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Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity of Elk (*Cervus elaphus*) Velvet Antler

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Abstract

Angiotensin I-converting enzyme (ACE) inhibitory activities of elk antler hydrolysates prepared with three kinds of proteases, pepsin, trypsin and α-chymotrypsin, were investigated. The ACE inhibitory activity of the pepsinolytic hydrolysate was the highest with an IC_{50} value of 9.3 μg/mL. In addition, three kinds of pepsinolytic hydrolysates with relatively high molecular weights (over 10,000 Da), medium molecular weights (5,000 to 10,000 Da), and low molecular weights (below 5,000 Da) were fractionated using an ultrafiltration membrane system. The below 5,000 Da hydrolysate exhibited the highest ACE inhibitory activity. These results indicate that the pepsinolytic hydrolysates of elk velvet antler could be a good source of peptides with ACE inhibitory activity.

Key words: angiotensin I-converting enzyme (ACE), antler, proteolysis, hydrolysate, peptide

INTRODUCTION

Hypertension is one of the most common cardiovascular diseases, affecting 15~20% of adults, especially in developed countries. Hypertension is the most common serious chronic health problem because it carries high risk factors for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (1). Angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase, which catalyzes the formation of antiotensin II, a strong pressor, from angiotensin I and inactivates bradykinin which has depressor action (2,3). ACE belongs to the class of zinc proteases that require zinc and chloride for its enzyme activity, and is located in the vascular endothelial lining of the lungs. Many synthetic ACE inhibitors such as captopril, enalapril, lisinopril and alacepril are available for clinical medicine (4,5). However, most ACE inhibitors have undesirable side effects such as cough, loss of taste, renal impairment and angioneurotic oedema (6). Therefore, a search for ACE inhibitors from natural materials has become a major field of research. In recent years, many ACE inhibitory peptides have been isolated from various food proteins such as cheese whey (7), casein (8), zein (9), tuna muscle (10), sardine (11), corn gluten (12) and bovine blood plasma (13) as well as from some fermented foods such as soy sauce (14).

Antlers have been used for thousands of years in oriental countries like Korea, China, Taiwan and Mongolia. The first report of antler used as a medicine appeared on a silk scroll unearthed from a Han tomb in China dated 100 BC. Since that time it has been used as a popular medicine in these countries. There are many medical and pharmaceutical uses of antler. It contains large variety biochemical components such as lipids, proteins, carbohydrates, and other organic substances (15). Its putative beneficial effects include modulation of various disorders such as kidney deficiency, gastrointestinal disorders, cardiovascular disorders and sexual disorders in men and menstrual disorders and menopause in women. It has also been used to promote rapid healing, and to treat weight loss, slow growth in children, strengthen weak bones, and alleviate cold hands and feet (16).

The aim of the present study was to investigate antler protein as a source of new peptides exhibiting ACE inhibitory activity and their abilities to act as ACE inhibitory compounds after digestion with proteolytic enzymes in human gastrointestinal tract.

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MATERIALS AND METHODS

Materials

The antler was obtained from adult male elks that were bred at Sam-Woo Deer Farm (Chungju, Korea). Each antler was equally divided into four sections (tip, upper, middle and bottom), and the tip section of antler was used for sample in this study. The sample was freeze-dried, homogenized, and stored at -20°C until used. Angiotensin I-converting enzyme (from rabbit lung), a substrate peptide (Hip-His-Leu; HHL), trypsin (from bovine pancrease, type II), α-chymotrypsin (from bovine pancrease, type II) and pepsin (from porcine stomach mucosa) were purchased from Sigma Co. (St Louis, MI, USA). All other chemicals were of analytical grade.

Preparation of antler hydrolysates

The enzymatic reaction mixture was prepared by adding 300 mL of buffer solution with pH 2.0 (pepsin), pH 7.6 (trypsin) and pH 7.8 (α-chymotrypsin) to 3 g of lyophilized antler. The mixtures were then initially pre-incubated in a 25°C shaking water bath and then sequentially digested with pepsin, trypsin and α-chymotrypsin. The enzymatic hydrolysis with pepsin (pH 2.0), trypsin (pH 7.6) and α-chymotrypsin (pH 7.8) was performed in the presence of 1% enzyme at 25°C for 24 h. One hundred millimolar phosphate buffer was used to hydrolyze with trypsin and α-chymotrypsin, and 100 mM potassium chloride/hydrochloric acid buffer was used for hydolysis of pepsin. During the hydrolysis, an aliquot (20 mL) was taken after 0, 10, 20, 40, 60, 120, 240, 360, 720 and 1440 min, the hydrolysates were subsequently boiled for 10 min to inactivate the enzyme after the solution was adjusted to pH 7.0, and then the ACE inhibitory activities of the hydrolysates were measured.

Fractionation of pepsinolytic hydrolysates

The pepsinolytic hydrolysates were fractionated in cartridges with molecular weight cut-offs (MWCO) of 10 and 5 kDa (Millipore Pelicon XL Biomax, Bedford, MA, USA). The cartridges were assembled in the ascending order of MWCO increment, and the hydrolysates were pumped with pressure (Millipore Labscale TFF system, Millipore System Division). The resultant fractions were assayed for ACE inhibitory activity.

Assay for ACE inhibitory activity

ACE inhibitory activity was measured by the method of Cushman and Cheung (17) with slight modifications implemented by Watanabe et al. (18). HHL was dissolved in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. A 200 μL volume of 5 mM HHL solution was mixed with 80 μL of sample solution followed by pre-incubation for 3 min at 37°C. The reaction was started by adding of 20 μL of ACE solution in distilled water (100 mU/mL), and the reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 1.0 M HCl (250 μL), and the liberated hippuric acid was extracted with 1.7 mL of ethyl acetate. After centrifugation (800×g, 15 min), 1.0 mL of the upper layer was transferred into a test tube and evaporated at room temperature for 2 h in a vacuum evaporator. The hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance was measured at 228 nm using an UV/VIS spectrophotometer (Opron 3000, Hanson Tech. Co. Ltd., Korea). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Preparation of a pepsinolytic hydrolysate of antler for measuring ACE activity

One gram of the pepsinolytic hydrolysates was separately incubated with trypsin (0.01 g) and α-chymotrypsin (0.01 g) at 37°C for 1 h. In addition, one gram of the hydrolysate was incubated with a mixture of trypsin and α-chymotrypsin (0.01 g, pH 8.0) for 1 and 2 h at 37°C. Finally, the reaction mixtures were boiled for 10 min and the ACE inhibitory activity was measured.

RESULTS AND DISCUSSION

Preparation of antler hydrolysates

The lyophilized elk tip antler was independently hydrolyzed with pepsin, trypsin and α-chymotrypsin, respectively, in order to select suitable proteases to prepare antler protein hydrolysates. As shown in Fig. 1, 2 and 3, ACE inhibitory activity of the antler hydrolysates increased after hydrolysis with various enzymes such as pepsin, trypsin, and α-chymotrypsin. Pepsin hydrolysate exhibited the highest ACE inhibitory activity followed by the trypsin and α-chymotrypsin. However, the ACE

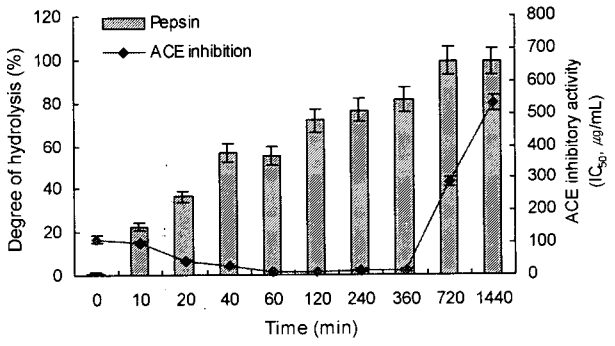

Fig. 1. Degrees of enzymatic hydrolysis of lyophilized antler at 37°C and pH 2.0 with pepsin, and ACE inhibitory activities of the hydrolysates. Mean±SD, n=3.

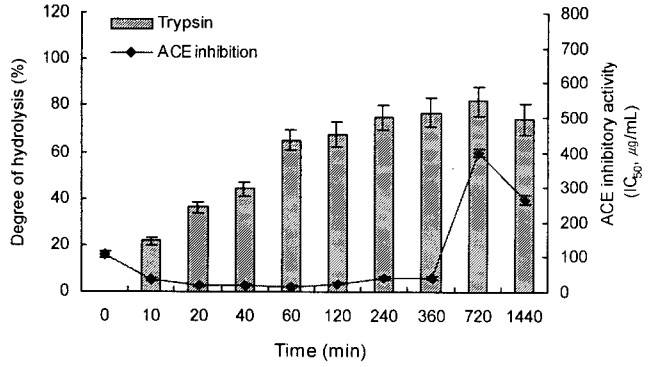

Fig. 2. Degrees of enzymatic hydrolysis of lyophilized antler at 25°C and pH 7.6 with trypsin and ACE inhibitory activities of the hydrolysates. Mean±SD, n=3.

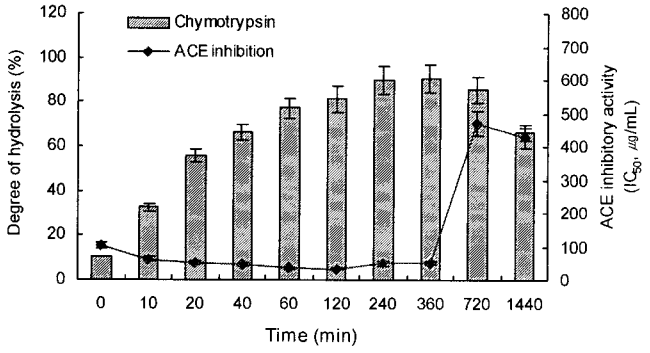

Fig. 3. Degrees of enzymatic hydrolysis activity of lyophilized antler at 25°C and pH 7.8 with chymotrypsin and ACE inhibitory activities of the hydrolysates. Mean±SD, n=3.

inhibitory activity of the three different kinds of hydrolysate decreased drastically after hydrolysis time exceeded 360 min. This suggests that the ACE inhibitory peptides undergo further digestion with prolonged incubation time. Among the hyrolysates obtained by pepsinolytic hydrolysis, the 1 h hydrosylate exhibited the highest ACE inhibitory activity (IC_{50}, 9.3 μg/mL) as shown in Fig. 1. Therefore, the pepsinolytic hydrolysate was selected for further experiments.

Antler is primarily composed of collagen as the major protein consisting primarily of glycine, alanine, proline and hydroxyproline (20,21). When pepsin in the stomach cleaves peptide bonds of protein, pepsin attacks the C-terminal side of tyrosine, phenylalanine and tryptophan residues, which contain aromatic side chains. It cleaves long polypeptide chains into shorter lengths. In the duodenum, trypsin cleaves peptide bonds on the C-terminal side of arginines, lysines, and α-chymotrypsin cleaves on the C-terminal side of tyrosine, phenylalanine and tryptophan residues as well as leucine, methionine, asparagine, and glutamic acid groups in a slower reaction. It was reported that the C-terminal amino acid of peptides made the most important contribution to substrate binding at the ACE active site (22). Many ACE inhibitory peptides have been discovered from enzymatic hydrolysates of various animal proteins such as casein (8), fish protein (23), porcine muscle (24), and beef protein (25). This indicates that many ACE inhibitory peptides with diverse molecular properties are present in various animal proteins. However, some ACE inhibitory peptides can be directly isolated from food materials without enzymatic hydrolysis (26). It was reported that 70% ethanol extract of deer antler reduces blood pressure in spontaneously hypertensive rats after oral administration (27). Therefore, antler hydrolysates have potential efficacy for decreasing blood pressure.

Fractionation of pepsinolytic hydrolysates

The pepsinolytic hydrolysate prepared with pepsin for 1 h under optimal conditions was fractionated by an ultrafiltration system with 5 kDa and 10 kDa membranes. Three kinds of fractionated pepsinolytic hydrolysates were below the 5 kDa hydrolysates, which had passed through the 5 kDa membrane; 5~10 kDa, hydrolysate, which passed through the 10 kDa membrane but not through the 5 kDa; the over 10 kDa hydrolysate, which are not passed through the 10 kDa membrane, followed by ACE inhibitory activity assays. The below 5 kDa hydrolysate showed the highest ACE inhibitory activity (IC_{50} 7.1 μg/mL) among the three kinds of fractions (Fig. 4). It was reported that two major fractions with ACE inhibitory activity was fractionated from soy sauce by gel filtration chromatography (14). The fraction with high molecular weights reduced blood pressure in spontaneously hypertensive rats, but the fraction with low molecular weights exhibited no activity. The ACE inhibitory activity of the fragmented hydrolysate from Alaska Pollack (*Theragra chalcogramma*) skin was markedly increased with decreasing molecular weights (23). In this study, the below 5,000 Da hydrolysate exhibited the highest ACE inhibitory activity. The results of ACE in-

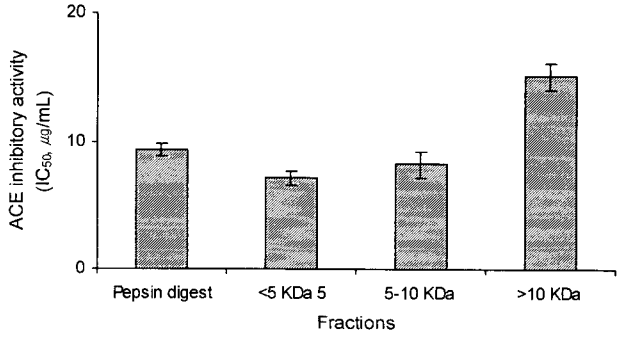

Fig. 4. ACE inhibitory activity (IC_{50} μg/mL) according to molecular weight distribution of pepsinolytic digeste (PD) of antler. The lyophilized antler was for 1 h with pepsin and fractionated using ultrafiltration membrae system. Mean±SD n=3.

hibitory activities of Antler and Alaska Pollack hydrolysates indicate that peptides with low molecular weights may be responsible for their antihypertensive effects.

Further hydrolysis experiments for pepsinolysic hydrolysate from antler

The pepsinolytic hydrolysate prepared from antler was further hydrolyzed with trypsin and α-chymotrypsin, and the activities of the hydrolysates were compared with those of before and after the hydrolysis. As shown in Fig. 5, the ACE inhibitory activity of the hydrolysates was slightly decreased with increasing enzymatic hydrolysis time. The IC_{50} values for further hydrolysis by trypsin (1 h), α-chymotyrpsin (1 h), mixture of trypsin and α-chymotrypsin (1 h), and mixture of trypsin and α-chymotrypsin (2 h) were 12.2, 12.4, 12.1 and 16.7 μg/mL, respectively. These results indicate that the ACE inhibitory activities of the hydrolysates significantly decreased after the further hydrolysis. Resistance to hydrolysis by gastrointestinal proteases is important for the anti-hypertensive effects of ACE inhibitory peptides. It was found that di- and tri-peptides derived from β-conglycinin were absorbed intact through the small intestinal membrane of rats (28). Many peptides can serve as competitive substrates for ACE although they exhibit ACE inhibitory activity. However, they are digested into inactive peptides or free amino acids when they are orally administered in some occasions. To exhibit an antihypertensive effect in the body, the ACE inhibitory peptides must be absorbed in their intact form in intestines. In fact, some ACE inhibitory peptides, especially di- and tri-peptides, could directly pass through the intestine without being decomposed by digestive enzymes (29-31).

The ACE inhibitory activities of peptides do not always correlate with their antihypertensive effects. It is known that some peptides with potent ACE inhibitory activity in the body are inactive or exhibit more inhibitory activity after oral administration. Furthermore, ACE inhibitory peptides can be classified into three groups such as inhibitor type, pro-drug type and substrate type depending on the nature of the interaction with ACE. The inhibitor type of ACE inhibitory activity of a peptide is not changed after pre-incubation. In addition, pro-drugs are also substrates for ACE, but they are converted to true inhibitors by ACE or gastrointestinal proteases. Finally, the substrate type is hydrolyzed by ACE to yield inactive peptides (32).

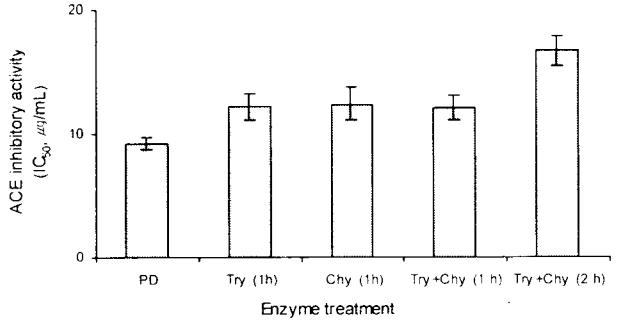

Fig. 5. Hydrolysis experiment (digestive stability) for pepsinolytic digest (PD) of antler. The lyophilized antler was digested for 1 h with pepsin and subjected to further digestion test with trypsin and chymotrypsin for 1 or 2 h. Mean ± SD, n=3.

In this study, antler hydrolysate exhibited high angiotensin I-converting enzyme inhibitory activity although the activity was slightly decreased with further digestion experiments (Fig. 5).

In conclusion, the pepsinolytic hydrolysate derived from the tip section of antler may enhance antiotensin I-converting enzyme inhibitory activity. In addition, the ACE inhibitory peptide derived from antler may be absorbed from the gastrointestinal tract in active forms to exhibit the antihypertensive function in the human body. Therefore, the results obtained in this study suggest that antler is a potential candidate for the treatment of hypertension. However, further study is needed to purify the compound responsible for antihypertensive effects in antler hydrolysate.

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