Full Length Research Paper

Therapeutical properties of cow milk fermented with *Lactobacillus plantarum* and *Lactococcus casei*

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Milk is an excellent source of well balanced nutrients and also exhibits a range of biological activities that influence digestion metabolic responses to absorbed nutrients, growth and development of specific organs, and resistance to disease. The angiotensin converting enzyme inhibitory activity of cow milk was evaluated. In the present study, the high ACE-inhibitory activity of 79% was found in milk fermented with the combination of *Lactobacillus plantarum* and *Lactococcus casei*. Our results clearly demonstrate that the cow milk hydrolysate have antioxidant activity. It showed strong scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical with IC₅₀ value of 231 µg/ml. And in the total reducing assay method, milk hydrolysate showed significant antioxidant activity. The fermented milk hydrolysate also inhibits the growth of *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*.

Key words: Cow milk hydrolysate, angiotensin converting enzyme inhibition, antioxidant activity, anti-bacterial activity.

INTRODUCTION

The importance of proteins in the diet has been increasingly acknowledged as a result of new scientific findings in the field of nutrition over the last two decades. The value of proteins as an essential source of amino acids is well documented, but recently it has been recognized that dietary proteins exert many other functionalities in *vivo* by means of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and can be released by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). Milk proteins are precursors of many different biologically active peptides. Milk protein-derived bioactive peptides may function as exogenous regulatory substances with hormone-like activity on the different intestinal and peripheral target sites of the mammalian organism (Gobbetti et al., 2000). Opiate, antithrombotic, anti-hypertensive, immunomodulating, antibacterial, anti-gastric, human immunodeficiency virus type 1 protease inhibitory, and mineral carrying are some properties that have been attributed to several of the bioactive sequences identified (Meisel, 1997, 1998; Meisel and Bockelmann, 1999; Smacchi and Gobbetti, 2000). The production and properties of milk protein-derived bioactive peptides have been reviewed in many articles (Korhonen and Pihlanto, 2006, 2007).

Cow milk contains many constituents including electrolytes, proteins, and peptides, which could affect blood pressure beneficially. In general, the major protein fractions in bovine milk include α-lactalbumin, β-lactoglobulin, caseins, immunoglobulins, lactoferrin, protease-peptide fractions (heat-stable, acid soluble phosphoglycoproteins), and minor whey proteins, such as, transferrin and serum albumin (Pihlanto, 2006; Lopez-Exposito and Recio, 2008). Hypertension is a major risk factor for the development of cardiovascular diseases, which is one of

Abbreviations: LAB, Lactic acid bacteria; SHR, spontaneous hypertensive rats; HA, hippuric acid; ACE, angiotensin-I-converting enzyme; HHL, hippuryl-histidyl-leucine.

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of the main causes of mortality in Western countries (Duprez et al., 2002). Diet and lifestyle modification represent effective tools in the prevention of hypertension.

In the treatment of the disease, these diet and lifestyle changes can decrease requirements of antihypertensive medication, as well as have beneficial effects related to hypertension not remedied by most drugs (Hermansen, 2000). In this respect, functional foods with blood pressure lowering properties have recently received considerable attention.

Angiotensin-I-converting enzyme (ACE) is a key enzyme in the regulation of peripheral blood pressure. ACE is a dipeptide-liberating carboxypeptidase (peptide-lydipeptide hydrolase, EC 3.4.15.1), classically associated with the renin-angiotensin system, and converts angiotensin I into angiotensin II, a highly potent vasoconstrictor molecule (Skeggs et al., 1956). The ACE inhibitors are well established in the therapy of hypertension and heart failure and have been shown to exert organ protective effects (Parmley, 1998). ACE inhibitory peptides have been produced by the enzymatic hydrolysis of milk proteins and by fermentation with lactic acid bacteria (FitzGerald et al., 2004). Several milk peptides inhibit ACE in vitro (Nakamura et al., 1995; Maeno et al., 1996; Abubakar et al., 1998; Pihlanto et al., 2000). The antihypertensive effect of the milk fermented with a starter containing Lactobacillus helveticus and Saccharomyces cerevisiae, is due to the presence of the ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro, contained in the primary structure of β-casein, and β-casein and κ-casein, respectively (Takano, 1998).

It is generally accepted that the total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and nonimmunoglobulin defense proteins or peptides. This may be due to the synergistic activity of naturally occurring proteins and peptides, in addition to peptides generated from inactive protein precursors (Clare and Swaisgood, 2000). Antimicrobial peptides are observed throughout nature. Casecidin, obtained by chymosin digestion of casein at neutral pH, was among the first defense peptides actually purified and exhibited activity in vitro against Staphylococcus, Sarcina, Bacillus subtilis, Diplococcus pneumoniae, and Streptococcus pyogenes (Lahov and Regelson, 1996). Milk also contains peptides that exhibit antifungal properties. Antifungal activity of lactoferrin or its peptides (example, lactoferricin B), in combination with azole antifungal agents, has been demonstrated with Candida albicans (Bellamy et al., 1993; Wakabayashi et al., 1996).

Free radicals are produced in the body in the course of regular metabolism but when exposed to xenobiotic agents from foods and environment, the risk of radical production significantly increases. The most important are the free radicals derived from oxygen. Provided the antioxidant system of the organism does not manage to neutralize them rapidly enough, they can cause destructive or lethal changes (such as, apoptosis) through oxidation of membrane lipids, proteins, enzymes and deoxyribonucleic acid (DNA). The cellular and subcellular damage caused by free radicals plays an important role in the pathogenesis of cancer, cardiovascular diseases, allergies, atherosclerosis, and other civilization diseases (Halliwell and Gutteridge, 1989; Bergendi et al., 1999; Agerholm et al., 2000).

The antioxidant activity of several species and strains of milk bacteria contained in fermented milk can significantly affect human health. Sour milk compared to nonfermented milk has shown important improvement of the overall antioxidant activity of blood, as well as antioxidant status, prolonged resistance of lipoprotein fraction to oxidation, reduced level of peroxide lipoproteins and oxidized LDL cholesterol, reduced level of glutathione reduct ratio, and increased overall antioxidant activity. Some lactobacilli produce antioxidant factors also in the human gastrointestinal tract (Ljungh et al., 2002).

The majority of milk bacteria show antioxidant behavior (eliminating the excess oxygen free radicals) producing superoxide dismutase, or glutathione. Taking this into account, several laboratories are working on the optional use of the milk bacteria in the form of food supplements that enhance also the antioxidant status of an individual. The antioxidant activity exerts also various peptides derived from α-lactalbumin, β-lactoglobulin and α-casein (Fitzgerald and Murray, 2006). Recently, people have become increasingly interested in the physiological functionality of foods, in terms of antioxidative activity and anti-hypertensive effect.

The aim of the present study was to evaluate the possible antihypertensive effect of fermented milk preparation, and also studied their role in antibacterial and antioxidant activities.

MATERIALS AND METHODS

Bacterial strains

The lactic acid bacteria (LAB) isolated from different dairy products was used for the present study. MRS media (Himedia, India) was used for the isolation of lactic acid bacteria. The isolated bacteria were purified by random selection of colonies from the MRS agar plates and transformed onto MRS agar. Preliminary tests were employed on the isolates to ensure that they belong to LAB. The isolated bacteria were identified as Lactobacillus plantarum and Lactococcus casei according to the methods described by Harrigan and Mccane (1976).

Production of fermented milk

Cow milk used for the present study was collected from nearest dairy farm, Coimbatore, India and sterilized by heating at 93°C for 20 min under constant stirring. L. plantarum and L. casei were used to inoculate (1%, v/v) 50 ml of pasteurized milk. Inoculation was carried out under sterile conditions and the milk was kept for ferme-
Preparation of milk hydrolysates

For the determination of the ACE inhibitory activity of the milk after fermentation, the whey fraction was used. The whey fraction was obtained as follows: The pH of milk was adjusted to 3.4 by the addition of 50% lactic acid, and then the milk was centrifuged at 6000 g for 10 min; 10 N NaOH was added to the supernatant to raise the pH to 8.3, and then the supernatant was centrifuged at 6000 g for 10 min. Milk hydrolysate was finally ultrafiltered through a 10 kDa cut-off membrane in a stirred ultrafiltration cell module (Amicon-Ultra15, Millipore). Ultrafiltered permeates were analyzed at this step and also after a second step of fractionation through a 3 kDa cut-off filter (Amicon-Ultra15, Millipore) with centrifugation (3200 x g for 40 min at 15°C). The final supernatant was used as the whey fraction (Nakamura et al., 1995).

Measurement of ACE inhibitory activity

The ACE inhibitory activity assay was by the method of Cushman and Cheung (1971) with some modifications. The Hip-His-Leu was dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. Then, 200 µl of 5 mM Hip-His-Leu solution was mixed with 80 µl (1 mg/ml of stock) of milk hydrolysate (the pH of which was adjusted to 8.3) and then preincubated for 3 min at 37°C. The reaction was initiated by addition of 20 µl of ACE. The mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 250 µl of 1 N HCl. The hippuric acid liberated by ACE was extracted with 1.7 ml ethyl acetate, dissolved by addition of 1 ml of distilled water after removal of ethyl acetate by vacuum evaporation, and measured spectrophotometrically at optical density of 228 nm. The extent of inhibition was calculated as follows:

\[(B - A)/(B - C) \times 100\]

Where, A is the optical density in the presence of ACE and ACE inhibitory component; B is the optical density without ACE inhibitory component and C is the optical density without ACE.

**In vitro antioxidant activity of milk hydrolysate by 2,2, diphenyl 1-picryl hydrazyl (DPPH) radical scavenging assay**

The antioxidant activity of the cow milk hydrolysate was checked by DPPH radical scavenging activity. 0.3 ml of different concentration (100, 200, 300, 400 and 500 µg) of cow milk hydrolysate was taken and made up to 0.4 ml with distilled water. To this added 0.6 ml of 100 M DPPH reagent in methanol. The reaction mixture was incubated for 20 min under dark and the reading was taken at 517 nm. The decrease in absorbance at 517 nm was taken as the antioxidant capacity of the hydrolysate. L-ascorbic acid was taken as standard (Srinivas and Prakash, 2008).

**Total reducing power assay**

The reducing power was determined according to the method of Lin et al. (2009). Different concentration (100, 200, 300, 400 and 500 µg) of cow milk hydrolysate (0.25 ml) was mixed with 0.25 ml of 200 mM sodium phosphate buffer (pH 6.6) and 0.25 ml of 1% potassium ferricyanide. Then the mixture was incubated at 50°C for 20 min and 0.25 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 3000 x g for 10 min. The supernatant (0.5 ml) was mixed with 0.4 ml of deionized water and 0.1 ml of 0.1% ferric chloride solution, allowed to stand for 10 min, and the absorbance was measured at 700 nm. Higher absorbance indicated higher reducing power. L-ascorbic acid was taken as standard.

**Determination of antibacterial activity of milk hydrolysates**

The bacterial cultures (S. aureus, E. coli and B. subtilis) were grown in nutrient broth medium at 37°C. After 6 h of growth, each microorganism, at a concentration of 10⁶ cells/ml was swabbed on the surface of Mueller-Hinton agar plates. Subsequently, sterile discs (6 mm in diameter) saturated with four different concentration (5, 10, 15 and 20 µg/ml) of milk hydrolysate was placed on the surface of each inoculated plate. The plates were incubated at 37°C for 24 h and observed for zone of inhibition.

**RESULT AND DISCUSSION**

**ACE inhibitory activities of milk hydrolysates**

ACE inhibitory activity was evaluated by the production level of hippuric acid (HA) from hippuryl-histidyl-leucine (HHL) by enzymatic activity of ACE. Figure 1 shows the ACE inhibitory activity of milk hydrolysates fermented with L. plantarum (55%) and L. casei (63.8%). Combination of these two bacteria showed inhibition of 79%. Due to increasing inhibition percentage, combination of these two organisms was used for further studies. From 11 isolated lactic acid bacteria, L. casei and L. plantarum in combination showed considerable ACE inhibitor activity after fermentation of cow milk. Yamamoto et al. (1994) demonstrated the ACE inhibitory activity of fermented milk produced by most strains of L. helveticus and it significantly lowers the blood pressure in spontaneous hypertensive rats (SHR) upon oral administration, while milk fermented by other species of lactic acid bacteria, among which was a L. acidophilus strain does not display significant antihypertensive effects. According to the same study, ACE inhibitory activity in most of the whey fractions of the milk fermented with L. helveticus is also higher than in the other fermented milks. Nevertheless, ACE inhibitory peptides have been isolated from different dairy products started by lactic acid...
bacteria: For example, Lactobacillus delbrueckii subsp. bulgaricus SS1, Lactococcus lactis subsp. cremoris FT4, Lactobacillus acidophilus, Bifidobacteria, and Streptococcus thermophilus (Gobbetti et al., 2000; Saito et al., 2000; Ryhanen et al., 2001).

Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important activity. In this study, we identified that the cow milk hydrolysate have a good ACE inhibitory activity. Recently, certain functional foods containing ACE inhibitory peptides have been shown to act as an additional or alternative treatment in hypertension. Daily administration of Calpis sour milk to hypertensive human subjects significantly reduces their blood pressure. Takano (1998) studied the antihypertensive effect of this milk fermented with a starter containing Lactobacillus helveticus and Saccharomyces cerevisiae, is due to the presence of the ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro, contained in the primary structure of β-casein, and β-casein and κ-casein, respectively.

Yamamoto et al. (1993) reported that casein hydrolyzed by proteinase from L helveticus CP790 that was isolated from the Calpis sour milk starter showed ACE inhibitory activity and also antihypertensive activity in SHR rats.

Antioxidative activity of milk hydrolysate

To obtain the information about the antioxidant effects of the milk hydrolysate, their radical scavenging effects was examined by measuring changes in absorbance of DPPH radical at 517 nm. Milk hydrolysate showed a concentration dependant scavenging of DPPH radicals. The activity was compared to L-ascorbic acid which is employed as the standard, and results were plotted against L-ascorbic acid equivalence in µg/ml (Figure 2). The IC₅₀ value for milk hydrolysate and L-ascorbic acid is 231 and 135 µg/ml, respectively.

The antioxidant activities of natural components might have a reciprocal correlation with their reducing powers. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant sample. Reducing power of a compound served as a significant indicator of its potential antioxidant activity (Figure 3).

Liu et al. (2007) studied the antioxidant nature of bovine milk β-Lactoglobulin. Several studies of evidence suggest that β-lactoglobulin (β-LG) plays a key antioxidant role in milk. First, the β-LG-deleted milk possessed about 50% less antioxidant activity than that of whole milk. Korhonen and Pihlanto (2003) in their studies have shown that antioxidative peptides can be released from caseins in hydrolysis by digestive enzymes and in fermentation of milk proteolytic LAB strains. Most of the identified peptides are derived from casein and have been shown to possess free radical scavenging activities and to inhibit enzymatic and non-enzymatic lipid peroxidation (Rival et al., 2001a; 2001b).

Evaluation of the antimicrobial potential of milk hydrolysate

The data pertaining to the antimicrobial potential of the milk hydrolysate are presented in Table 1. The antibacterial activity of the milk hydrolysate was studied using the disc diffusion method. Growth inhibitory zone of E. coli, S. aureus, and B. subtilis were followed in presence of hydrolysates, and were compared with Gentamicin except for S. aureus (use Methicillin) as standard (Figure 4).

Casocidin-I (bovine milk), a cationic αs2-CN derived peptide inhibited the growth of E. coli and S. carnosus (Minervini et al., 2003). Isracidin, an N-terminal segment of αs1-CN B, protected mice against S. aureus and
Table 1. Antibacterial activity of fermented cow milk hydrolysates.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Microorganism</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard</td>
</tr>
<tr>
<td>1</td>
<td><em>Escherichia coli</em></td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td><em>Staphylococcus aureus</em></td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus subtilis</em></td>
<td>13</td>
</tr>
</tbody>
</table>

Figure 4. Antibacterial activity of fermented cow milk hydrolysates on selected bacterial species.

**Candida albicans.**

Minervini et al. (2003) studied Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase hydrolyzed caseins of milk from six species. It showed a very large spectrum of inhibition against Gram-positive and negative bacteria, including species of potential clinical interest, such as *Enterococcus faecium*, *Bacillus megaterium*, *E. coli*, *Listeria innocua*, *Salmonella* spp., *Yersinia enterocolitica*, and *Staphylococcus aureus*.

In a literature, studies indicated that lactoferricin B was active against clinical isolates of enterohaemorrhagic *E. coli* 0157:H7 at concentrations significantly less than either the lactoferrin hydrolysate or lactoferrin, itself (Shin et al., 1998). A potent bactericidal peptide specifically generated by pepsin degradation of lactoferrin, so named lactoferricin B, also displayed antimicrobial activity towards both Gram-positive and Gram-negative microorganisms (Jones et al., 1994; Tomita et al., 1991).

**Conclusion**

The present study investigates the ACE inhibition, antioxidant activity and antimicrobial activity of hydrolysate from cow milk. Recently, much attention has been paid by consumers towards natural bioactive compounds as functional ingredients and hence it can be suggested that milk derived ACE inhibitors are alternatives tools that can contribute to consumer’s well-being, by being a part of novel nutraceuticals or pharmaceuticals replacing synthetic drugs. Food bioactive compounds are often effective in promoting health and lead to the reduction of disease risk. However, further study is needed to identify the particular peptide from milk and to study it’s *in vitro* and *in vivo* hypertensive activity.

**REFERENCES**


