Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Suppl 5, 2011

Research Article

ANTIHYPERTENSIVE AND ANTICANCER EFFECT OF COW MILK FERMENTED BY *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS CASEI*

[1]BAHULEYAN VASANTHA PRAVEESH, [2]JAYARAMAN ANGAYARKANNI, [3]MUTHUSAMY PALANISWAMY*

[1]Department of Microbiology, Karpagam Arts and Science College, Coimbatore 641021,[2]Department of Biotechnology, Bharathiar University, Coimbatore 641046, [3]Department of Microbiology, School of Life Sciences, Karpagam University, Coimbatore 641021, Tamil Nadu, India. Email: m.palaniswamy@gmail.com

Received: 3 Aug 2011, Revised and Accepted: 30 Sep 2011

ABSTRACT

Milk proteins are precursors of many different biologically active peptides. These peptides are inactive within the sequence of the precursor proteins but can be released during microbial fermentation. In the present study, the cow milk hydrolysate showed multifunctional properties like angiotensin converting enzyme inhibition, antioxidant and anticancer activity. The optimized fermentation conditions for angiotensin converting enzyme inhibitor production by the combination of *Lactobacillus plantarum* and *Lactobacillus casei* were also studied. Angiotensin converting enzyme inhibition activity (69.28±0.37%) to be highest at 37ºC with inoculum concentration of 1% after 24 hours of incubation. The milk hydrolysate exhibited scavenging potential with IC_{50} value of 185.87 µg/ml for 2, 2-diphenyl-1- icrylhydrazyl. And also the cow milk hydrolysate showed *in vitro* anticancer activity against HeLa cell with IC_{50} of 150 µg/ml.

Keywords: Cow milk hydrolysate, Angiotensin converting enzyme inhibition, Antioxidant activity, Anticancer activity.

INTRODUCTION

Hypertension, (i.e.) high blood pressure, is a key factor in the development of cardiovascular diseases such as myocardial infarction, stroke and heart failure. Life style modifications such as weight reduction, moderation of alcohol consumption, reduction in salt intake, and increase in physical activity, cessation of smoking and healthy eating patterns are recommended for treatment of mild hypertension without risk factors for cardiovascular diseases[1]. Further, more approaches like these can contribute to the primary prevention of hypertension. In clinical practice vasodilators, diuretics, calcium channel blockers, angiotensin II receptor blockers and angiotensin I-converting enzyme (ACE) inhibitors are normally used.

ACE plays an important role in the regulation of peripheral blood pressure in the human body. The dipeptidylcarboxypeptidase, ACE catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin. The ACE inhibitors are well established in the therapy of hypertension and heart failure and have been shown to exert organ protective effects[2]. Synthetic ACE inhibitors such as captopril, enalapril, alecepril and lisinopril are used extensively in the treatment of essential hypertension. These drugs may cause several side effects, like hypotension, reduced renal function, cough, and fetal abnormalities. This has stimulated the search for natural ACE inhibitors, and consequently several peptides have been identified in a range of food proteins[3].

For many years, food researchers have extensively studied peptides derived from food proteins as potential nutraceuticals in respect to the development of functional foods. Many research groups have combed for novel ACE inhibitors in natural products and in microbial sources. ACE inhibitory peptides have been discovered in various food sources such as milk[4,5], gelatine[6], maize[7,8], soybean[9], and wheat[10]. Among the food sources, milk proteins are a good source of bioactive peptides[11,12]. Milk is a good source of protein and essential amino acids, minerals and vitamins (especially calcium, magnesium, zinc, vitamin A and vitamin B12). The bioactive peptides encrypted in intact milk proteins can be liberated through the action of proteolytic enzymes from various sources, e.g. during the manufacture of dairy products or upon enzymatic hydrolysis of milk proteins *in vitro*[13].

Many biologically active peptides have been identified from milk proteins and dairy products by proteolysis with digestive enzymes or microbial enzymes or by fermentation[11,14]. The single most effective way to increase the concentration of bioactive peptides in fermented dairy products is to ferment or co-ferment with highly proteolytic strains of lactic acid bacteria. The types of lactic acid bacteria starter used are one of the main factors that influence the synthesis of hypertensive peptides in dairy products. Hence the strain selection is one of the main factors that influence the release of ACE inhibitors in dairy fermentations[15-17]. Nakamura et al.[17] purified and characterize angiotensin 1- converting enzyme inhibitors from sour milk. The inhibitory activity of angiotensin 1 – converting enzyme in milk increased during fermentation with the calpis sour milk starter containing Lactobacillus helveticus and Saccharomyces cerevisiae. Chen et al.[18] observed that fermentation of milk with a commercial starter culture mixture of five lactic acid bacteria (LAB) strains followed by hydrolysis with a microbial protease increased ACE inhibitory activity of the hydrolysate. Two strong ACE-inhibitory tripeptides (Gly-Thr- Trp) and (Gly-Val-Trp) were identified and an antihypertensive effect of the hydrolysate containing these peptides was demonstrated in an animal model study using spontaneously hypertensive rats (SHR).

Many milk-derived peptides are multifunctional, (i.e.,) specific peptide sequences have more than one biological activity[4]. In addition to antihypertensive property milk proteins also showed antioxidant and antibacterial property.

Many human diseases are caused or negatively affected by free radicals. The natural defense of the human against free radicals is not always sufficient mainly due to the significant exposition to free radicals from external sources in the modern world. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. The dietary intake of antioxidants plays an important role in the protection of the human against free radicals. Many clinical and epidemiological studies show a connection between the antioxidant activity of the substances present in the diet and the prevention from such diseases as cardiovascular diseases or carcinogenesis[19-21]. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods. A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods.

The imbalance between free radical formation (chemical compound that contains one or more unpaired electrons) and the mechanisms involved in their elimination results in oxidative stress, which lies at the baseline of many diseases, such as degenerative diseases associated with aging. It has been reported that milk proteins and fermented milks are among the dietary sources of natural antioxidants, in the form of antioxidant peptides. For example, αs1-casein f (144-149) hexapeptide corresponding to the amino acid sequence YFYPEL was found to possess a potent superoxide anion radical scavenging activity. Other peptides derived from tryptic cleavage of β-casein exhibited potent inhibition of lipooxygenase activity. Quenching of free radicals by oxidation of amino acid residues in casein is thought to be involved in the mechanisms of action of bovine casein derived antioxidant peptides. A κ-casein derived peptide with 2, 2-diphenyl-1-picrylhidrazyl activity was recently isolated from milk fermented with *Lb. delbrueckii* subsp. *bulgaricus*. Another peptide corresponding to the amino acid sequence SKVLPVPQ was identified from two commercial Spanish fermented milk drinks manufactured with *Lb. helveticus* and *Saccharomyces cerevisiae*, which, based on structure exhibited antioxidant activities[22]. A fragment of SQSKVLPVPQ, with the amino acid sequence VLPVPQK was identified previously as an antioxidant peptide.

Milk fat contains a number of individual components that can be described as having anticarcinogenic properties. In particular, conjugated linoleic acid (CLA) and sphingomyelin have been suggested to have important anticancer properties[23-26]. McIntosh *et al.*[27] demonstrated a protective role for dietary dairy proteins against tumour development, showing that dietary whey protein and casein were more protective against the development of intestinal cancers in rats than was red meat or soy bean protein. They concluded that dietary proteins differ in their ability to protect against cancer development and that the proteins in dairy foods, particularly the whey proteins, appear to play a significant role in cancer prevention[27]. Daily ingestion of foods containing peptides with potent ACE-inhibitory activities may be effective at keeping the human blood pressure low. The aim of this study was to detect an ACE-inhibitory cow milk hydrolysate and its *in vitro* antioxidant activity and *in vitro* anticancer activity.

MATERIALS AND METHODS

Microorganisms

Lactobacillus plantarum and *Lactobacillus casei* isolated from milk product was used in the present investigation. Identification of the organism was made based on their morphological and cultural characteristics.

Screening of different milk

The cow and buffalo milk were used to screen based on their ACE inhibitory activity. The raw milk was collected from nearest dairy farm and sterilized by heating at 93ºC for 20 min under constant stirring for the study.

Production of fermented milk

Combination of *Lactobacillus plantarum* and *Lactobacillus casei* were used to inoculate (2%, vol/vol) 50 ml of sterilized milk. Inoculation was carried out under sterile conditions and the milk was kept for fermentation at 37ºC for 24 h.

Preparation of milk hydrolysates

For the determination of the ACE inhibitory activity of the milk during fermentation, the whey fraction was used. The whey fraction was obtained as follows. The pH of milk was adjusted to 3.4 by addition of 50% lactic acid, and then the milk was centrifuged at 6000 x g for 10 min; 10N NaOH was added to the supernatant to raise the pH to 8.3, and then the supernatant was centrifuged at 6000 x g for 10 min. Milk hydrolysate were finally ultrafiltered through a 10 kDa cut-off membrane in a stirred ultrafiltration cell module (Millipore USA). Ultrafiltered permeates were analyzed at this step and also after a second step of fractionation through a 3 kDa cut-off filter (Millipore, USA) with centrifugation (3200 x g for 40 min at 15ºC). The final supernatant was used for the study[28].

Measurement of ACE inhibitory activity

The ACE inhibitory activity was assayed by the method of Cushman and Cheung[29] with some modification. 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 of milk hydrolysate (the pH of which was adjusted to 8.3) and then preincubated for 3 min at 37ºC. The reaction was initiated by addition of 20 µl of ACE. And the mixture was incubated for 30 min at 37ºC. The reaction was stopped by addition of 250 µl of 1N 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 = the optical density in the presence of ACE and ACE inhibitory component,

B = the optical density without ACE inhibitory component and

C = the optical density without ACE.

Effect of fermentation conditions on ACE inhibitory activity

Various process parameters influencing ACE inhibitor production during fermentation were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments**.** The various conditions include temperature (28, 31, 34, 37, 40 and 43ºC), inoculum size (0.5, 1, 1.5, 2, 2.5, and 3%) and incubation period (12, 24, 36 and 48 hr). After fermentation milk hydrolysates were prepared for ACE inhibitory assay.

In-vitro antioxidant activity of milk hydrolysate by DPPH radical scavenging assay

The antioxidant activity of the milk hydrolysate was checked by DPPH (2, 2-diphenyl-1- icrylhydrazyl) radical scavenging activity. 0.3 ml of different concentration (100, 200, 300,400 and 500 µg/ml) 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 cow milk hydrolysate. Ascorbic acid was taken as standard[30].

Total reducing power assay

The reducing power was determined according to the method of Lin *et al.*[31]. Different concentration (100, 200, 300,400 and 500 µg/ml) 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. After 0.25 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 3000 rpm 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. Ascorbic acid was taken as standard.

In vitro anticancer study

The human cervical cancer cell lines (HeLa), obtained from National Centre for Cell Science (NCCS), Pune, India was used for this work. The HeLa cells were grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). For screening experiment, the cells were seeded into 96-well plates in 100 µl of medium containing 5% FBS, at plating density of 10,000 cells/well and incubated at 37ºC, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of hydrolysate. The hydrolysate were solubilized in 0.2 M phosphate buffer (pH, 7) diluted in serum free medium. After 24 h, 100 µl of the medium containing the hydrolysate at various concentrations (15.625, 31.25, 62.5, 125 and

250 µg/ml) was added and incubated at 37ºC, 5% CO_2, 95% air and 100% relative humidity for 48 h. Triplicate was maintained and the medium containing without sample were served as control.

Anti-cancer activity

The anticancer effect of the samples against HeLa cells was estimated by the 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay according to Hansen *et al.*[32]. The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5 x 10^4 cells / well) were incubated with various concentrations of the compound at 37ºC for 48 h in a FBS-free medium, before submitted to MTT assay. The absorbance at 570 nm was measured using micro plate reader. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to the respective control. The half maximal growth inhibitory concentration IC_{50} values were calculated.

Statistical analysis

The SPSS software (10.0 versions) was used for the major data processing throughout this work. All results were expressed as the mean ± SD.

RESULTS AND DISCUSSION

Screening of different milk with ACE inhibitory activity

Among the two types of milk screened for ACE inhibitory activity, cow milk showed maximum inhibiting percentage of 51.99±0.21% (Fig.1). Buffalo milk showed ACE inhibitory of 50.22±0.23%. Cow milk was selected for further work because of high ACE inhibitory activity and its availability. Cow's milk contains many constituents including electrolytes, proteins, and peptides, which could affect blood pressure beneficially.

Effect of temperature on ACE inhibiting activity of cow milk

Temperature has an important role in the fermentation process. The effect of fermentation temperature (28-43ºC) on the ACE inhibition activity was studied. It was observed that during fermentation the ACE inhibition was highest (52.45±0.42%) at temperature 37ºC (Fig. 2). Negligible amount of ACE inhibitory activity was produced between 28 and 43ºC. Pan and Guo[5] reported maximum ACE inhibition activity at 39ºC. The temperature is one of the most critical parameters that have to be controlled in a bioprocess. The process temperature should ensure the optimum growth of organism and production of ACE inhibitor.

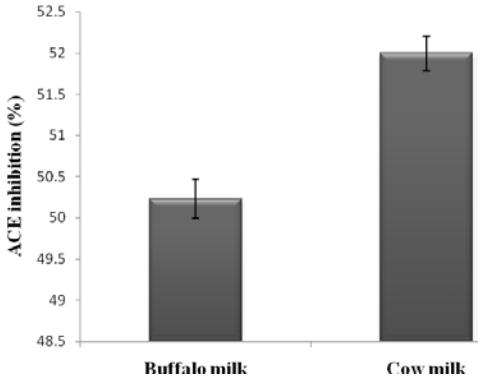

Fig. 1: Screening of different milk with ACE inhibitory activity

Effect of inoculum size on ACE inhibitor production

The effect of inoculum concentration (0.5-3%) on ACE inhibitor production by combination of *Lactobacillus plantarum* and *Lactobacillus casei* was studied. The maximum activity of 64.78±0.15% was obtained in the cow milk containing 1% inoculum (Fig.3). Reduction of ACE inhibition activity was noticed at higher concentration of inoculum. This indicates that low inoculum concentration is a good for ACE inhibition activity. Gobbetti *et al.*[33] studied the production of ACE inhibitory peptides in fermented milk with 1% inoculum of *Lactobacillus delbrueckii* and *Lactobacillus lactis*.

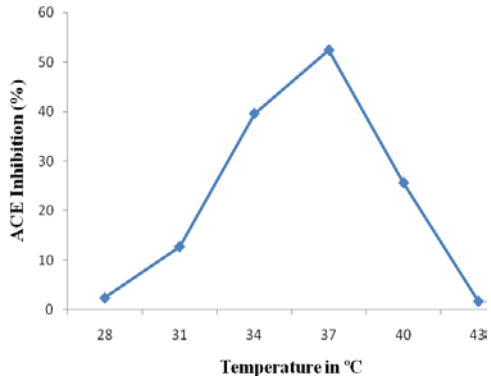

Fig. 2: Effect of temperature on ACE inhibitor production

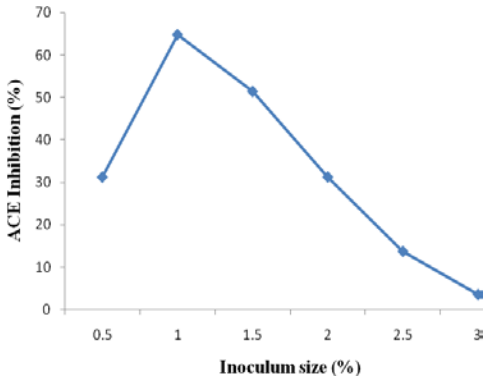

Fig. 3: Effect of inoculum size on ACE inhibitor production

Effect incubation period on ACE inhibitor production:

To evaluate the effect of different incubation period on ACE inhibitor production, the incubation period of medium range was varied from 12 to 48 h. With a rise in incubation period, the ACE inhibition activity increased and optimum activity was recorded at 24 h (Fig. 4). With a further increase in incubation period, there was a decrease in activity. Maximum ACE inhibitory activity of 69.28±0.37% was observed. ACE inhibitory activity increased between the log and stationary phase indicating that the ACE inhibitory peptides were produced during fermentation. Thereafter, the ACE inhibitor production started decreasing (Fig. 4).

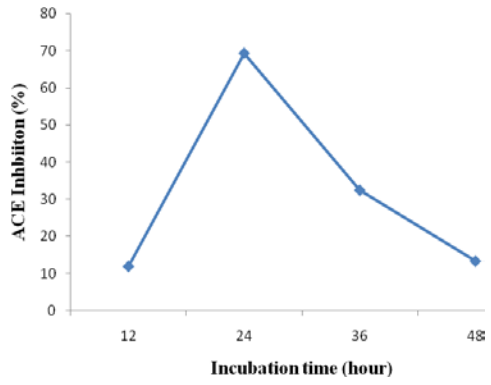

Fig. 4: Effect of incubation time on ACE inhibitor production

Antioxidant activity of cow milk hydrolysate

In vitro antioxidant activities of cow milk were studied using DPPH scavenging and total reducing power assay. The observed result showed that cow milk fermented with the combination of *Lactobacillus plantarum* and *Lactobacillus casei* have good antioxidant property. The milk hydrolysate and ascorbic acid exhibited scavenging potential with IC_{50} value of 185.87 µg/ml and 131.25 µg/ml respectively for DPPH (Fig. 5). Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and biological systems. The reduction of DPPH absorption is indicating the capacity of the milk hydrolysate to scavenge free radicals, independently of any enzymatic activity. The antioxidant activity of several species and strains of milk bacteria contained in fermented milk can significantly affect human health. This has been confirmed also by clinical studies of milk fermented with a starter culture *Lactobacillus fermentum* ME-3 [34,35].

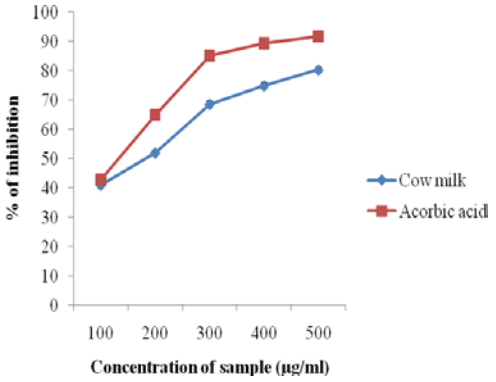

Fig. 5: DPPH scavenging activity of cow milk hydrolysate

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 activity. The hydrolysate showed dose dependant increase in reducing power that was compared with standard ascorbic acid (Fig. 6).

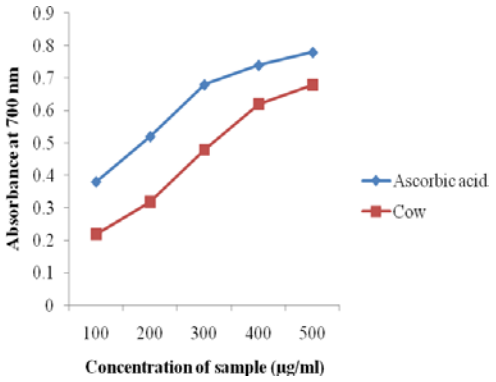

Fig. 6: Reducing power activity of cow milk hydrolysate

***In vitro* cell-line study**

In MTT assay, the cow milk hydrolysate showed IC_{50} value of 150 µg/ml against cervical cancer cell line, HeLa (Fig.7). HeLa cells were subjected to ACE inhibitors and the cell viability assayed by MTT (expressed as percentage over control). Cell growth was decreased with the increasing concentration of ACE inhibitor. In order to show the concentration dependant action of ACE inhibitor in HeLa cells were treated with different (15.625, 31.25, 62.5, 125 and 250 µg/ml) concentration of sample. Milk fat contains a number of individual components that can be described as having anticarcinogenic properties. In particular, conjugated linoleic acid (CLA) and sphingomyelin have been suggested to have important anticancer properties[23-26].

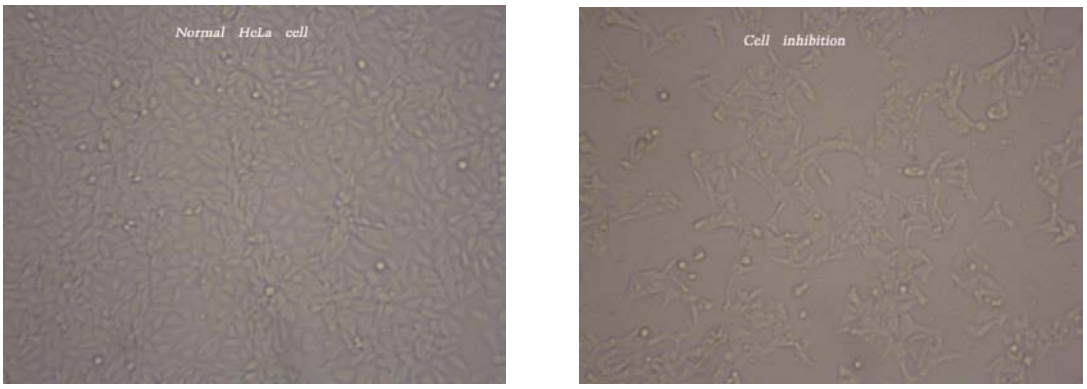

Fig. 7: In vitro Anticancer activity of cow milk hydrolysate against HeLa (Cervical cancer) cells (IC_{50} 150 µg/ml)

CONCLUSIONS

Prevention of diseases may in the future be just as important as treatment of diseases. Interest on the possible health-promoting properties of food is increasing and more and more research is targeted at the search for new biologically active compounds in different food products. Milk and dairy products have traditionally been an important part of human nutrition. Milk peptides have multi functional properties. In our study, cow milk hydrolysate showed ACE inhibitory, antioxidant and anticancer activity. The result suggests that due to the inhibition of ACE the increasing blood pressure could be partially prevented by taking the milk derived peptides. And also being a natural product milk derived peptides may have less side effects. However, more studies are required to isolate peptides and to use these peptides as medicine.

ACKNOWLEDGEMENT

This work was supported by Department of Science and Technology (DST) New Delhi, India, major research grant F. No. SR/SO/HS-135/2007.

REFERENCES

1. Hyun C, Shin H. Utilization of bovine blood plasma proteins for the production of angiotensin I-converting enzychem me inhibitory peptides. Process Biochem 2000; 36: 65-71.

2. ParmLey WW. Evolution of angiotensin-converting enzyme inhibition in hypertension, heart failure, and vascular protection. Am J Med 1998; 105: 27S–31S.
3. Ariyoshi Y. Angiotensin-converting enzyme inhibitors derived from food proteins. Trends Food Sci Technol. 1993; 4: 139-144.
4. Meisel H. Multifunctional peptides encrypted in milk proteins. Biofactors 2004; 21:55–61.
5. Pan D, Guo Y. Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate. Int Dairy J 2010; 20: 472-479.
6. Oshima G, Shimabukuro H, Nagasawa K. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatine by bacterial collagenase. Biochim Biophys Acta 1979; 566: 128-137.
7. Maruyama S, Miyoshi S, Kaneko T, Tanaka H. Angiotensin-I-converting enzyme inhibitory activities of synthetic peptides related to the tandem repeated sequence of a maize endosperm protein. Agric Biol Chem 1989; 53: 1077-1081.
8. Miyoshi S, Ishikawa H, Kaneko T, Fukui F, Tanaka H, Maruyama S. Structures and activity of angiotensin-converting enzyme inhibitors in an alpha-zein hydrolysate. Agric Biol Chem 1991; 55: 1313-1318.
9. Chen JR, Yang SC, Suetsana K, Chao JCJ. Soybean protein-derived hydrolysate affects blood pressure in spontaneously hypertensive rats. J Food Biochem 2004; 28: 61-73.
10. Matsui T, Li CH, Osajima Y. Preparation and characterization of novel bioactive peptides responsible for angiotensin I-converting enzyme inhibition from wheat germ. J Pept Sci 1999; 5:289-297.
11. Meisel H. Overview on milk protein derived peptides. Int Dairy J 1998; 8: 363-373.
12. Saxelin M, Korpela R, Mayra-Makinen A. Introduction: classifying functional dairy products. In: Mattila-Sandholm T, Saarela M, editors. Functional dairy products. Cambridge, UK: Woodhead Publishing Ltd; 2003. p 16.
13. Lopez FR, Otte J, van Camp J. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. Int Dairy J 2006; 16: 1277-1293.
14. Korhonen H, Pihlanto-Leppal A. Milk protein-derived bioactive peptides. Novel opportunities for health promotion. Bull IDF 2001; 363: 17–26.
15. Takano T. Anti-hypertensive activity of fermented dairy products containing biogenic peptides. Antonie Van Leeuwenhoek 2002; 82:333–340.
16. Korhonen H, Pihlanto A. Food-derived bioactive peptides–opportunities for designing future foods. Curr Pharm Des 2003; 9: 1297–1308.
17. Nakamura Y, Masuda O, Takano T. Disease of tissue Angioensin converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats. Biosci Biotechnol Bioche 1996; 60: 488-489.
18. Chen GW, Tsa JS, Pan BS. Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease facilitated lactic fermentation. Int Dairy J 2007; 17: 641–647.
19. Hughes DA. Dietary antioxidants and human immunity function. Nutrition Bulletin 2000; 25: 35–41.
20. Kris-Etherton PM, Hecker K, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. Bioactive compounds in food: Their role in the prevention of cardiovascular disease and cancer. Am J Med 2002; 133: 71–88.
21. Lindsay DG, Astley SB. European research on the functional effects of dietary antioxidants. Molecular Aspects of Medicine 2002; 23: 1–38.
22. Hernandez LB, Amigo L, Ramos M, Recio I. Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. J Agric Food Chem 2004; 52: 1504–1510.
23. Ip C, Chin SF, Scimeca JA, Pariza MW. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res 1991; 51: 6118-6124.
24. Dillehay DL, Webb SK, Schmelz E, Merrill AHJr. Dietary sphingomyelin inhibits 1, 2-dimethylhydrazine-induced colon cancer in CF1 mice. J Nutr 1994; 124: 615-620.
25. Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res 1994; 54: 1212-1215.
26. Ip C, Briggs SP, Haegele AD, Thompson HJ, Storkson J, Scimeca JA. The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. Carcin 1996; 17: 1045-1050.
27. McIntosh GH, Regester GO, Le Leu RK, Royle PJ, Smithers GW. Dairy proteins protect against dimethylhydrazine induced intestinal cancers in rats. J Nutr 1995; 125: 809-816.
28. Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S, Takano T. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. J Dairy Sci 1995; 78: 777–783.
29. Cushman DW, Cheung JCJ. Spectrophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. Biochem Pharmacol 1971; 20: 1637–1648.
30. Srinivas S, Prakash V. Effect of cosolvents on the stabilization of biological active peptides from bovine milk alpha casein. Prot Pep Lett 2008; 15:371–374.
31. Lin ES, Yang CT, Chou HJ, Chang TT. Screening of antioxidant activities by the edible Basidiomycete *Antrodia cinnamomea* strains in submerged culture. J Food Biochem 2010; 34: 1141-1156.
32. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth cell kill. J Immunol Methods 1989; 119: 203-210.
33. Gobbetti M, Ferranti P, Smacchi E, Goffredi F, Addeo F. Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4. Appl Environ Microbiol 2000; 66: 3898-3904.
34. Kullisaar T, Songisepp E, Mikelsaar M, Zilmer K, Vihalemm T, Zilmer M. Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects. Brit J Nutr 2003; 90: 449–456.
35. Songisepp E, Kals J, Kullisaar T, Mandar R, Hutt P, Zilmer M, Mikelsaar M. Evolution of the functional efficacy of an antioxidative probiotic in healthy volunteers. Nutr J 2005; 4: 22–31.