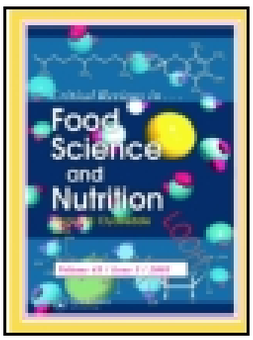

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Production of angiotensin I converting enzyme inhibitory (ACE-I) peptides during milk fermentation and their role in reducing hypertension

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Abstract

Fermented milk is a potential source of various biologically active peptides with specific health benefits. Angiotensin converting enzyme inhibitory (ACE-I) peptides are one of the most studied bioactive peptides produced during milk fermentation. The presence of these peptides is reported in various fermented milk products such as yoghurt, cheese, sour milk, etc, which are also available as commercial products. Many of the ACE-I peptides formed during milk fermentation are resistant to gastrointestinal digestion and inhibit angiotensin converting enzyme (ACE) in the rennin angiotension system (RAS). There are various factors, which affect the formation ACE-I peptides and their ability to reach the target tissue in active form, which includes type of starters (lactic acid bacteria, yeast, etc), substrate composition (casein type, whey protein, etc), composition of ACE-I peptide, pre and post fermentation treatments, and its stability during gastrointestinal digestion. The antihypertensive effect of fermented milk products has also been proved by various *in-vitro* and *in-vivo* (animal and human trials) experiments. This article reviews the literature on fermented milk products as a source of ACE-I peptides and various factors affecting the production and activity of ACE-I peptides.

Keywords : Milk, lactic acid bacteria, yeast, fermentation, peptides, Angiotensin converting enzyme, hypertension.

1. Introduction

Fermented milk is a rich source of easily digestible proteins with high nutritional value and wide range of bioactive properties. The health promoting properties of fermented milk are either due to the ingested live organism (probiotic) or the microbial metabolite (cell free fraction) produced during the fermentation process. Fermented milk products such as yoghurt and cheese have been used as a popular delivery system for probiotics (Prasanna et al. 2014). In recent years, several fermented milk products have been used as functional food, which provide various health benefits beyond their nutritional value (Rai and Jeyaram, 2015). Dried cell free fraction of fermented milk is also a potential functional additive for the food industry to bring functionality in ordinary foods (Vinderola, 2008). Among the bioactive metabolites formed during milk fermentation, peptides are emerging as an important tool for the treatment of various diseases. These peptides vary from 2-20 amino acids and some of them are multifunctional and can exert more than one functional property (Saito et al. 2000; Schanbacher et al. 1998; Yamamoto et al. 1994). In the last few years, peptides have shown to exhibit different biological properties depending on the sequence of amino acids, which includes antihypertensive, antioxidant, antibacterial, antithrombic, immunomodulatory, opiate like, mineral binding and hypocholesterolemic activites (Arai et al. 2001; Choi et al. 2012; Haque and Chand, 2008; Sanjukta et al. 2015; Smacchi and Gobbetti, 2000; Urisata et al. 2011).

Hypertension (blood pressure> 140/90) is one of the risk factors for coronary heart disease and stroke; and is one of the main causes for mortality in developing countries (Lin et al. 2012). Research and various clinical trials during the past decade on angiotensin converting enzyme inhibitory (ACE-I) property of fermented products or enzymatic hydrolysis of food

protein has provided extensive scientific evidence for the application of food derived peptides for the prevention and treatment of hypertension (Nakamura et al. 1995a; Miguel et al. 2009; Quiros et al. 2007; Rodriguez – Figueroa et al. 2013). ACE plays a key physiological role in controlling blood pressure by the rennin angiotensin pathway (Hartmann and Meisel, 2007). ACE-I peptide inhibits the activity of ACE and prevents the conversion of Angiotensin I to Angiotensing II, which is a potent vasoconstrictor. Milk is good source in ACE-I peptides, which are released during fermentation by proteolytic hydrolysis from the native protein (Rai and Jeyaram, 2015). Most of the reports on bioactive peptides derived from milk protein are either by fermentation or enzymatic hydrolysis using proteases. This paper reviews the updated knowledge on the presence of ACE-I peptides in fermented milk products, factors affecting ACE-I peptides production during milk fermentation and their activities in animal model.

2. Bioactive peptides from milk

It is well established that milk protein may act as a precursor for bioactive peptides with various physiological properties. Presently, fermented milk is the main source of various bioactive peptides and daily intake of milk and fermented milk products have proven to be important for both adults and neonates (Korhonen, 2003). The main milk proteins are α_{s1}-casein and β-casein, which have the capacity to liberate more than 20000 peptides each on hydrolysis (Yamamoto and Takano, 1999). Biologically active peptides from milk protein can be produced by following ways: (a) fermentation of milk using proteolytic starter (lactic acid bacteria, yeast) (b) hydrolysis of milk protein by enzymes derived from microorganisms (eg; alcalase, fungal proteases) and plants (eg; papain) (c) hydrolysis by digestive enzymes (eg; trypsin, pepsin, chymotrypsin). Hydrolysis of milk with individual specific proteases results in the formation of

peptide of different size and sequence of amino acids. Researchers have even used combination of the above methods for the production of novel bioactive peptides, which is also discussed later in this review.

Fermented milk has been studied as a potential source of different types of bioactive peptides (Table 1). Among the different types of bioactive peptides in fermented milk, ACE-I peptides have been extensively studied due to the high incidence of hypertension, which is currently considered to be one of the most serious chronic illnesses. Different types of fermented milk products have been studied for ACE-I properties, which include different varieties of cheese products (Singh et al. 1997; Ong et al. 2007; Meira et al. 2012), sour milk (Nakamura et al. 1995a), *dahi* – yoghurt consumed in the Indian subcontinent (Ashar and Chand, 2004a), *kefir* (Quiros et al. 2005), *koumiss* – fermented mare milk (Chen et al. 2010), sheep milk yogurt (Papadimitriou et al. 2007), fermented camel milk (Moslehishad et al. 2013), and fermented goat milk (Minervini et al. 2009). ACE-I properties of some of these products has also been proved by animal experiments and clinical trials.

3. Importance of ACE-I peptides

Currently, synthetic drugs such as, captoptril, enalaptril or ramipril are used in treatment of cardiovascular diseases, whose actions are based on the inhibition of ACE-I activity. Unfortunately, use of these drugs for prevention of hypertension may cause serious side effects, such as cough, skin rashes, nausea, vomiting and dizziness (Lin et al. 2012). This has led researchers to search for other, more secure, innovative and cheaper ACE inhibitors, which can be used both in the prevention and treatment of cardiovascular diseases (Wijesekara et al. 2011). The first ACE-I peptide reported for *in vivo* antihypertensive effect was from snake venom

(Ferreira et al. 1970). Although, snake venom peptides were potential inhibitors of ACE, they had limited pharmacological applications due to their lack of oral activity. Apart from synthetic drugs and snake venom, several other bioactive components from food source, such as peptides formed during fermentation/enzymatic hydrolysis (Schanbacher et al. 2012; Quiros et al. 2005; Meira et al. 2012), γ-amino butyric acid (GABA) in fermented milk (Inoue et al. 2003), calcium (McCarron et al. 1999) and polyphenols from tea and cocoa (Taubert et al. 2007) have shown antihypertensive properties by different mechanisms. Among all natural sources of ACE inhibitors, peptides derived during food fermentation are one the emerging nutraceuticals for reducing blood pressure. Fermented milk products are natural source of ACE-I peptides, which are formed on hydrolysis of milk protein by microbial proteases, produced by the starter culture (Figure 1). These peptides might also be released in the process of enzymatic hydrolysis *in vivo* (in the gastrointestinal tract) of the fermented milk products by the action of pepsin, trypsin and chymotrypsin (Figure 1).

4. ACE-I peptides in different milk products

Milk from different sources (cow, buffalo, goat, sheep, camel, yak, etc) is used for the production of fermented milk products (Ao et al. 2012; Moslehishad et al. 2013; Fadda et al. 2010; Papadimitriou et al. 2007). The starter culture and the fermentation process decide the biological activity of the peptide produced. Globally, several types of fermented products are available according to the method of preparation and nature of the final product. In this review fermented milk products have been categorized and discussed in two groups (i) fermented milk type products (liquid), and (ii) cheese type products (solid).

4.1. Fermented milk type products (liquid)

Milk fermentation for the production of ACE-I peptides is catalyzed by lactic acid bacteria (LAB) or yeast starter. These peptides are not only produced during fermentation but also during storage of fermented products. Selected fermented milk products, their starters and sequence of ACE-I peptides formed after fermentation is presented in Table 2. Yoghurt is a classical example of a fermented milk product produced by lactic acid fermentation. Cow's milk is used for yogurt preparation, but milk from buffalo, goat, ewes, mares, sheep, camel and yaks is also used in various part of the world. Worldwide, several authors have studied ACE-I properties of yogurt prepared with different LAB starter (Ramchandran and Shah, 2011; Lim et al. 2011; FitzGerald et al. 2004). Yogurt has been of greater interest in the past two decades due to its probiotic properties. Apart from being probiotic, researchers have also focused on ACE-I peptides formed during milk fermentation for yogurt production. Papadimitriou et al. (2007) have characterized several ACE-I peptides in probiotic traditional Greek sheep milk yogurt prepared using normal yogurt culture (*Lb. delbrueckii* and *Streptococcus thermophilus*) and yogurt culture mixed with probiotic culture (*Lb. paracasei*), respectively. In another study, eight ACE-I peptides were characterized in yoghurt prepared with yoghurt starter culture and probiotic organism originating from α_{S2} – casein, κ – casein and β- casein (Donkor et al. 2007). ACE-I peptide (SKVYP) originating from β-casein has also been reported in *dahi*, a form of yoghurt consumed in the Indian subcontinent (Ashar and Chand, 2004a).

The milk products fermented with both yeast and LAB are generally sour to taste and have an alcoholic flavor. Calpis is a Japanese soft drink made by fermentation of skimmed milk by *L helveticus* CP790 and *Saccharomyces cerevisiae* (Namakura et al. 1995a). Calpis has been reported to contain two ACE-I dipeptides (VPP and IPP) (Namakura et al. 1995a). Kefir is an

ancient fermented milk beverage that has an exotic sour and slightly alcoholic flavor, and has also been reported to posses ACE-I properties (Quiros et al. 2005). The two most potent ACE-I peptides isolated from Caprine Kefir were PYVRYL and LVYPFTGPIPN. LAB and yeast are also responsible for fermentation and production of ACE-I peptides in *Koumiss*, a slightly alcoholic fermented milk beverage (Mu et al. 2012). Apart from these traditional fermented milk products ACE-I peptides are reported in milk fermented with LAB such as novel probiotic-fermented milk by *Bifidobacterium bifidum* MF 20/5 (Gonzalez-Gonzalez et al. 2013), cow milk fermented with *Enterococcus durans* and *Lactobacillus acidophilus* (Nejati et al. 2013), camel milk fermented by *Lactobacillus rhamnosus* (Moslehishad et al. 2013) and bovine skim milk fermented by *E faecalis* (Gutiez et al. 2013). ACE-I activities have also been reported in herbal yoghurts fermented with mixed starter consisting *Bifidobacterium bifidum* Bb- 12, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* LC-01 and *Streptoccus thermophilus* Th-4 (Amirdivani and Baba, 2011; Shori and Baba, 2013).

4.2.Cheese type products (solid)

Cheese is another high protein content milk product, which is a natural source of bioactive peptides because of the diversity of the proteolytic system during cheese ripening (Ryhanen et al. 2001). Selected Cheese products and their ACE-I peptides are presented in Table 3. The ACE-I property of the peptide depends on the proteolytic property of the starter culture and ripening condition applied post fermentation. ACE-I activity has been reported in cheddar cheese prepared with lactococci and probiotic strain of *Lb. casei* and Lactococci (Ong et al. 2007; Ong and Shah, 2008). Here, they suggested that ACE-I activity in cheddar cheese was dependent on proteolysis during ripening. Ryhanen et al. (2001) have reported ACE-I activity of

low fat Festivo cheese made in Finland prepared by a mixture of LAB. They have observed that the ACE-I activity increased during ripening and remained active for a limited period of time. Pripp et al. (2006) have studied the relationship between proteolysis and ACE-I activity in Gamalost, Castello, Brie, Pultost, Kesam, Port Salut and Noevegia Cheese. Peptide fractions having different ACE-I activities were isolated from Italian cheese products such as Crescenza (37% ACE inhibition), Mozzarella (59% ACE inhibition), Gorgonzola (80% ACE inhibition) and Italico (82% ACE inhibition) (Smacchi and Gobbetti, 1998). ACE-I activity has also been reported in manchego cheese (Gomez-Ruiz et al. 2006), Fungal enriched cheese (Apostolidis et al. 2007), probiotic cheese (Ong and Shah, 2008) and general cheeses such as Camembert, Edam, Roquefort and Parmesan cheese (Meisel et al. 1997; Okamoto et al. 1995).

The two ACE-I peptides, IPP and VPP were also reported in various cheese products. Butikofer et al. (2007) analyzed 44 different varieties of cheese products of Swiss origin and found that the content of VPP ranged from 0 – 224 mg/kg whereas IPP ranged from 0 – 95.4mg/kg. Later, Meyer et al. (2009) studied the content of VPP and IPP in 7 Swiss cheese varieties including semi hard cheeses (Appenzeller 1/4 fat, Tilsiter, Vacherin fribourgeois and Tete de Moine) and hard cheeses such as Berner Hobelkase, Le Gruyere and Emmentaler. They also showed positive correlation between the ACE inhibition and concentration of IPP + VPP at advanced stage of ripening because of the lower susceptibility of IPP + VPP for further degradation compared to other ACE-I peptides.

5. Factors affecting the synthesis and activity of ACE-I peptides

There are various factors, which effects the formation of ACE-I peptides during milk fermentation and their ability to reach the target tissue in active form. The important factors are

the starter culture and substrate (casein and whey protein) used for fermentation, amino acid composition of ACE-I peptides, pre and post fermentation treatments, and the stability of ACE-I peptides to gastrointestinal digestion.

5.1.Starter cultures

ACE-I peptides are produced during hydrolysis of milk protein by the proteolytic enzymes produced by the starter culture (Figure 1). The site of action of microbial protease on the polypeptide chain of casein decides the activity, size and sequence of the bioactive peptides formed during fermentation. ACE-I activity depends on the degree of protein hydrolysis till a particular level, beyond which, the activity reduces due to extensive proteolytic degradation of protein including ACE-I peptides (Ong et al. 2007). The starter cultures generally used for milk fermentation can be grouped as bacterial and yeast starter cultures. The different types of starter used for the production of ACE-I peptides are discussed below.

5.1.1. Bacterial starter cultures

Most of the reports on ACE-I peptides in fermented milk products are developed by LAB. The starter LAB group mainly includes *Lc. Lactis*, *Leuconostoc* spp, *Streptococcus thermophilus*, *Lb. delbrueckii* and *Lb helviticus* (Settanni and Moschetti, 2010). The proteolytic system of this LAB is composed of cell envelop proteinases and more than 10 different intracellular peptidases (Exterkate, 1995; Haandrikman et al. 1991). The strain of LAB used as a starter is one of the major factor that influences the activity and sequence of the peptides (Gobbetti et al. 2002; Rodríguez-Figueroa et al. 2010), as the extent of proteolysis and specificity will depend on the LAB strain used as a starter culture. LAB isolated from both dairy and non dairy sources have been used as a starter for the production of ACE-I peptides (Gutiez et al.

2013; Kilpi et al. 2007). Among different LAB, *Lactococcus lactis* is mostly used for the preparation of fermented dairy products due to its ability of fast lactose fermentation and flavor production (Kuipers, 2001). Rodríguez-Figueroa et al. (2010) have showed ACE-I activity of water soluble fraction of milk fermented with *Lc. lactis* isolated from different ecological niches, such as green beans, corn, dairy starter cultures, beetroot, whey, curd and Chihuahua cheese. *Lactobacillus helveticus* is one of the most popular LAB with a strong proteolytic activity for production of ACE-I peptides IPP and VPP in various fermented milk products (Pan and Guo, 2010; Yamamoto et al. 1994). In another study, *Lb. jensenii* showed higher ACE-I properties when compared to other *Lactobacillus* sp (Philanto et al. 2010).

The activities of the peptides formed are comparable if same starter is used for different products. VPP and IPP are reported in different milk products where *Lb. helviticus* is used as a starter culture such as sour milk (Nakamura et al. 1995a,b), soft and hard cheese (Bütikofer et al. 2007; Meyer et al. 2009) and yogurt (Kajimoto et al. 2002). The elevated concentration of VPP and IPP during cheese ripening was also associated with the presence of *Lb. helveticus* in the starter culture (Meyer et al. 2009). Higher activity of ACE-I peptides in milk fermented with *Lc. lactis* D1BCA2 than other *Lactobacillus* species was reported by Nejati et al. (2013). Strain of *Enterococcus* genera has also been reported in several fermented milk products. Quiros et al. (2007) have identified several novel ACE-I peptides in milk fermented with *Enterococcus faecalis* CECT 5727. Among the ACE-I peptides, two of the identified peptides, originating from *β*-casein were LHLPLP *f*(133–138) and LVYPFPGPIPNSLPQNIPP *f*(58–76), showing ACE-I activity (IC_{50}) values as low as 5 μM.

The proteolytic system of different LAB even in the same genus target different types of milk proteins (α_{s1}, α_{s2} and β-casein) for the formation of ACE-I peptides. Milk fermented with *Lb helveticus* CPN4 produces ACE-I peptide (YP) on the hydrolysis of whey protein (Yamamoto et al. 1999). In another study, milk fermented with *Lb. delbrueckii* resulted in production of ACE-I peptide (SKVYPFPGPI) from β-casein (Ashar and Chand, 2004b). Different strains of same species as a starter culture also affects the ACE-I activity of the product. Milk inoculated with different strains of *Lc. lactis* showed difference in ACE-I properties in the fermented milk products (Rodriguez-Figueroa et al. 2010). The *Lc. lactis* strains isolated from artisanal dairy products showed higher ACE-I activity, whereas lowest activity was observed in milk fermented with *Lc. lactis* strains isolated from vegetables. Similarly, Quiros et al. (2007) has isolated novel ACE-I peptides with higher activity from milk fermented with *E. faecalis* strain isolated from milk source than the non milk sources. Chen et al. (2014) studied ACE-I activity of fermented milks produced by *Lb. helveticus* strains isolated from traditional Chinese and Mongolian fermented foods. Among all the isolates, milk fermented by *Lb. helveticus* strain H9 (IMAU60208) had the highest *in vitro* ACE-I activity (86.4 ± 1.5%), and detectable concentrations of VPP (2.409 μ*M*) and IPP (1.612 μ*M*). Recently, Chen et al. (2015) isolated 38 *Lb. helveticus* strains from traditional fermented dairy products showing more than 50 % ACE-I activity in fermented milk, and 3 strains (IMAU80851, IMAU80852, IMAU80872) among the isolates showed higher activity. Fermented Goat milk prepared using multiple starter culture (includes LAB) has shown to posses good ACE-I activity (Minervini et al. 2009). Non-starter LAB also contributes to ACE-I activity of fermented milk products. Recently, non-starter LAB,

Lb. casei PRA205 and *Lb. rhamnosus* PRA331, isolated from cheese showed strong ACE-I activity (IC_{50}) of 55.57 µg/ml and 212.38 µg/ml, respectively (Solieri et al. 2015).

In a recent study, milk fermented with *Bifidobacterium bifidum* MF20/5 showed higher ACE-I activity in comparison to other LAB including *Lactobacillus helveticus* DSM 13137 (Gonzalez-Gonzalez et al. 2013). Previously, Ramachandran and Shah (2008) have also shown similar results in milk fermented with *Bifidobacterium longum* in comparison to other species such as *Lactobacillus acidophilus* and *Lactobacillus casei*.

5.1.2. Yeast starter cultures

Yeasts are believed to play a major role in fermentation of various milk products such as *Koumis, Kefir, Kurut, Amasi, Viili, Longfil* and *Laban* (Chaves-Lopez et al. 2012). Yeast fermentation can influence the flavor production and/ or accelerate ripening. The ability of yeast protease for the production of bioactive peptides has been reported in various fermented products (Addis et al. 2001; Didelot et al. 2006; Chaves-Lopez et al. 2012). In addition to LAB, yeast has also been used as a co-starter for production of fermented product possessing ACE-I activity (Bai et al. 2010). Higher population of yeast in *Kumis* (a traditional, low alcoholic fermented cow milk of south west Colombia) is reported to produce peptides with ACE-I properties (Chaves-Lopez et al. 2012). *Clavispora lusitaniae, Galactomyces geotrichum, Candida tropicalis* and *Pichia kudriavzevii* were the dominant yeast species isolated from Colombian kumis (Chaves-Lopez et al. 2012). Among the various yeast isolated from Colombian Kumis, milk fermentation with *P. kudriavzevii* KL84A and *Kluyveromyces marxianus* KL26A resulted in higher ACE-I activity. In naturally fermented cow milk in the Tibetan platue of China, *Kluyveromyces*

marxianus, Saccharomyces cerevisiae and *Pichia fermentans* were the most common cultivable species reported (Bai et al. 2010). Namakura et al. (1995a) co-cultured *Saccharomyces cerevieseae* and *Lactobacillus helveticus* for the formation of ACE-I peptides in sour milk. In a recent study, a combination of proteolytic co-culture of *P. kudriavzevii* KL84A, *Lb. plantarum* LAT3 and *E. faecalis* KL06 showed highest ACE-I activity during milk fermentation (Chaves-Lopez et al. 2014).

Several studies have shown the role of yeast during cheese ripening (Padilla et al. 2014; Fleet et al. 2007) as they affect the quality of cheese in relation to their biological activities. The major yeasts reported in the ripening process of different cheese varieties are *Trichosporon cutaneum* (Corbo et al. 2001), *Candida zeylanoides* (Fadda et al. 2010), *Debaryomyces hansenii* (Padilla et al. 2014) and *Geotrichum candidum* (Tornadijo et al. 1998). In comparison to LAB, reports on formation of ACE-I peptides in yeast fermented milk products are less and there may be a higher possibility of discovering novel peptides on yeast proteolysis.

5.2.Substrate – casein and whey protein

Milk contains 3.5 – 6 % protein depending on its source, which consists of casein (80%) and whey protein (20%) (Madureira et al. 2007). Casein comprises the major component of milk protein that represents the product of four genes that differ considerably in their relative proportion across species (Ginger and Grigor, 1999). Bovine caseins are composed of four phosphoproteins, α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, which are found in milk at approximate proportions of 4:1:4:1 (Bevilacqua et al. 2006). The two major whey proteins are α-lactalbumin and β-lactoglobulin (Weimann et al. 2009). Each of these milk proteins are present in different allelic forms, controlled by codominant genes (Farrell et al. 2004). There is

considerable variation in the primary sequence of α_{s1}, α_{s2} and β-casein with rapidly evolving genes that are proposed to have common precursor (Ginger and Grigor, 1999). There is a greater possibility of producing bioactive peptides with various structures and functions on hydrolysis of milk proteins of different breeds or bovine species (Benkerroum, 2010). Fermented products are available from milk of different sources such as "*Kefir*" from camel milk, "*Churphi*" from cow and yak milk, "Cheeses" from cow and camel milk (Didelot et al. 2006; Moslehishad et al. 2013; Padilla et al. 2014). Although, there are significant differences in physiochemical characteristics of milk obtained from different sources (cow, sheep, goat, yak and camel) there is a great homology in the sequence of milk protein (Meira et al. 2012). A minor difference in the protein sequence can lead to the production of different types of bioactive peptides. The genetic polymorphism of casein protein also determines the type of bioactive peptide formed during hydrolysis (De Noni et al. 2009). The genetic variant of bovine κ-casein A, B, C, E, F1, F2, G1, G2, H, I, and J can result in the formation of different type of ACE-I peptides (Weimann et al. 2009). Camel milk fermented with *Lb. rhamnosus* PTCC1637 have shown to possess higher ACE-I activity compared to bovine milk fermented with the same starter culture. Sheep milk and its fermented products are very important in Greece and they have been studied for various health benefits and have good sensory properties (Papadimitriou et al. 2007). Some of the rare sources of milk have to be studied for the formation of ACE-I peptides during fermentation, as slight difference in amino acid sequence of the parent protein can result in the formation of novel ACE-I peptides.

5.3.Composition of ACE-I peptides

ACE-I peptides are found to be competitive substrate inhibitors of ACE (Miguel et al. 2006), which normally contain 2-12 amino acids (Phelan and Kerins, 2011). There are two active sites of somatic ACE, the N- and C-domain, out of which the C-domain plays a major role in blood pressure regulation (Fuchs et al. 2008) and is therefore a promising pharmacological target to reduce blood pressure (Lunow et al. 2015). Several authors have described the presence of hydrophobic amino acids, such as Try, Phe, Trp, Ala, Ile, Val, and Met or positively charged amino acids such as Arg and Lys as well as Pro at the C terminal position of the peptides and demonstrated that these have better affinity with ACE (Haque and Chand, 2008; He et al. 2012). Fermented milk products with *L. delbrueckii* and *Lc. lactis* contained ACE-I peptides that had higher proportion of hydrophobic residues (Gobbetti et al. 2000). In their recent study, Rodríguez-Figueroa et al. (2012) showed that peptide fraction 1 (F1) obtained from *Lc. lactis* NRRL B-50571 from fermented milk had Pro at the C-terminal position (peptide sequence HPHPHLSFMAIPP), which has a hydrophobic nature, responsible for higher ACE-I activity. They also concluded that presence of positively charged amino acid residue His at C-terminal position of the peptide DDQNPH is responsible for its lower IC_{50} value of ACE-I activity. The composition and sequence of the peptide is dependent on the above factors such as type of starter used and protein composition of the milk used for fermentation.

5.4. Pretreatment of milk before fermentation

Cheese made from raw milk resulted in higher ACE-I activity than that made from pasteurized milk, which could be due to wide range of proteolytic microflora, particularly non-starter lactic acid bacteria (Butikofer et al. 2007; Gomez-Ruiz et al. 2002). Heat pretreatment may also promote the degree of protein hydrolysis during fermentation. In another study,

skimmed milk subjected to pasteurization (90°C for 30 min) and heat pretreatment (135°C for 5 sec) before fermentation with *Lb. helveticus* resulted in 6.5 and 6 fold increase in VPP content, respectively (Sieber et al. 2010). This may be due to the changes in conformation of β-casein due to heat treatment. Pretreatment of milk can also result in uniformity of ACE-I property in fermented milk product due to controlled proteolysis. Pre-heat treatment has shown to improve bioactive properties due to increase in hydrolysis of protein from animal sources (Rai et al. 2009)

5.5. Post fermentation treatments

Post fermentation treatment is a very important factor, which affects the bioactivity of the peptide before it is consumed. Proteolytic microorganisms in the final fermented products are responsible for formation of additional ACE-I peptides during storage or ripening.

5.5.1. Storage of fermented milk products

Most of the fermented milk products are stored before consumption, which may lead to changes in various bioactive components. During storage, there is a possibility of hydrolysis of peptides present in the initial stages of storage (in the final fermented product) and formation of new ones due to secondary proteolysis during storage. ACE-I activity of fermented milk using *Lc. lactis* and *Lb. helveticus* was increased during cold storage for 7 days (Nielsen et al. 2009). In contrast, no specific trend was observed with respect to change of ACE-I activity in water soluble extract of bovine milk fermented with *Lb. rhamnosus* during 21 days of cold storage (Moslehishad et al. 2013). However, ACE-I activity value was found to increase during cold storage when the same starter was used for the fermentation of camel milk (Moslehishad et al. 2013). Storage studies of sheep milk yogurt at 4°C for 26 days also showed increase in ACE-I activity and peptide content in water soluble extract (Papadimitriou et al. 2007). Storage of

fermented milk products can also result in reduction of ACE-I property due to hydrolysis of potent ACE-I peptide into low activity peptide.

5.5.2. Ripening of Cheese

Cheese ripening is a very important technological process, which affects the quality of the product due to significant change in microbiological and biochemical parameters (Pachlova et al. 2012). Peptides can be produced on proteolysis of milk protein by proteases originated from milk, LAB, non LAB and yeasts that are involved in the process of ripening. Population of starter LAB reduces during cheese ripening, whereas non starter LAB increases during ripening process (Settanni and Moschetti, 2010). The enzymes produced during the ripening process hydrolyze the milk protein or the peptides formed during fermentation resulting in formation of new ones. Carboxypeptidase activity in lactobacilli and in nonstarter LAB was reported to hydrolyze α_{s1}-CN (f 24-32) to α_{s1}-CN (f 24-30) and smaller fragments during Feta cheese ripening (Michaelidou et al. 1998). Several studies have shown the effect of ripening on changes in profile of peptides responsible for ACE inhibition (Gomez-Ruiz et al. 2004; Lignitto et al. 2010). Several authors have shown positive impact of ripening on the formation of ACE-I peptides (Meyer et al. 2009; Ong and Shah, 2008). Ong and Shah (2008) reported that most of the ACE-I peptides were accumulated at the early stage of ripening, and on further proteolysis, some of the peptides were hydrolyzed into smaller fragments. They also showed that cheeses made with the addition of *Lb. casei* 279, *Lb. casei* LAFTI® L26 or *Lb. acidophilus* LAFTI® L10 had significantly higher ($P < 0.05$) ACE-I activity in comparison to cheese made without any probiotic adjunct after 24 weeks at 4 and 8 °C. Meyer et al. (2009) showed that VPP and IPP concentration above 100mg/Kg in semi hard cheese is attainable after a ripening period of about

4 to 7 months. Moreover, these ACE-I peptides are expected to be stable for several weeks. It is also reported that short and medium cheese ripening has more potent ACE-I peptides in comparison to long term ripening process (Hayes et al. 2007; Ardo et al. 2007; Saito et al. 2000), which may be due to the breakdown of ACE-I on further exposure to microbial enzymes. ACE-I activity of Manchango cheese prepared with various starters, decreased within the first 4 months of ripening, was maximum at 8 months, which further diminished towards 12 months of ripening process (Gomez-Ruiz et al. 2002). However, the change in concentration of ACE-I peptides during ripening is cheese specific and is affected by various other factors such as temperature, starter, and time of ripening (Ong and Shah, 2008; Meyer et al. 2009).

5.6.Stability of ACE-I Peptides to gastrointestinal digestion

Dietary proteins are hydrolyzed at all stages of gastrointestinal digestion before reaching the basolateral side of intestinal enterocytes. It is hence important that any bioactive peptide released on fermentation (*in vitro*), should be resistant to gastrointestinal digestion so as to reach the cardiovascular system in an active form (Figure 1). Some of the peptide sequences are inactive even after fermentation and are activated during gastrointestinal digestion by pepsin, trypsin and chymotrypsin. Pepsin plays important role in primary digestion, resulting in long chain peptides followed by secondary digestion by trypsin and chymotrypsin, resulting in formation of oligopeptides (Goodman, 2010). A recent study by Qureshi et al. (2013) has shown that more peptides are formed during gastric digestion in comparison to duodenal digestion. *In vitro* ACE-I activity of a peptide does not always imply that the peptide will exhibit *in vivo* antihypertensive effect. Thus, it is very difficult to establish a direct relationship between *in-vitro* and *in-vivo* ACE-I activity of a peptide.

Fujita et al. (2000) has classified ACE-I peptides into three groups: a) true inhibitors (Type I) – ACE-I (IC_{50} value) of the peptide are not affected by gastrointestinal proteases or preincubation with ACE, (b) Substrate type (Type II) – ACE-I peptides that are converted to low activity peptides on digestion by gastrointestinal proteases, and (c) pro drug (Type III) – peptides that are converted to potential ACE inhibitors by the action of gastrointestinal proteases (Figure 1). Effect of gastrointestinal digestion of ACE-I activity of different fermented milk products is presented in Table 3. Peptide containing (hydroxyl) proline residue is generally resistant to gastrointestinal digestion (Vermeirssen et al. 2004). Chen et al. (2010) have reported a prodrug type of ACE-I peptides in koumiss, traditional fermented mare milk. ACE-I properties of a peptide (TQPKTNAIPY) in Manchego cheese increased 6 times on gastrointestinal digestion (Gomez-Ruiz et al. 2004). VPP and IPP are highly resistant to gastrointestinal digestion and thus reach the small intestine in intact form (Meyer et al. 2009). Parrot et al. (2003) studied the effect of gastrointestinal digestion on ACE-I activity of water soluble extracts of Emmental cheese, which was found to decrease by 33 ± 13% and 32 ± 13%, after digestion by pepsin and trypsin, respectively. *In silico* digestion of LVYPFP present in *B. bifidum* MF 20/5 fermented milk has suggested that it can be hydrolysed under gastrointestinal conditions (pepsin digestion) into peptide fragments with higher potency, which would enhance the antihypertensive properties (Gonzalez-Gonzalez et al. 2013).

A fermented milk product can have a mixture of different ACE-I peptides, which will differ in their ability to survive on gastrointestinal digestion (Gomez-Ruiz et al. 2004). However, the bioavailability of any ACE-I peptide, which is resistant to gastrointestinal enzymes is also strongly affected by its absorption. In a study conducted on oral administration of Calpis sour

milk, 4.0% and 5.0% of ingested VPP and IPP reached the aorta of spontaneously hypertensive rats (Masuda et al. 1996). Also, Jauhiainen et al. (2007a) used radiolabelled tripeptide IPP for oral dose to rats and observed partly intact absorption from the gastrointestinal tract. They also reported that the excretion of IPP was slow, which was not even completely excreted after 48 hours and radioactivity was observed in aorta, liver and kidney.

6. Animal studies and clinical trials of fermented milk products

There is always a possibility that a peptide exhibiting *in-vitro* ACE-I activity may not show *in-vivo* effect, as peptides are prone to gastrointestinal digestion before reaching the site of action. It has been shown that some peptides showing *in-vitro* ACE inhibition are inactive after oral administration (Saito et al. 1994; Aihara et al. 2005; Yamamoto et al. 1999). Therefore, several animal studies and clinical trials have been conducted to prove antihypertensive properties of various fermented milk products (Nakamura et al. 1995b; Seppo et al. 2003; Xu et al. 2008).

6.1.Animal studies

The spontaneously hypertensive rat (SHR) model is the most extensively and successfully used animal model to test medicines for their effectiveness in lowering blood pressure, and also to study the mechanisms in established hypertension (Doggrell and Brown, 1998; Rodriguez – Figueroa et al. 2013). The bioactive peptides VPP and IPP in Calpis have shown to reduce the systolic blood pressure after 6-8 hours when fed to SHR at a dosage of 5 mL/kg of body weight (Nakamura et al. 1995b). A significant decrease in abdominal aorta ACE activity on administration of sour milk rich in VPP and IPP was showed by Nakamura et al. (1996). Further, the presence of ACE-I peptides (VPP and IPP) in the extract of aorta was also demonstrated

(Masuda et al. 1996). Long-term intake of IPP and VPP, or sour milk containing these tripeptides attenuated the development of hypertension in SHR (Sipola et al. 2002). In another study, a yogurt like product fermented with *Lb. helveticus* CPN4 rich in di-peptide (Tyr-Pro) has shown to possess antihypertensive property in SHR (Yamamoto et al. 1999). Milk fermented with *Enterococcus faecalis* exhibited antihypertensive activity when orally administered to SHR. The antihypertensive property of milk fermented by *Enterococcus faecalis* was related with the peptides LHLPLP, LVYPFPGPIPNSLPQ-NIPP, VLGPVRGPFP, and VRGPFPIIV (Muguerza et al. 2006), LHLPLP and LVYPFPGPIPNSLPQNIPP (Quiros et al. 2007). The peptides derived from the β-casein of milk fermented with *Lb. jensenii* showed transient reduction of blood pressure in SHR (Philanto et al. 2010). In another study, milk fermented with *Lactococcus lactis* NRRL B50571 showed similar antihypertensive properties in comparison to captopril (40 mg/ Kg of body weight) in SHR (Rodriguez – Figueroa et al. 2013). In a recent study, a single oral dose of *Lb. helveticus* H9 fermented milk significantly attenuated the systolic, diastolic, and mean blood pressure of SHR by 15 to 18 mm Hg after 6 to 12 h of treatment (Chen et al. 2014). Further, long-term (7 weeks) daily intake of *Lb. helveticus* H9 fermented milk resulted in significant antihypertensive effect to SHR by lowering 12 ad 10 mm Hg systolic and diastolic blood pressure in comparison to control rats.

6.2.Human Trials

Antihypertensive property of peptides in fermented milk products has also been proven by several human trails. In a placebo-controlled study, Hata et al. (1996) showed antihypertensive effect of sour milk in hypertensive patients. Sour milk was also studied for its hypotensive effects on patients with mild or moderate hypertension and resulted in a significant decrease in

blood pressure (Kajimoto et al. 2002). In a placebo-controlled double blind study, consumption of yoghurt had significant effect in reducing blood pressure of mild hypertensive patient without causing any adverse effect (Kajimoto et al. 2002). Fermented milk (Evolus) showed hypotensive properties in 8 weeks double-blind, placebo-controlled, randomized trial including 17 subjects with mild hypertension (Seppo et al. 2002). Further, in a long-term study (21 weeks) conducted on hypertensive patients, milk fermented with *Lb. helveticus* LBK-16H was able to significantly reduce systolic and diastolic blood pressure in comparison to control group (Seppo et al. 2003). In another study, daily ingestion of tablets containing fermented milk with *L. helveticus* CM4 reduced the elevated blood pressure in subjects with high blood pressure and mild hypertension (Aihara et al. 2005). Ambulatory blood pressure measurement for 24 hours showed that consumption of milk fermented with *Lb. helveticus* resulted in lowering blood pressure in hypertensive subjects (Jauhiainen et al. 2005) and reduced the arterial stiffness in hypertensive subjects (Jauhiainen et al. 2007b). A meta-analysis of 12 randomized controlled trials (total 623 participants) showed that milk derived tripeptides (VPP and IPP) have hypotensive effect in hypertensive and pre-hypertensive subjects (Xu et al. 2008). The effect of VPP and IPP was more evident in Asian patients based on meta-analysis of 18 clinical trials (Cicero et al. 2011). Recently, a cross sectional study was conducted to examine whether the frequency of Gamalost cheese consumption was associated with blood pressure in a Norwegian population (Nilsen et al. 2014). The result of the study concluded that each increase in frequency unit of Gamalost intake corresponded to a reduction in systolic blood pressure of 0.72 mmHg, after controlling factors such as, sex, age, education, waist circumference, physical activity, smoking status, and dairy

food intake. These animal experiments and human trials confirm the ability of peptides derived during milk fermentation as an effective food component for reduction of hypertension.

7. Commercial milk products with ACE-I property

The dairy industry is a dynamic sector with many new products with additional benefits appearing every year. There are various milk derived commercial products (sour milk, yoghurt, cheese etc.) having ACE-I properties and their market value has significantly increased in the last decade. These products are either in the form of milk protein hydrolysate or fermented milk product. Fermented milk products with health benefits are gaining widespread popularity and acceptance in developed countries such as Europe, Japan and United States. Europe has experienced rapid growth in commercial production of fermented foods, and fermented milk products with ACE-I properties are widely consumed (Stanton et al. 2001; Hernandez-Ledesma et al. 2005). The active peptides (IPP and VPP) present in fermented milk products with brand name of Calpis (Calpis Co., Japan) and Evolus (Valio, Finland) are commercially available in the global market. Japan has the most advanced market where nutraceutical dairy products represent 44% of the total dairy market. In Japan, the functional food market is over $16 billion and is increasing at the rate of 12% per year for the last 10 years (Bhadoria and Mahapatra, 2011). In USA, per capita consumption of refrigerated yoghurt has increased from 6.5 pounds in 2000 to 13.5 pounds in 2010 (IDFA, 2011). The increase in market value in recent years is encouraging for researchers to come up with novel fermented products and peptides with wide range of health benefits.

8. Conclusions and future

Recent studies have provided conclusion that ACE-I peptides can play a vital role in human health by reducing the risk of hypertension. The possibility of designing fermented milk based new functional foods and nutraceuticals to reduce hypertension is a promising area of research. Improvement of antihypertensive properties has been achieved in selected fermented milk products by optimizing fermentation conditions for controlled proteolysis and formation of ACE-I peptides. There are numerous traditional fermented milk products, which still need to be studied for the production of ACE-I peptides. Future direction of research should be oriented towards (i) selection of starter culture producing fermented milk with higher ACE-I properties and (ii) development of mixed starter culture for providing several health benefits in same product. However, future studies on molecular mechanism, impact of peptides on gene expression has to be done to optimize the concentration of a peptide needed to get optimum health benefits. Studies on bioactive peptides in fermented milk products will also lead to interesting opportunity to dairy industry for their expansion and promotion of various traditional milk products with potential health benefits.

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Table 1. Biological activities of fermented milk derived bioactive peptides

Activity	Milk product	Starter	Specific bioactive compound	Reference
Peptide mediated biological activity				
ACE inhibitory	Cheese	LAB*	FFVAP, TTMPLW	Kajimoto et al. 2002; Korhonen, 2009
	Yoghurt	LAB and Yeast*	VPP, IPP	
Antioxidant	Yoghurt	LAB	VPYPQ, IPIQY, KAVPYPQ	Sabeena et al. 2010
Antimicrobial	Sodium caseinate fermented	*Lactobacillus acidophilus* DPC6026	IKHQGLPQE, VLNENLLR	Hayes et al. 2006
Anticancer	Fermented goat milk	*Lb. plantarum* and *Lb. paracasei.*	Peptide not characterized	Nandhini and Palaniswamy, 2013
Immunomodulation	Fermented milk	*Lb. helveticus* R389	Peptide not characterized	LeBlanc et al. 2002
Antitumor	Fermented	*Lb. helviticus*	Peptide not	LeBlanc et al.

	milk	R389	characterized	2002
Lipid lowering	Fermented milk	*Lc. lactis* NRRL B-50571	YPSYGL, TVQVTSTAV, SLPQNIPPL	Galland et al. 2014

LAB – lactic acid bacteria, "*" – there are many examples throughout the manuscript

Table 2. Selected fermented milk and cheese products, their starter and ACE inhibitory peptides

Milk products	Starter	Sequence	Protein origin	Reference
Fermented milk type products				
Skimmed milk	*Enterococcus faecalis* CECT5727	LHLPLP, LVYPFPGPIPNSLPQNIPP	β-casein	Quiros et al. 2007
Fermented bovine Skim milk	*Enterococcus faecalis* BCS27	VVVPPF, ENLLRF	β-casein	Gutiez et al. 2013
Dahi	*Lactobacillus delbrueckii*	SKVYP	β-casein	Ashar and Chand, 2004a
Yoghurt	*Lb. helveticus* and *S. cerevisiae*	VPP, IPP	β-casein	Kajimoto et al. 2002
Kefir	*Lc. lactis, Lc. cremoris, Lb plantarum, Lb. casei, Kluyveromyces marxianus*	PYVRYL, LVYPFTGPIPN	α_{s2}-casein β-casein	Quiros et al. 2007
Sheep milk yogurt	*Lb. delbrueckii, Streptococcus*	DKIHPFAQ, TQTPVVVP	β-casein	Papadimitriou et al. 2007

	thermophilus and *Lb paracasei*			
Koumiss (mare milk)	Lactic acid bacteria and yeast	YQDPRLGPTGELDP-ATQPIVAVHNPVIV	β-casein	Chen et al. 2010
Sour milk (Calpis)	*Lb. helveticus*, *Saccharomyces cereviseae*	IPP, VPP	β-casein	Nakamura et al. 1995a
Probiotic fermented milk	*Bifidobacterium bifidum* MF 20/5	LVYPFP	β-casein	Gonzalez-Gonzalez et al. 2013
Probiotic Yogurt	*Lb. delbrueckii ssp. Bulgaricus*, *Streptoco ccus thermophilus*	8 peptides	α_{s2}-casein , κ-casein and β-casein	Dunkor et al. 2007
Fermented milk	*Kluyveromyces marxianus* Z17	VLSRYP, LSFF	κ-casein, α_{s2}-casein	Li et al. 2015
Cheese type products				
Cheddar cheese	*Lactobacillus casei*	RPKHPIKHQ, DKIHPF	α_{s1}-casein β-casein	Ong et al. 2007
Manchango	Mixed LAB starter	RPKHPIKHQ	α_{s1}-casein	Gomez-Ruiz

				et al. 2004
	Mixed LAB starter	VRYL	α_{s1}-casein	
Fresco	*Enterococcus*	EVLNENLLRF	α_{s1}- casein	Torres Llanez
cheese	*faecium*			et al. 2011
	Lactococcus lactis	YQEPVLGPVRGPFPI	β-casein	
Crescenza	*S. thermophilus, Lb.*	LVYPFPGPIHNSLPQ	β-casein	Smacchi and
	delbrueckii ssp.			Gobbetti,
	bulgaricus;			1998
Festivo	*Lactobacillus*	RPKHPI, RPKHPIK	α_{s1}-casein	Ryhanen et
	acidophilus and			al. 2001
	Bifidobacteria			
Gouda	Mixed starter	RPKHPIKHQ	α_{s1}-casein	Saito et al.
				2000
		YPFPGPIPN	β-casein	

LAB – lactic acid bacteria

Table 3. Effect of Gastrointestinal digestion on activity of ACE-I peptide in selected fermented milk products

Milk product	Starter	Enzymes used	Peptides	Effects	Reference
Caprine Kefir	Mixed lactic acid bacteria	Pepsin, Corolase	DKIHPF	8 fold	Quiros et al. 2005
Colombian Kumis	*Clavispora lusitaniae* KL4A	Pepsin, Pacreatin	NI	+ 1.1 fold	Chaves-Lopez et al. 2012
	Torulaspora delbrueckii KL66A	Pepsin, Pacreatin	NI	+ 1.2 fold	
Manchango Cheese	Mixed lactic acid bacteria	Pepsin, Pancreatic extract	TQPKTNAIPY	+ 6 fold	Gomez-Ruiz et al. 2004
Fermented milk	*Lactobacillus casei*	Pepsin, Corolase	GPV, HLPLP, PFPGPIPN	2-3 fold	Hernandez-Ledesma et al. 2004
Fermented milk	*Enterococcus faecalis*	Pepsin, Corolase	LVYPFPGPIP NSLPQNIPP	+ 17 fold	Quiros et al. 2009
			VRGPFPIIV	- 0.5 fold	

Gamalost Cheese	Lactic acid bacteria and *Mucor mucedo* (ripening)	Human Gastric and duodenal juice	Many peptides	Varied with peptides	Qureshi et al. 2013

NI- Not identified

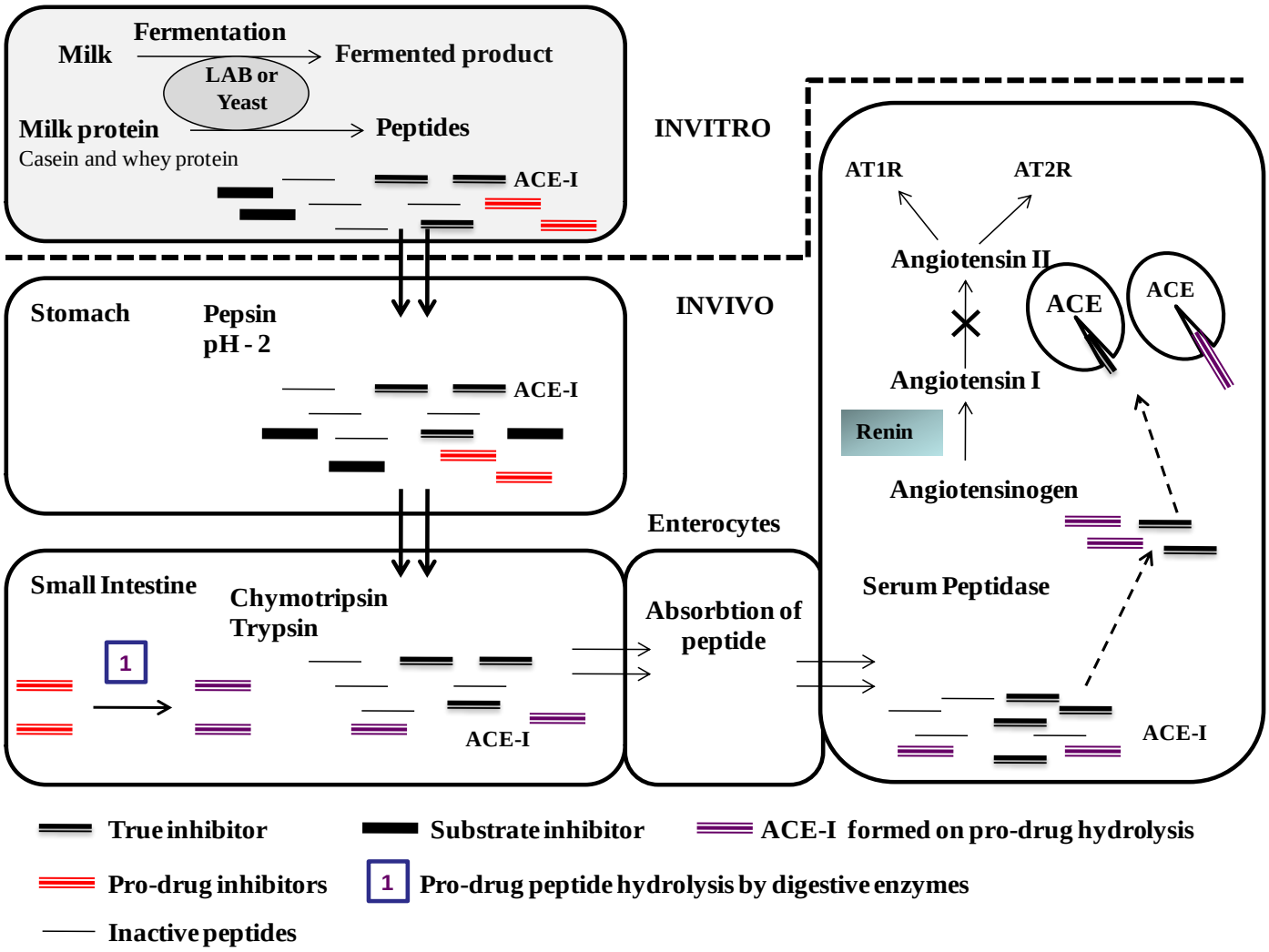

Figure 1. Formation and fate of angiotensin converting enzyme inhibitory (ACE-I) peptide before reaching the site of action