Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 4, 2012

Research Article

ANGIOTENSIN CONVERTING ENZYME INHIBITORY ACTIVITY AND ANTIOXIDANT PROPERTIES OF GOAT MILK HYDROLYSATES

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Received: 13 Aug 2012, Revised and Accepted: 22 Sep 2012

ABSTRACT

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) plays a dual role in the regulation of hypertension. It catalyzes the production of the vasoconstrictor angiotensin II and it inactivates the vasodilator bradykinin. Milk proteins are a good source of bioactive peptides, and ACE inhibitory peptides have been produced by the enzymatic hydrolysis of milk proteins and by fermentation with lactic acid bacteria. Several milk peptides inhibit ACE *in vitro*. The aim of this study was to investigate the antioxidant activity and angiotensin converting enzyme inhibitory activity of goat milk fermented using *Lactobacillus plantarum* incubated at 37°C for 48h. Angiotensin converting enzyme inhibitory activity of hydrolysates was found to be 88.96%. Antioxidant activity of goat milk hydrolysates was measured using three different methods: DPPH radical scavenging activity, lipid peroxidation assay and hydroxyl radical scavenging activity. Goat milk hydrolysates displayed significantly greater antioxidant activity. These findings have suggested that goat milk hydrolysates may be considered among the most promising food components in term of preventing oxidative damage and cardiovascular diseases.

Keywords: Goat milk hydrolysates, *Lactobacillus plantarum*, Angiotensin converting enzyme inhibitor, Antioxidant property.

INTRODUCTION

Over the last century, the definition of health has no longer been restricted to the absence of disease, but includes physical fitness as well as mental and physiological well-being. For the development, growth and maintenance of the body, food is required, but food is also recognized to play a key role in the quality of life. Functional foods are those foods that positively affect one or more target functions in the body, beyond the basic nutritional function, in a way that it is relevant to either an improved state of health and well-being and/or reduction of risk of disease[1]. One of the components of functional foods may be bioactive peptides, as they exert a physiological effect in the body[2]. These peptides are short chains of amino acids that are inactive within the sequence of parent protein but can be released during gastrointestinal digestion or food processing. A wide range of biological activities has been ascribed to peptides such as antihypertensive (ACE inhibitory), antithrombotic, antioxidative, immunomodulating, opioid, antimicrobial, anticarcinogenic or mineral binding activity[3,4,5]. Moreover, some protein hydrolysates and peptides have an extra advantage of being multifunctional, as they initiate two or more different biological activities[6]. For example, multifunctional hydrolysates exerting both ACE inhibitory and antioxidant activities have been reported in commercial fermented milk in Europe[7], in egg white protein hydrolysates[8], and in extracts prepared from Chum salmon cartilage and skin[9].

Angiotensin converting enzyme (ACE) is a key enzyme in the regulation of blood pressure in humans; ACE catalyses the hydrolysis of the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, an octapeptide[10]. Moreover, ACE cleaves bradykinin, a vasodilator, into inactive fragments. The result is an increase in blood pressure[11]. Synthetic ACE inhibitors have been developed as antihypertensive medicine but the use of such inhibitors can cause serious side effects[12]. The search for natural alternatives has resulted in the identification of numerous ACE inhibitory peptides derived from various food sources. Food proteins from vegetable (such as soybean and maize) and animal (such as milk, porcine and beef muscle) origin are reported as good sources of ACE inhibitory peptides[13].

Antioxidants may have a positive effect on human health as they can protect our body against damage by reactive oxygen species (ROS), which attack membrane lipids, protein and DNA and play an important role in many diseases such as cardiovascular diseases, diabetes mellitus, cancer and Alzheimer[14, 15]. In addition, lipid peroxidation is a great concern to the food industry as deterioration of food quality has been identified due to oxidation of lipids and formation of secondary lipid peroxidation products. The use of synthetic antioxidants is under strict regulation due to the potential negative health effects caused by such compounds. The substitution of synthetic antioxidants by natural ones is gaining interest due to these health concerns and due to consumer's preferences. Antioxidant activity has been demonstrated in various hydrolysates, e.g. the peptide digests of jumbo squid gelatin[16], chum salmon cartilage[9], Alaska pollack skin[17] porcine muscle[18], soybean[19] and milk[7, 20].

In the present study, we compared antioxidant activity of milk hydrolysates with antioxidant activity of butylated hydroxyansiole (BHA). BHA is one of the most commonly used antioxidant. BHA preserved fats is used in food and cosmetic industry. It has undergone the additive application and review process required by the Food and Drug Administration. However, the same reaction may combat oxidative stress. There is evidence that certain persons may have difficulty metabolizing BHA resulting in health and behavior changes[21]. These are all reasons why scientists are searching for new natural antioxidants.

MATERIALS AND METHODS

Microorganisms and culture condition

Test bacterial strain *Lactobacillus plantarum* was isolated from commercially available dairy products and routinely propagated lactobacilli in MRS broth for 24 h at 37°C. Twenty-four-hour-old cells of *Lactobacillus* sp., was used to inoculate (2%, v/v) 10 ml of ultra-high temperature- treated (UHT) skim milk (protein, 3.15%; fat, 0.3%; lactose, 4.95%), which was incubated for 24 h at 30°C. These milk cultures were used to produce fermented milk.

Fermentation of goat milk

At the start of the fermentation, the selected microorganisms were added to the fermentation medium under sterile conditions in a concentration of 6.3 cfu/ml of *Lactobacillus plantarum*. Samples at the start of the fermentation were taken for analysis. Then the inoculated fermentation medium was incubated at 37°C for 48 h, after which fermented samples were used for further study.

Preparation of hydrolysates

For determining the ACE inhibitory activity of the goat milk hydrolysates, the following hydrolysis was performed. The pH of

fermented goat milk hydrolysates was adjusted to 3.5 using 50% lactic acid and centrifuged (5,000 × *g*, 10 min) and the supernatant was collected. 10N NaOH was added to the supernatant in order to raise the pH to 8.5 and then the supernatant was centrifuged at 6000 × *g*, 10 min. The final supernatant was used as milk hydrolysates (whey fraction).

Assay for ACE activity

For each assay, a sample solution of ACE inhibitor (20µl) with 50 µl of 5mM N-Hippuryl-His-Leu tetrahydrate (HHL) in 100mM sodium borate buffer (pH 8.3) containing 300mM NaCl was preincubated at 37°C for 5 min. The reaction was initiated by the addition of 10 µl of ACE solution from rabbit lung and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 100 µl of 1M HCl. Sodium borate buffer (pH 8.3) was then added to the reaction mixture to a volume of 0.5 ml. The HA released from HHL by ACE was monitored according to the method of Wang *et al.*, [22]. All measurements were performed in triplicates. The inhibition activity was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \frac{B - A}{B - C} \times 100$$

(A-sample, B- control, C- blank)

Antioxidant activity of goat milk hydrolysates

Antioxidant activity of goat milk hydrolysates[23] was measured using three different methods: DPPH radical scavenging activity, lipid peroxidation assay and hydroxyl radical scavenging activity. The antioxidant activity of hydrolysates was tested and compared with a standard BHA.

DPPH radical scavenging activity

Scavenging activity on DPPH free radicals by the samples were assessed according to the method reported by Blois[24]. 100µl of samples solution was mixed with 1ml of 0.1mm DPPH in ethanol solution and 450µl of 50 mm Tris-HCl buffer (pH 7.4) was added. The solution was incubated at 37ºC for 30min and reduction of DPPH free radicals was measured by reading the absorbance at 517nm. Tube without extract solution served as control. The percentage of DPPH scavenging activity was calculated according to the following equation.

$$\frac{\text{Control absorbance- sample absorbance}}{\text{Control absorbance}} \times 100$$

Lipid peroxidation assay

The sample (0.1ml) and distilled water (0.4 ml) was mixed with 0.5ml of egg yolk solution. Then this solution was vortexed well with 0.07ml of $FeSO_4$ (10mM) and incubated for 30 min at room temperature. After adding 1.5ml of thiobarbituric acid solution (0.8% thiobarbituric acid in 1.1% sodium dodecyl sulphate). Samples were mixed well and heated for 60 min. After samples were cooled, 5ml of butanol was added. The samples were centrifuged for 10 min at 3000 rpm. Supernatant was used and absorbance of sample was measured at 532 nm. 95% ethanol was used as a control. The antioxidant activity was given as an inhibition percentage and calculated as:

$$\frac{\text{Control absorbance- sample absorbance}}{\text{Control absorbance}} \times 100$$

Hydroxyl radical scavenging activity

An aliquot of the sample (0.075ml) was mixed with 0.45ml of sodium phosphate buffer (0.2 M, pH 7), 0.15 ml of 2-deoxyribose (10mM), 0.15ml of EDTA (10mM), 0.15ml of FeSO4 (10 mM), 0.15 ml of hydrogen peroxide (10mM) and 0.525ml of distilled water. Samples were then incubated at 37°C for 4 h. After incubation, 0.75ml of trichloro acetic acid (2.8%) and thiobarbituric acid (0.1%) were added. Then samples were kept in boiling water for 10 min. The absorbance of each sample was measured at 520 nm and ethanol was used as a control. The antioxidant activity was given as an inhibition percentage and was calculated as:

$$\frac{\text{Control absorbance- sample absorbance}}{\text{Control absorbance}} \times 100$$

RESULTS

ACE inhibitory assay

ACE inhibition assay based on the direct spectrophotometric measurement of HA released from HHL by ACE, because this method did not require a large amount of test samples. In this study ACE inhibitory activity of milk hydrolysates was found to be 88.96%.

Antioxidant activity of the goat milk hydrolysates

DPPH method

Antioxidant activity of goat milk hydrolysate determined by the DPPH assay was showed in Figure 1. The DPPH stable free radical method is an easy, rapid and sensitive way to evaluate the antioxidants property to scavenge free radicals.

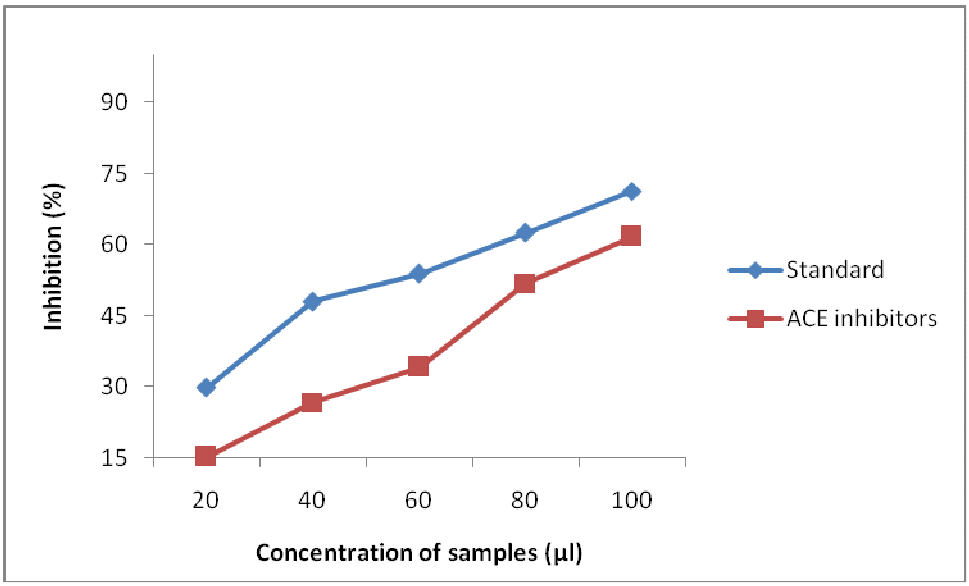

Fig. 1: Antioxidant activity of goat milk hydrolysates using DPPH scavenging method

Lipid peroxidase assay

Inhibition of lipid peroxidation increased with increasing concentration of peptide sample. The inhibition ranges of the hydrolysates were compared with butylated hydroxy toluene standards. Comparison of the inhibition of lipid peroxidation is shown in Figure 2. The percentage of inhibition of lipid peroxidation of sample was less when compared to butylate hydroxy toluene standards.

Hydroxyl radical scavenging activity

Hydroxyl radical is an extremely reactive free radical formed in biological systems and has been cited as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. ACE inhibitor milk hydrolysates showed efficient hydroxyl radical scavenging activity on par with the ascorbic acids. The hydroxyl radical scavenging of the hydrolysates was more or less equal to the standard and it is shown in the Figure 3.

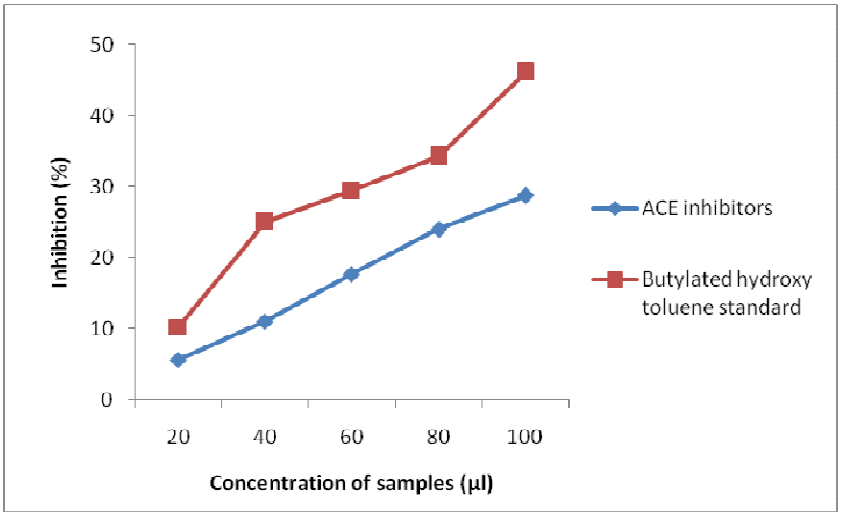

Fig. 2: Antioxidant activity of goat milk hydrolysates using lipid peroxidase assay

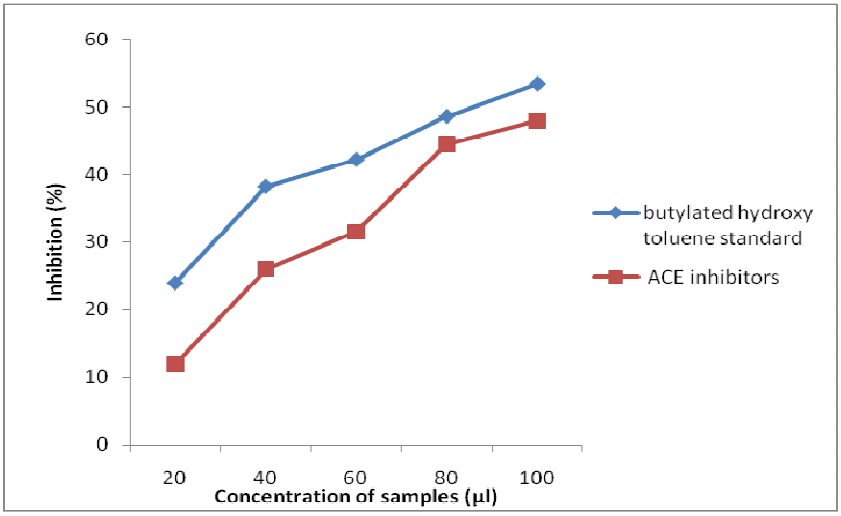

Fig. 3: Antioxidant activity of goat milk hydrolysates using hydroxyl radical assay

DISCUSSION

In the present study goat milk fermented with *Lactobacillus plantarum* showed ACE inhibitory activity of 88.96%. Yamamoto *et al.*,[25] observed that the two strains of *L. helveticus* produce competitive inhibitors (maximum inhibition according to the fit is approximately 100%), which is in accordance with earlier reports[26] on the mode of action for milk-derived inhibitors of ACE.

We also tested the antioxidant property of goat milk. The antioxidant activity of goat milk hydrolysates (100µl) were found to be 61.7% for DPPH assay, 46% for lipid peroxidase activity and 48% for hydroxyl scavenging activity. Milk samples inhibited lipid peroxidation and peroxyl/superoxide radical generation. Lactoferrin in milk can bind iron and inhibit Fe-induced lipid peroxidation[27,28,29,30]. Also lactoperoxidase appears to have strong antioxidant properties[31] and finally hydrolysates from milk were found to be antioxidative and some of them have been patented[32, 33]. These examples show that several components are active in preventing lipid peroxidation and maintaining milk quality, and also point to their potential usage as ingredients in foods and pharmaceuticals to provide products for enhanced consumer health. Harper[34] has discussed that the antioxidant activity of the unhydrolysed whey due to cysteine and glutamate, precursors of glutathione, which in turn mitigates oxidative stress. He has also reported that lactoferrin inhibits the oxidation by scavenging free iron, thereby limiting its availability to catalyze oxidative reactions. Chen *et al.*,[35], reported that the increased scavenging activity of fermented milk may be related to milk protein. Antioxidant properties might, therefore not be uniquely attributed to chelating metals by phosphoseryl residues but also to scavenging of free radicals[36].

The activities have, so far, only been tested in simple *in vitro* screening trials and no evidence is available at present which proves that they are also effective in humans. It is anticipated that in the near future such targets will be related to various lifestyle-related disease groups, such as cardiovascular diseases, cancers, osteoporosis, stress and obesity. Physiologically active peptides derived from milk proteins offer a promising approach to prevent, control and even treat such disease conditions through a regulated diet. There are several reports on the ACE inhibitory activity of peptides from milk protein[13]. It is well recognized that milk proteins treated with digestive enzymes or fermented with lactic acid bacteria can generate a number of peptides having potential biological activities. ACE inhibitory peptides have been found in many food proteins sources[37], among which milk protein is a particularly good source of bioactive peptides. 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*[38].

CONCLUSION

This study has therefore tried to understand the antioxidant property of the goat milk hydrolysates and also the angiotensin converting enzyme inhibitory activity. It is reasonable to expect that goat milk hydrolysates contain more antioxidant compounds and angiotensin converting enzyme inhibitor compounds. These findings have suggested that goat milk hydrolysates may be considered among the most promising food components in terms of preventing oxidative damage and also against cardiovascular diseases.

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