro-Pro	Seppo et al. (2003)
Skim milk hydrolysate	Val-Pro-Pro, Ile-Pro-Pro	Pan et al. (2005)
ß- casein, αs1-casein	Tyr-Pro-Phe-Pro, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Thr-Met-Pro-Leu-Trp	Gobbetti et al. (2002)
ß- casein k-casein	Multiple fragments	Gobbetti et al. (2000)
ß- casein	Ser-Lys-Val-Tyr-Pro	Ashar and Chand (2004)
ß- casein	Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly Pro-Ile	Ashar and Chand (2004)
ß- casein	Asp-Lys-Ile-His-Pro-Phe, Tyr-Gln-Glu-Pro-Val-Leu, Val-Lys-Glu-Ala-Met-Ala-Pro-Lys	Herna´ndez et al. (2004)

Table 4: Anti-microbial peptides derived from milk proteins (McCann et al., 2006; Lopez et al., 2006)

Parent protein	Peptide fragment	Releasing mechanism
Lactoferrin	Bovine LF *f*(17--41/42), human LF *f*(1--11)S-S(12--47), caprine LF *f*(14--42)	Hydrolysis with pepsin or chymotrypsin
a-Lactalbumin	*a* -La *f*(1--5), *a* -La *f*(17--31)S-S(109--114)	Hydrolysis with trypsin
a Lactalbumin	*a* -La *f*(61--68)S-S(75--80)	Hydrolysis with chymotrypsin
β --Lactoglobulin	*β* -Lg *f*(15--20), *f*(25--40), *f*(78--83), *f*(92--100)	Hydrolysis with trypsin
a S1-Casein	*a* S1-Casein *f*(99--109)	Hydrolysis with pepsin
a S2-Casein	*a* S2-Casein *f*(150--188)	Heated and acidiWed milk
a S2-Casein	*a* S2-Casein *f*(164--179)	Hydrolysis with pepsin
a S2-Casein	*a* S2-Casein *f*(183--207), *f*(164--207), *f*(175--207), *f*(181--207)	Hydrolysis with chymosin
k-Casein	*k* -Casein *f*(106--169)	Hydrolysis with chymosin
k-Casein	*k* -Casein *f*(18--24), *f*(30--32),	Hydrolysis with peptic

	f(139--146)	enzyme
k-Casein	Human *k* -casein *f*(43--97)	Hydrolysis with pepsin
β-Casein	*β* -Casein *f*(184--210)	Hydrolysis with proteinase of *LAB. helveticus* PR4

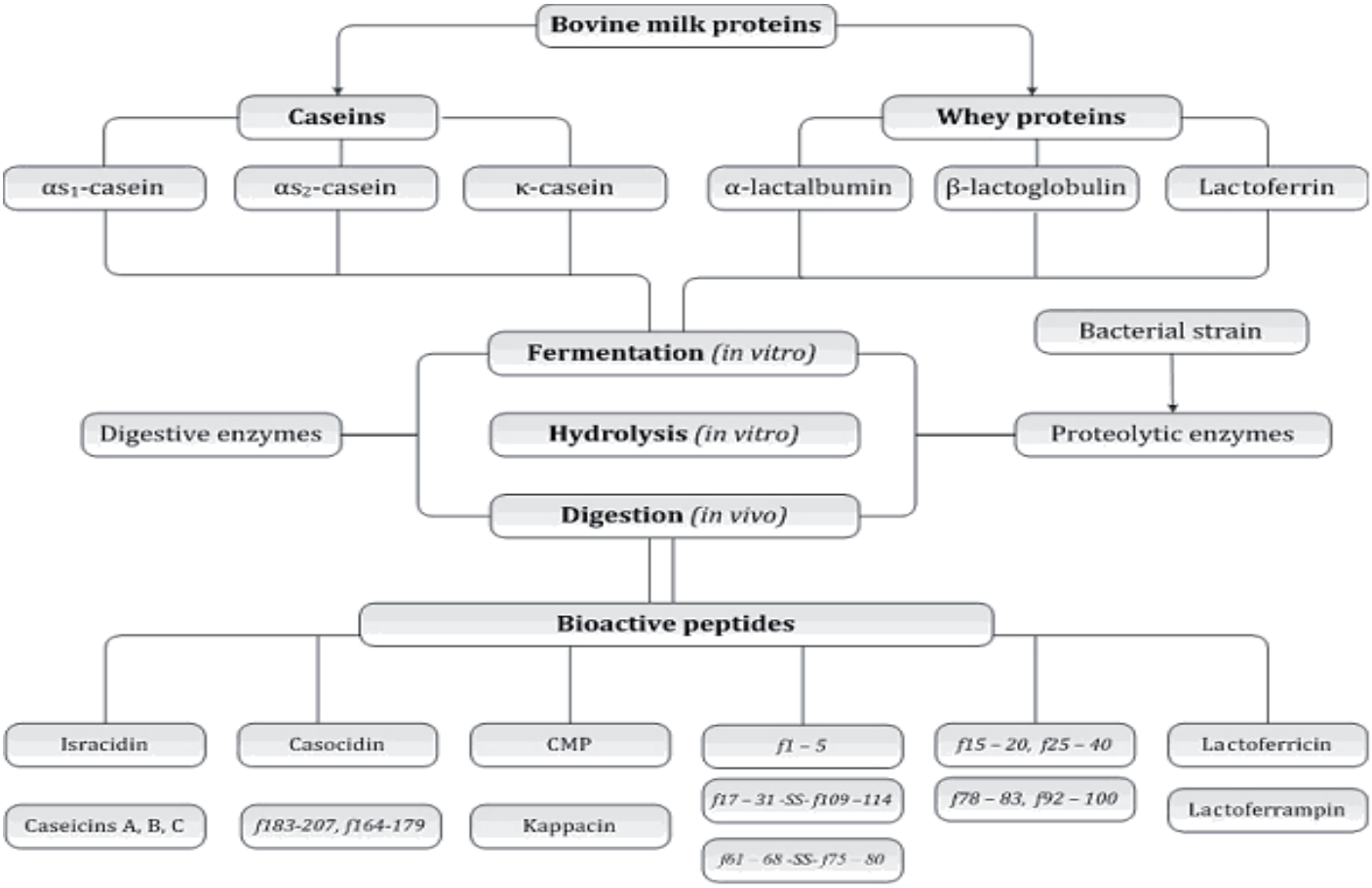

Figure 1: Production of bioactive peptides (Korhonen and Pihlanto, 2006)

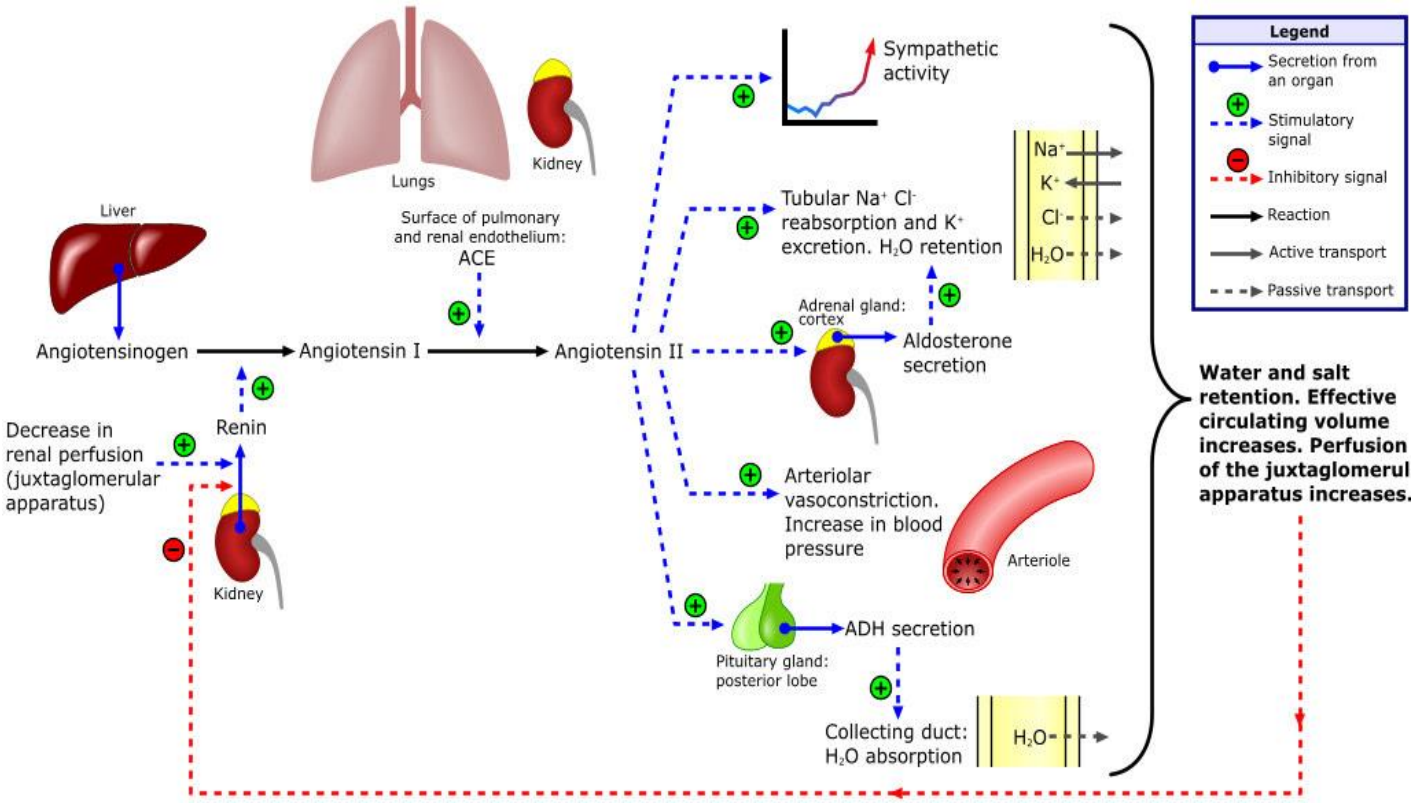

Figure 2: Mechanism of Angiotensin Converting Enzyme activity (Tavares et al., 2011)