

INHIBITORY ACTIVITY OF ANGIOTENSIN CONVERTING ENZYME FROM GOAT MILK PROTEINMan Kyu Huh^{1*} and Yong Lim²¹Food Science & Technology Major, Dong-eui University, Busan 47340, Republic of Korea.²Department of Clinical Laboratory Science, Dong-eui University, Korea.***Corresponding Author: Dr. Man Kyu Huh**

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ABSTRACT

Goat milk is the milk of domestic goats. Due to unavailability of cow milk, goat milk and its products are important daily food sources of protein. Angiotensin converting enzyme (ACE) inhibitors plays a critical role in treating hypertension. The ACE activity was evaluated by determining the hydrolysis rate of substrate, hippuryl-L-histidyl-L-leucine (HHL), using captopril. Four products were arbitrarily selected that are sold in Korea because they advertised and contained the most goat milk protein ingredients. Water, ethanol, and methanol were used as the extraction solvent. Hot water extract of the goat's milk protein was evaluated 42.7% on 50 mM and that of the ethanol extract was 40.1% at same concentration. Methanol extract was 39.3% at same concentration. ACE inhibitory activity was no significant difference among four products at low concentrations (25, 12.5, and 6.25 mg/L) ($p > 0.05$). However, there was significant difference among 50 mg/L ($p < 0.05$). We prepared four products as ACE inhibitor and compared its positive control inhibitory activity with captopril. Products A, B, C, and D were 42.7%, 40.1%, 39.3%, and 39.1% on the concentration of 50 mg/L, respectively. Overall, goat milk proteins of four products were not enough to generate the inhibitory activity of ACE. **KEYWORDS:** Angiotensin converting enzyme (ACE), Goat milk protein, extraction solvent.

INTRODUCTION

High blood pressure (hypertension) is quietly damage. So it is called a silent, invisible killer that rarely causes symptoms.^[1] 26.4% adults aged 25 and above of the world's population in 2000 suffered hypertension and approximately 40% had been diagnosed with hypertension.^[1] Hypertension is one of the most prevalent pathologies in America affecting approximately 75 million adults or one in three US adults.^[2] It is predicted that this rate would increase by 60% in 2025.^[3] Raised blood pressure is a serious warning sign that significant lifestyle changes are urgently needed. To raise this kind of awareness, countries need systems and services in place to promote universal health coverage and support healthy lifestyles: eating a balanced diet, reducing salt intake, avoiding harmful use of alcohol, getting regular exercise and shunning tobacco. Access to good quality medicines, which are effective and inexpensive, is also vital, particularly at the primary care level. As with other noncommunicable diseases, awareness aids early detection while self-care helps ensure regular intake of medication, healthy behaviors and better control of the condition. Hypertension puts strain on the heart, leading to hypertensive heart disease and coronary artery disease if not treated.^[4] Hypertension is also a major risk factor for stroke, aneurysms of the arteries, peripheral arterial disease and is a cause of chronic kidney disease.

There are a number of choices for the treatment of hypertension. Some treatments include diuretics, β -blockers, calcium channel blockers and angiotensin II receptor blockers, the most common of which is angiotensin converting enzyme inhibitors.^[5]

Angiotensin converting enzyme (ACE) is a di-peptidyl carboxypeptidase (EC 3.4.15.1) converts the angiotensin I (inactive decapeptide) to angiotensin II (a potent vasoconstrictor), and bradykinin (a hypotensive peptide) to inactive components.^[6] ACE conversion of angiotensin I to angiotensin II is a normal regulatory process in the body. High ACE activity leads to increased concentration of angiotensin II and hypertension. Therefore, development of agents that inhibit the conversion of angiotensin I to angiotensin II, and bradykinin to inactive components began as a therapeutic strategy to treat hypertension. ACE inhibitors such as captopril and lisinopril play key roles in treating hypertension and maintaining the electrolyte balance.^[7] This process has been targeted by the development of drugs called ACE inhibitors that are commonly used in treating hypertension and diabetes. These drugs inhibit the conversion process, keeping the blood vessels more dilated and the blood pressure lower.^[8] These peptides/hydrolysates may be classified as functional food ingredients and nutraceuticals due to their ability to

provide health benefits i.e. as functional food ingredients in reducing the risk of developing a disease and as nutraceuticals in the prevention/treatment of disease.

Goat milk is closer to breast milk than other dairy, and even has a higher level of immunoglobulin than breast milk.^[9] Goat milk has a casein-to-whey protein ratio of 78:22 but a higher α -LA-to- β -LG percentage characterizes this whey protein compared to whey protein in cow milk.^[10] The incubation conditions of goat milk fermented by *Lactobacillus bulgaricus* LB6 were optimized to increase the angiotensin converting enzyme inhibitory activity.^[11]

The aim of the current study was to isolate and characterize the constituents responsible of the ACE activity of the aqueous, ethanol, and methanol extract of goat milk protein.

MATERIALS AND METHODS

Sample extract

Four products were arbitrarily selected that are sold in Korea because they advertised and contained the most goat milk protein ingredients. For example, product A contains 3.7 g total protein, carbohydrate 1.2 g, glucose 1.0 g and minor components (sodium 13.8 mg, cholesterol 3.6 mg) per 6 g. A sufficient amount of total protein is prepared for this study. Total 110 g are required for one experiment and 6 cans are required for three repetitions.

Water, ethanol, and methanol were used as the extraction solvent. The powders of goat milk protein was stirred with hot distilled water or 80% ethanol or methanol. An aliquot was further mixed with 100 mM Tris-HCl buffer (pH 7.4). The mixture of each group was further stirred with a magnetic bar at room temperature for 60 minutes. The sample was treated with ultrasound at room temperature for 60 minutes. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was shaken vigorously for one hour at room temperature. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

ACE inhibition assay

Angiotensin converting enzyme (ACE) from rabbit lung, hippuryl-L-histidyl-L-leucine (HHL), sodium borate buffer, hippuric acid (HA) and captopril were purchased from Sigma-Aldrich Co. (England). Inhibition modes of the goat milk protein extracts against ACE were determined according to the method described by a Sharifi et al.^[12] We used sodium borate buffer instead of HEPES. Sodium borate buffer solution used in this assay was prepared by dissolving 50 mM sodium borate and 300 mM NaCl in 1000 ml water and adjusting the solution to pH 8.3 by 1 M NaOH solution.

The substrate solution (9 mM) was prepared by dissolving HHL (19.74 mg) in 5 ml of sodium borate buffer. Goat milk protein extract (1 mg) was dissolved in 1 ml of solvent containing buffer/DMSO (90:10, v/v) to provide 330 μ g/ml concentration.

First, ACE solution (25 μ l) (80 mU/ml) was added to 25 μ l of inhibitor solution (or solvent as negative control). After 3 min pre-incubation at 37°C, 25 μ l substrate solution was added and the mixture was incubated at 37°C for 30 min with shaking at 300 rpm in an Eppendorf thermomixer. After 30 min, the reaction was stopped by addition of 50 μ l of 1 M HCl and then the reaction mixture was subjected to RP-HPLC. The mobile phase was an isocratic system consisting of a mixture of 10 mM KH_2PO_4 (adjusted to pH 3 with H_3PO_4) and methanol (50:50, v/v). The flow rate was 1 ml/min and the injection volume was 20 μ l. Analytcs were detected by a PDA detector at the wavelength of 228 nm.

All experiments were done in triplicate.

ACE inhibition was calculated on the ratio of the area under curve (AUC) of ACE peak in an inhibitor sample to that of negative control sample as follows:

$$\text{ACE inhibition (\%)} = [1 - (\text{AUC inhibitor} / \text{AUC control})] \times 100$$

AUC inhibitor: AUC of the ACE peak with inhibitor

AUC control: AUC of the ACE peak of control sample without inhibitor

Control and repeat tests were analyzed by a one sample *t* test with values above the 95% confidence interval considered significant ($p < 0.05$). The difference in group mean values among in vivo treated groups were analyzed by one way analysis of variance followed by Student Newman Keuls (SNK) multiple comparisons test.^[13] In some cases the paired t-test was used for comparisons.

RESULTS

The company's products were marked with goat's milk protein and other ingredients (Table 1). It was marked as containing from 526.3 g to 625.0 g per 1000 g (total weight). It was extracted as a solvent per 100 g of goat product and then concentrated in a concentrator to obtain about 5.45 grams of powder (Table 2). In this study, the inhibitory effects of their goat milk protein extracts against ACE inhibition were investigated. It was observed that inhibition percentage values go on increasing with enhancements in concentration of research protein extracts in the assay mixture. Fig. 1 was shown ACE inhibition activity at various concentrations and three solvents of the product A. Hot water extract of the goat's milk protein was evaluated 42.7% on 50 mM and that of the ethanol extract was 40.1% at same concentration. Methanol extract was 39.3% at same concentration. Although water extract of the goat's milk protein was slightly higher in ACE inhibitory activity than those of ethanol and methanol, there was no significant difference among four concentrations ($p > 0.05$). Since the distilled water extract had the best ACE inhibitory activity, other products were also tested with distilled water extract.

ACE inhibitory activity was no significant difference among four products at low concentrations (25, 12.5, and 6.25 mg/L) ($p > 0.05$) (Table 3). However, there was significant difference among 50 mg/L ($p < 0.05$).

We prepared four products as ACE inhibitor and compared its positive control inhibitory activity with captopril. The result showed that product A inhibitory

rate of ACE was 42.7% on the concentration of 50 mg/L (Fig. 1). Products B, C, and D were 40.1% (Fig. 2), 39.3% (Fig. 3), and 39.1% (Fig. 4) on same concentration, respectively. Products A, B, C, and D were 30.3%, 29.3%, 30.6%, and 29.7% on the concentration of 6.25 mg/L, respectively.

Table 1: The extracted dry weight (mg) from 100 g samples of four samples.

Product	Producer location, Protein source	Component (1000g)				
		Protein	Carbohydrate	Glucose	Fat	Minerals
A	Chungcheong-do, Korea, Netherlands	616.7	200.0	166.7	0.0	2.3
B	Daegu, Korea, Netherlands	616.0	250.0	206.0	8.0	3.4
C	Kwangweon-do, Netherlands and U.S.A	526.3	315.8	78.9	78.9	3.7
D	Kwangweon-do, Netherlands, Singapore, and U.S.A	625.0	270.0	145.0	31.1	7.2

Table 2: The extracted dry weight (mg) from 100 g samples of four samples.

Concentration (mg/L)	Solvent		
	Water	Ethanol	Methanol
Product A	5.45±0.23	5.12±0.10	4.88±0.22
Product B	2.82±0.22	2.51±0.16	2.48±0.18
Product C	2.58±0.17	2.34±0.12	2.27±0.14
Product D	2.48±0.09	2.22±0.16	2.13±0.07

Data represent the mean ± SD from three replicates

Table 3. Compare *t*-test of difference in means of four products goat milk protein at same concentration

<i>t</i> -test	Concentration (mg/L)			
	50	25	12.5	6.25
<i>t</i>	4.290*	1.346	0.075	0.666
Significance	*	NS	NS	NS

NS: non-significance, *: $p < 0.05$.

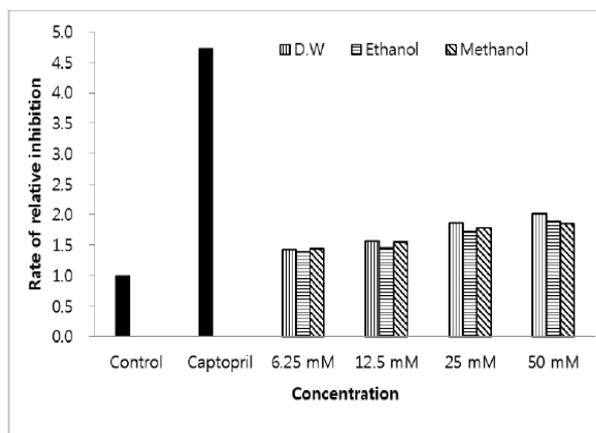


Fig. 1: ACE inhibition activity at various concentrations and three solvents of the product A. Control: distilled water. Captopril is positive control.

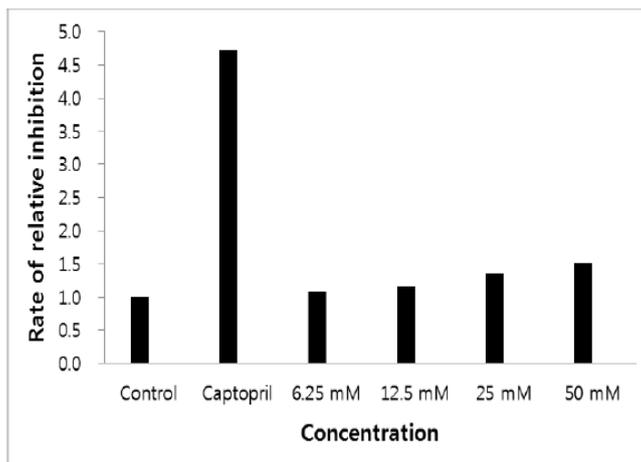


Figure 2: The relative inhibition rate of product B to the positive control group (captopril).

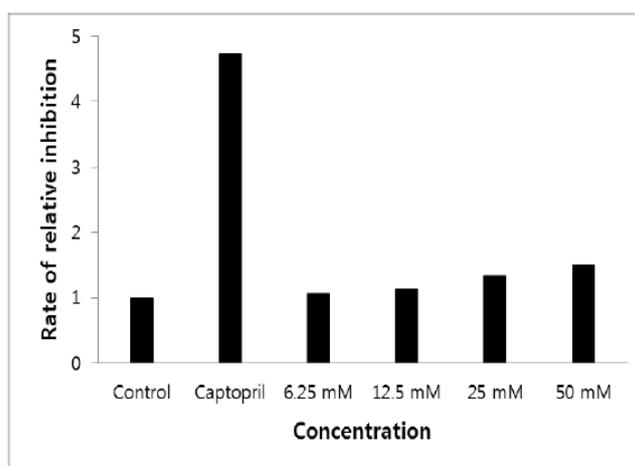


Figure 3: The relative inhibition rate of product C to the positive control group (captopril).

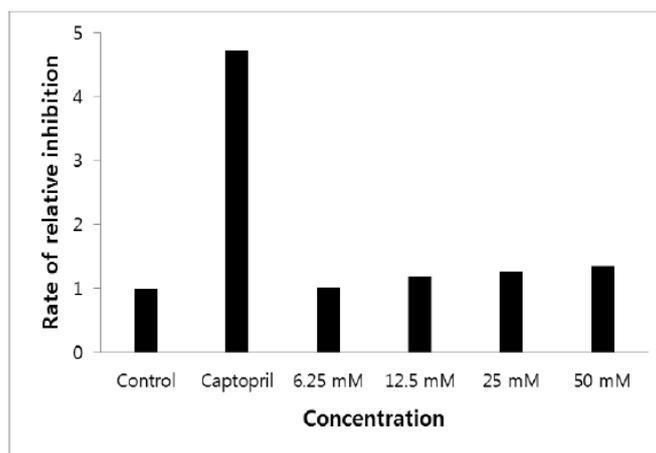


Figure 4: The relative inhibition rate of product D to the positive control group (captopril).

DISCUSSION

High blood pressure generally develops over many years, and it affects nearly everyone eventually. Accordingly while the Society remains committed to clinical investigation in hypertension and related vascular conditions; it is launching a renewed and vigorous focus on translational research. It's strategies, accordingly, are

to integrate not only investigators and physicians, but all hypertension health care providers-including primary care physicians, physician assistants, nurse practitioners and Pharm Ds-as well as patients themselves. One of the most effective medications for the treatment of hypertension is angiotensin converting enzyme inhibitors. Meanwhile, medicinal plants have been used

for treating illnesses. Therefore, they can be important resources to develop new drug candidates.^[14]

As illustrated in Table 2 and Fig. 1, extractions of goat milk protein of the product A showed inhibition activity more than 42.7%. The extractions of goat milk proteins of other products showed inhibition activity less than 39.1% (Figs. 2-4).

The basic nutrient composition of goat milk resembles cow milk, where both milks contain substantially higher protein and ash, but lower lactose content than human milk.^[15] Pihlanto- et al.^[16] demonstrated that the fermentation of milk with a cell crop is not enough to generate the inhibitory activity of ACE and that digestion with pepsin and trypsin is necessary. Bioactive peptides or hydrolysates of milk proteins have been considered as possible approach for use in nutraceuticals and pharmaceuticals for prevention and treatment of hypertension.^[17-19] Milk bioactive peptides constitute alternatives for this, serving directly as ACE inhibitors, or providing a scaffold for the engineering of novel molecules with clinical potential.^[20] In this study, goat milk contained ACE inhibitors like other milk (Figs 1-4).

Why are there differences in ACE inhibitory activity in each of the four products? First, there may be differences in the protein components of goat's milk used in the product. Both Goat and cow milks usually contain 30 to 35 g/L total protein, consisting of 80% casein and 20% whey.^[21] Although goat milk contains a similar amino acid profile to ewe milk, but the amino acid pattern in whey proteins differs from that in milk.^[22] These goat milk proteins are distinct from casein, both immunologically and chemically.^[23] Second, this is because the goat milk protein used in the product is expensive, so it is prepared by supplementing the content with vegetable proteins such as soy protein or fish protein. The company's product advertises high protein content in goat milk. Consumers are likely to overlook the fact that other proteins are mixed behind the advertisement that the product contains goat's milk protein.

CONCLUSIONS

Among four commercial products of a goat milk protein in Korea, high concentrations of milk proteins could be utilized for the treatment of ACE inhibition in a supplementary nutrient. However, four commercial products of a goat milk protein were not enough to generate the inhibitory activity of ACE.

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