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Attenuating Development of Cardiovascular Hypertrophy with Hydrolysate of Chicken Leg Bone Protein in Spontaneously Hypertensive Rats

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ABSTRACT : This study developed a natural ingredient as a functional food possessing properties of attenuation of hypertension and cardiovascular hypertrophy. In a previous study hydrolysates obtained from chicken leg bone protein using Alcalase strongly inhibited angiotensin I converting enzyme (ACE) *in vitro*. In particular, hydrolysate (A4H) from four hours of incubation exhibited the highest ACE inhibitory activity (IC_{50} = 0.545 mg/ml). A4H was selected as a potent ACE inhibitor and orally administrated to spontaneously hypertensive rats (SHR) for eight weeks to investigate attenuating effects on age-related development of hypertension and cardiovascular hypertrophy. Results showed that treatment with A4H of SHRs attenuated the development of hypertension as effectively as the clinical antihypertensive drug captopril. Moreover, a significantly lower heart to body weight ratio and thinness of coronary arterial wall was observed in SHRs that had been treated with A4H or captopril. The results suggest that A4H can be utilized in developing an ACE inhibitor as a potential ingredient of functional foods to alleviate hypertension and cardiovascular hypertrophy. (**Key Words :** Angiotensin I Converting Enzyme (ACE), Chicken Leg Bone Protein, Hydrolysate, Antihypertensive Effect, Cardiovascular Hypertrophy)

INTRODUCTION

The renin-angiotensin system is a vital regulator of blood pressure and fluid homeostasis (Laragh et al., 1972; Johnston et al., 1992). Angiotensin I converting enzyme, ACE (dipeptidyl carboxypeptidase, EC 3.4.15.1), a zinc metal peptidase, plays an important role in this system. ACE can increase the blood pressure both by catalyzing the conversion of decapeptide angiotensin I to the potent vasoconstricting octapeptide angiotensin II, and by inactivating the vasodilator bradykinin (Bhoola et al., 1992; Turner and Hooper, 2002). It was found that several antihypertensive agents such as captopril, lisinopril and enalapril owe their therapeutic efficacy to ACE inhibitory activities (Ondetti et al., 1977; Cohen, 1985). In addition, the ACE inhibitors inhibited hypertensive left ventricular hypertrophy more strongly than other first-line antihypertensive agents (Dhlof et al., 1992).

Globally, hypertension is regarded as a major health problem and constitutes a high risk factor for development of arteriosclerosis, stroke, coronary heart disease and myocardial infarction (Kannel, 1996; Brian and Rosario, 2005). Treatment of hypertension involves the chronic control of blood pressure under normal conditions. The ACE inhibitor is one of several classes of pharmacological agents that have been extensively employed in hypertensive therapy. Natural peptides with ACE inhibitory activity were discovered first in snake venom (Ondetti et al., 1971) and have driven the development of synthetic ACE inhibitors. Potent ACE inhibitors were designed in the 1980s and are currently in use. However, they are also known to have a significant adverse effect. Therefore, peptides with ACE inhibitory activities that are obtained by the enzymatic hydrolysis of food proteins have drawn considerable attention. Currently, many natural ACE inhibitors have been produced by enzymatic hydrolysis of various food proteins. Some have demonstrated efficacy in reducing systolic blood pressure in spontaneously hypertensive rats (SHRs) following their administration (Li et al., 2004; Vercruysse et al., 2005; Jang and Lee, 2006; Jung et al., 2006). In addition, research has increasingly focused on natural products with ACE inhibitory peptides in recent years (Wu and Ding,

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Table 1. Chemical composition of chicken leg bone and its hydrolysate produced by incubation with Alcalase for four hours (A4H)

Constituent	Chicken leg bone	Hydrolysate (A4H)
	% (w/w)	
Moisture	38.34±2.26	7.79±0.12
Crude fat	22.02±2.66	2.19±0.60
Crude protein	23.54±2.21	80.18±0.20
Ash	11.80±1.64	6.04±0.13

2001; Katayama et al., 2003; Muguruma et al., 2003; Miguel et al., 2005; Lee et al., 2005). Considering cost, economy and application, hydrolysates with potent ACE inhibitory peptides and antihypertensive effects may be preferred due to the low recovery of purified ACE inhibitory peptides from enzymatic hydrolysates or fermentated food proteins.

In previous studies (Cheng et al., 2007a; Cheng et al., 2007b) of the utilization of chicken leg bones, which are byproducts of industrial chicken meat processing and are normally discarded, various proteases have been adopted to study antioxidative and ACE inhibitory activity of hydrolysates of chicken leg bone. The results of these studies showed that chicken leg bone contains large amounts of protein in the form of meat, cartilage and bone marrow, comprising about 23.5% crude protein. Following hydrolysis with Alcalase at 1% enzyme to substrate (E:S) ratio, the hydrolysate exhibited strong antioxidant and ACE inhibitory activities. Moreover, Alcalase-treated hydrolysates displayed the highest ACE inhibitory activity when the E:S ratio was increased to 2% and hydrolysis was continued for 4 h (A4H) (Cheng et al., 2008). Thus, the aim of this study was to investigate the effects of orally administering the A4H to SHRs and Wistar-Kyoto (WKY) rats on attenuation of the development of hypertension and cardiovascular hypertrophy.

MATERIALS AND METHODS

Preparation of hydrolysates of chicken leg bone protein

The method was based on the previous study (Cheng et al., 2008). Chicken leg bones (broiler) were obtained from a meat processing factory in Tai-Chung, Taiwan, and cut into small pieces. 100 g of chicken leg bone was ground with 200 ml of water using a blender (Waring Commercial, Torrington, USA) and then heated in a boiling water bath for 5 min. The chicken leg bone proteins were hydrolyzed for 4 h using Alcalase (P4860, Sigma, St. Louis, MO, USA) with E:S ratio of 2% at pH 8.0 and 50°C. The enzymatic hydrolysis was stopped by boiling for 10 min and the hydrolysates were collected. The hydrolysates were centrifuged at 10,000×g for 10 min, and the supernatant was recovered, filtered through a 0.45 μm pore sized filter, lyophilized and stored at -80°C.

Composition of hydrolysate of chicken leg bone protein (A4H)

Moisture, crude fat, crude protein, and ash were determined following methods of the Association of Official Analytical Chemists (AOAC, 1990).

Treatment of animals and blood pressure measurement

Twenty four male SHRs and eight male WKY rats, aged of seven weeks, were obtained from the National Laboratory Animal Center, Taipei, Taiwan, and raised in an air-conditioned room (25°C) with a 12 h light-dark cycle (lighting from 7:00 to 19:00). Laboratory diet (MF-18, Oriental Yeast Co., LTD, Tokyo, Japan) and tap water were available *ad libitum* throughout the experiment. After a week of acclimation, SHRs were randomly divided into three groups. Hydrolysate with the best ACE inhibitory activity (A4H) (IC_{50} = 0.545 mg/ml) was dissolved in 1 ml of deionized water and orally administered (50 mg/kg bw/day) to rats using a metal gastric zoned. Captopril (China Chemical & Pharmaceutical Co., Ltd., Taipei, Taiwan)(1.5 mg/kg BW/d) was administered as a positive control. Control SHRs and WKY rats were orally administrated the same volume of water only. Liveweights were monitored weekly, and both arterial blood pressure and heartbeat were measured every two weeks by the tail-cuff method using an indirect blood pressure meter (BP-98-A, Softron, Tokyo, Japan) after warming to 39°C, controlled by a thermostat, for 10 min. The blood pressure of each rat was calculated as the average of three individual measurements. At the end of the experiment (16th week of life), the rats were fasted for 12 h before being sacrificed to weigh their hearts.

Histology

The hearts of rats were immediately soaked in neutral buffered 10% formalin, embedded in paraffin, and cut into cross-sections (6 μm thick), which were stained with hematoxylin-eosin and mounted. The thickness of intramyocardial coronary vessel wall was quantified morphometrically using a Leica LB30 microscope (Leica, Göttingen, Germany) with Motic Images Plus2 software (Motic, Xiamen, China).

Statistical analysis

Data were analyzed by a GLM program and Duncan's new multiple range test using the SAS System for Windows V8 (SAS, 2000).

RESULTS AND DISCUSSION

Composition of hydrolysate (A4H)

Table 1 presents the composition of chicken leg bone and its hydrolysate. Chicken leg bone contained 38.3%

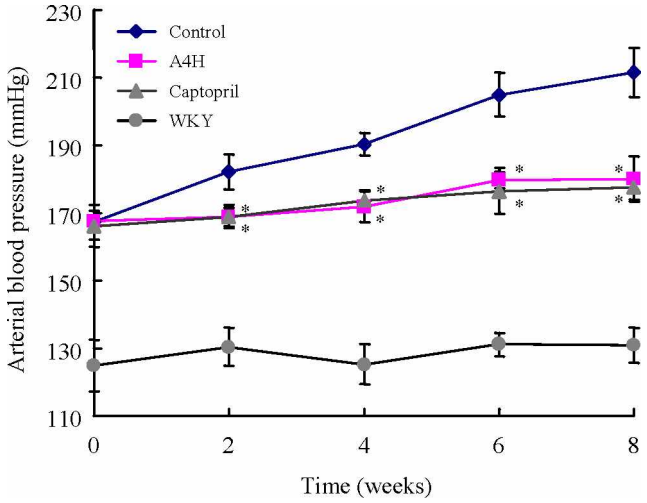

Figure 1. Changes in arterial blood pressure of SHRs treated with four hours of incubation hydrolysate (A4H) by oral administration (50 mg/kg BW/d). Captopril (1.5 mg/kg BW/d) was used as the positive control. WKY and control rats were orally administered with deionized water. *: different significantly from control ($p<0.05$). n = 8.

moisture, 22% crude fat, 23.5% crude protein and 11.8% ash. After hydrolysis by Alcalase for four hours, centrifugation, filtration and lyophilization, about 10% yield was obtained. A4H was a hydrolysate powder with excellent ACE inhibitory activity *in vitro* (IC_{50} = 0.545 mg/ml), and its color was light yellow. It contained 7.8% moisture, 2.2% crude fat, 80.2% crude protein and 6% ash. A4H exhibited lower amounts of moisture, crude fat and ash, and higher amounts of crude protein than chicken leg bone.

Attenuation of the age-related development of hypertension

Figure 1 presents changes in arterial blood pressures of rats with different treatments. As expected, SHRs without pharmacological therapy showed elevated, blood pressure with increasing age. Blood pressure was lowest in WKY rats and was maintained at about 130 mmHg throughout the experiment. After the second week, significant inhibition was evident in the blood pressure of rats that had been treated with A4H or captopril ($p<0.05$). At the end of the experiment (16th week of life), the control SHRs had higher blood pressure (211 mmHg) than the A4H-treated (180 mmHg) and captopril-treated SHRs (178 mmHg). Restated, a significant decrease of about 33 mmHg in blood pressure was finally measured in SHRs that were treated A4H or captopril ($p<0.05$).

Captopril is well known as a potent ACE inhibitor with excellent antihypertensive efficacy because of its ACE inhibitory activity (Ondetti et al., 1977). Hu et al. (2007) reported that early treatment with captopril prevents the development of hypertension by inhibiting the generation of angiotension II and smooth muscle contraction. This is consistent with our results that the captopril group attenuated significantly the early development of hypertension, as expected. Also, A4H exhibited an antihypertensive activity that was as strong as that of captopril, and which was considered as being related to its ACE inhibitory activity. Miguel et al. (2004) prepared hydrolysate from egg white that was treated with pepsin. Not only did this preparation exhibit ACE inhibitory activity *in vitro*, but it also had an acute antihypertensive effect in SHR due to its content of ACE inhibitory peptides (Miguel et al., 2005; Miguel et al., 2006). Besides, certain hydrolysates obtained from enzymatic hydrolysis of food protein, with strong ACE inhibitory activity *in vitro*, have been demonstrated to reduce systolic blood pressure and attenuate the development of hypertension by inhibiting ACE activity in SHR (Wu et al., 2001; Sipola et al., 2001; Yang et al., 2004). From the aspects of economy and natural products, hydrolysates with potent ACE inhibitory possess higher potential in application than natural purified ACE inhibitory peptides or synthetic ones. Furthermore, Vercruysse et al. (2005) reviewed the ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein, and suggested the need to focus on new sources of ACE inhibitory peptides. Chicken leg bones are a waste product of industrial chicken meat processing and are

Table 2. Changes of heartbeat in SHRs and WKY rats during treatment

Weeks	Heart rate (beats/min)			
	SHR			WKY
	Control	A4H	Captopril	Control
0	426.2±39.4^{ax}	433.7±17.1^{ax}	428.3±25.1^{ax}	330.3±33.5^{cy}
2	443.8±26.1^{ax}	430.8±11.7^{ax}	429.0±39.6^{ax}	352.7±23.3^{bcy}
4	434.0±38.7^{ax}	432.0±34.5^{ax}	436.5±31.3^{ax}	389.2±40.5^{ax}
6	430.8±40.7^{ax}	437.7±34.4^{ax}	431.0±32.1^{ax}	390.3±29.5^{ax}
8	435.8±36.4^{ax}	430.2±21.2^{ax}	437.5±34.2^{ax}	389.8±37.3^{ax}

Values are means±standard deviations (n = 8).
WKY = Wistar-Kyoto rats; SHR = Spontaneously hypertensive rats.
A4H: four hour of incubation hydrolysate.
$^{a-c}$ Means with different superscript letters in the same column are different significantly ($p<0.05$).
$^{x-y}$ Means with different superscript letters in the same row are different significantly ($p<0.05$).

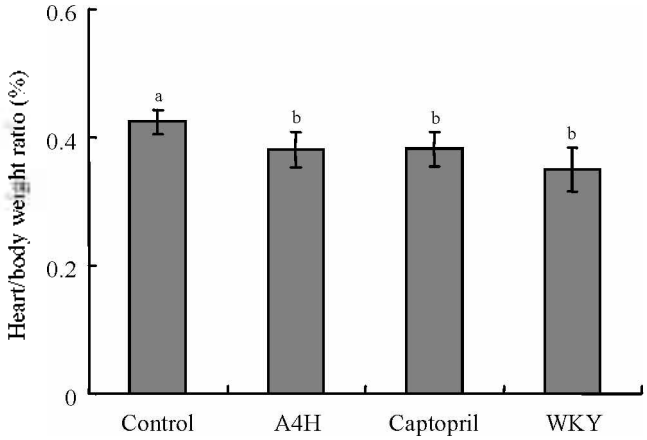

Figure 2. Heart to body weight ratio of rats on different treatments at the end of the experiment (16th week). Each value is expressed as mean±standard deviation. Different superscript letters indicate significant differences ($p<0.05$). n = 8.

produced in large quantities every year, especially in Asia. They are very suitable for utilization in developing functional foods.

The changes in heartbeat during the experimental period in SHRs and WKY rats are summarized in Table 2. Unsurprisingly, WKY rats had the lowest heart rates. Hypertensive patients have higher heart rates than normotensive patients, and such increases are related to cardiovascular disease. Hence, heartbeat is a main factor that should be monitored chronically in hypertensive patients (Perski et al., 1993; Habib, 1997). The heartbeats of SHRs under different treatments were measured herein to identify whether the administration of A4H or captopril was responsible for the physical changes. No significant differences in heartbeat were observed among the various treatments of SHRs. These results are consistent with other investigations. For instance, Materson et al. (1998); Materson et al. (1999) compared the effects of antihypertensive drugs on heartbeat and indicated that heart rate did not significantly change when hypertensive patients were treated with the ACE inhibitor captopril. Furthermore, Saiga et al. (2003) reported that when SHRs were administered orally with 1,000 mg/kg BW of extract that was prepared from the hydrolysis of a chicken breast muscle, a maximal reduction of 50 mmHg was found whereas there was no change in heartbeat.

Attenuation of the development of cardiovascular hypertrophy

Figure 2 plots heart to body weight ratio at the end of the experiment (16th week of life) in variously treated rats. It was not surprising that normotensive WKY rats had a lower heart to body weight ratio (0.35%) than SHRs ($p<0.05$). The control SHRs exhibited a significantly higher

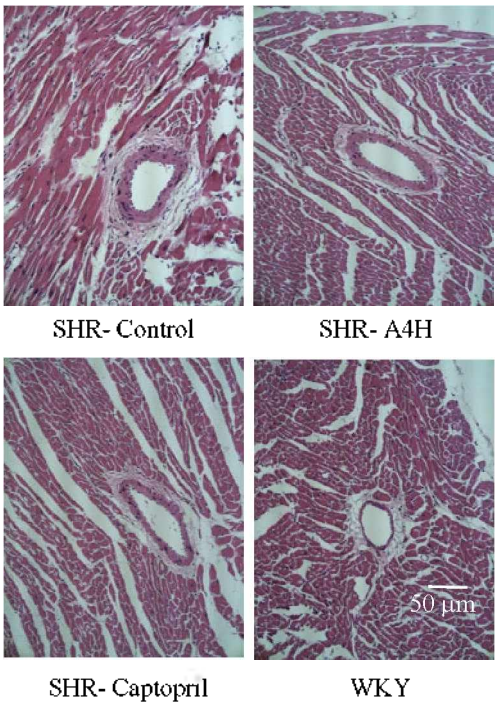

Figure 3. Histological characterization of intramyocardial coronary vessels from rats on different treatments at the end of the experiment. 200×. Bar indicates 50 μm.

heart to body weight ratio (0.42%) than the rats treated with A4H or captopril (0.38%), suggesting that A4H could inhibit cardiac hypertrophy as effectively as captopril. Generally, chronic hypertension increases the load on the heart, accelerates the synthesis of myocardium, and causes cardiac hypertrophy (Moalic et al., 1984; Tsutsui et al., 1999). However, it has been demonstrated that hypertensive patients can prevent cardiac hypertrophy by maintaining normal blood pressure (Chen et al., 1998). Hu et al. (2007) reported that the ACE inhibitor captopril not only had antihypertensive activity but also showed the ability to inhibit cardiac hypertrophy. Moreover, early captopril treatment of SHR exhibited great therapeutic effects in antihypertension and anti-cardiac hypertrophy (Freslon and Giudicelli, 1983; Chen et al., 1998). That observation is consistent with the results of this study.

Figure 3 shows the histological characterization of intramyocardial coronary vessels. Apparently, WKY rats showed thinner vessel walls than SHRs. SHRs treated with A4H or captopril obviously prevented the development of vascular hypertrophy. Measurements of wall thickness in intramyocardial coronary vessels are presented in Figure 4. Similarly, the thickness in WKY rats (15.8 μm) was significantly lower than SHRs ($p<0.05$). The thickness of wall in control SHRs was 26 μm which was 2.7 times that of WKY rats. In contrast, SHRs treated with A4H or captopril had wall thickness of 17 μm and 15 μm,

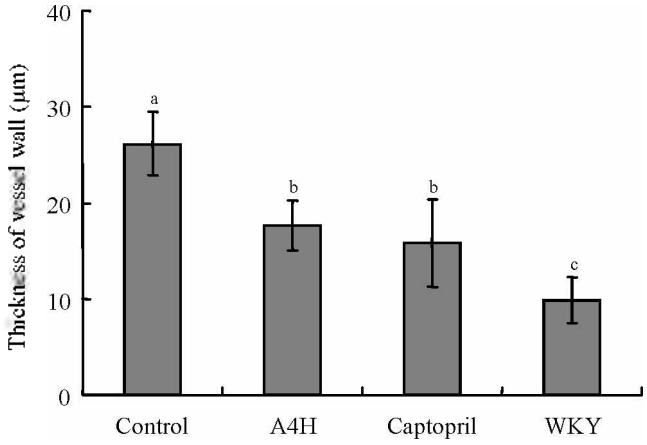

Figure 4. Thickness of coronary vessel walls from rats on different treatments at the end of the experiment. Each value is expressed as mean±standard deviation. Different superscript letters indicate significant differences ($p<0.05$). n = 8.

respectively, which were significantly lower than that of control SHRs ($p<0.05$). Currently, hypertension is one of the major health problems world-wide and a high-risk factor for diseases such as stroke, arteriosclerosis and coronary heart disease. Chronic hypertension not only results in cardiac hypertrophy but also stretches smooth muscle cells, eventually leading to proliferation of smooth muscle cells and wall thickening (Diez and Laviades, 1997; Hu et al., 2007). Treatment with ACE inhibitors has been reported to inhibit ACE activity, decrease generation of angiotensin II, reduce arterial blood pressure and attenuate cardiovascular hypertrophy in SHRs (Ikeda et al., 2000; Ishimitsu et al., 2006). The present observations were consistent with those results and demonstrated effects of A4H in attenuating development of age-related hypertension and cardiovascular hypertrophy as significant as the clinical drug captopril. In addition, A4H is a hydrolysate with strong ACE inhibitory activity derived from mixed competitive inhibitors that are contributed from several peptides within. Therefore, further research is necessary to investigate the mechanisms of ACE inhibition in SHRs.

CONCLUSION

In the present study, chicken leg bone protein hydrolyaste (A4H) exhibited excellent ACE inhibitory activity *in vitro* (IC_{50} = 0.545 mg/ml). After oral administration in SHR, A4H significantly attenuated the age-related development of hypertension and cardiovascular hypertrophy. It is noteworthy that chicken leg bones are waste byproducts of industrial chicken meat processing. The findings of this work will contribute to the utilization of chicken leg bones in the production of such valuable products as functional foods with antihypertensive and anti-cardiovascular hypertrophy effects.

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