Journal of Food and Drug Analysis, Vol. 15, No. 2, 2007, Pages 169-173 藥物食品分析 第十五卷 第二期

Hydrolysates from Tuna Cooking Juice as an Anti-hypertensive Agent

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(Received: September 12, 2006; Accepted: December 14, 2006)

ABSTRACT

Tuna cooking juice containing 4% protein was hydrolyzed using a commercial protease, Orientase from *Bacillus subtilis*, to prepare hydrolysate (OAH) that inhibits angiotensin I-converting enzyme (ACE). The systolic blood pressure (SBP) dropped by 18 mmHg in spontaneously hypertensive rats (SHR) 2hr after being orally administered the hydrolysate at 0.25g hydrolysate/kg body weight, and reduced, from 172 mmHg to 150 and 142 mmHg for those administered with lyophilized OAH at 0.5 and 1.0 g/kg body weight, respectively. This indicated that the lowering effect on SBP was dose-dependent. Constituents derived from the gel filtration of OAH with molecular weight ranging from 240-565 Da exerted an obvious effect on SBP. Although the reduced SBP in SHR gradually returned to the original level, the reduction effect lasted for 2-10 hr, depending on the dose. The SBP of SHR fed on a normal diet increased gradually to 215 mmHg by the age of 15 weeks, while it decreased, then increased to and stayed at, respectively, 200, 205 and 215 mmHg for those fed on diets containing 2.5, 1.25 or 0.25% lyophilized OAH. This result showed that proteins in tuna cooking juice are potential source of ACE-inhibiting agent, and thus may be useful to regulate the blood pressure.

Key words: tuna cooking juice, hydrolysates, anti-hypertensive properties, angiotensin I-converting enzyme (ACE)

INTRODUCTION

The angiotensin I-converting enzyme (ACE) catalyzes the formation of angiotensin II, a strong vasopressor from angiotensin I, and inactivates bradykinin, which exhibits hypotensive activity[(1)]. ACE inhibitors, such as the commercially anti-hypertensive drugs captopril and enalapril, block both of these actions, thus contributing to their potent anti-hypertensive activities in spontaneously hypertensive rats (SHR) or patients[(2)]. Recently, various peptides or hydrolysates derived from food proteins were found to inhibit ACE[(3-8)]. Many studies also demonstrated ACE inhibitors derived from tuna[(9-11)]. Some of these ACE-inhibiting substances, while given orally, did reduce the blood pressure in humans and SHR[(1,7,8,12)]. These findings suggest that those substances are absorbed through the digestive tract, inhibit ACE activity and subsequently reduce the blood pressure.

Tuna cooking juice, a protein-rich byproduct containing 4% water-soluble protein, is discarded as drainage in commercial canned tuna factories[(13)]. It is essential to recover the proteins in order to prevent water pollution and to achieve complete utilization of available proteins. Our previous study indicated that orientase, a commercial protease from *Bacillus subtillu*, effectively released ACE- inhibitory peptides and the absorption-enhancing treatments increased the antihypertensive activity of these peptides from tuna cooking juice *in vivo* in SHR[(14,15)]. In this report, the inhibitory effect of orientase hydrolysate (OAH) and its fractions on the development of hypertension in SHR during oral administration were investigated in the hope that the development of foods for blood pressure control could benefit.

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MATERIALS AND METHODS

I. *Materials and Reagents*

Tuna cooking juice containing 4% proteins was obtained from a tuna can plant in Chiayi County, Taiwan. Captopril was purchased from Sigma Chemicals (MO, USA). Orientase, an endopeptidase prepared from *Bacillus subtilis*, was purchased from Hankyu Bioindustry Co. (Osaka, Japan). All other chemicals were of analytical grade.

II. *Preparation of Tuna Cooking Juice Hydrolysate*

Orientase (1.0%, w/w) was added to the tuna cooking juice in a ratio of substrate/enzyme = 25 (v/v), i.e. E/S = 1/250. Enzymatic hydrolysis was performed at

50°C for 3 hr. The juice was then boiled for 10 min to inactivate the protease. The reaction mixture was centrifuged at 10,000 ×g for 10 min, and the supernatant was collected and lyophilized as OAH.

III. *Fractionation of OAH*

OAH (280 mg) dissolved in 1 mL of distilled water was applied on a Sephadex G-25 column (2.5 × 70 cm) The elution was effected with 0.05 M phosphate buffer (pH 6.5) at a flow rate of 45 mL/hr. The eluent was collected in the 5 mL of fraction. The absorbance at 280 nm and the ACE inhibitory activity of all fractions were measured. The distribution of molecular weight (MW) of hydrolysate was estimated using β-endothein (MW 2,573), neurotensin (MW 1,672.9), bradykinin (MW 1,060), [Sar^1, Ala^8] angiotensin-II (MW 926), N-formyl-Met-Leu-Phe-Lys (MW 565.7) and tryptophan (MW 204).

IV. *Single Oral Administration in SHR*

Eight-week-old male SHRs obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan, ROC) were fed on autoclavable rodent diet (Labdie Co., USA) consisting of 55.5% carbohydrates, 23% protein, 4.5% fat, 6.0% fiber and 3.0% minerals, and were given water ad libitum. All rats were housed for 7 days to help them acclimatize to their new environment. After being housed in cages controlled at 22 ± 3°C, RH 50 ± 10%, and 12 hr light/12 hr dark (lights on 06:00 hr), the rats were randomly given 0.25, 0.5 or 1.0 g/kg of body weight of OAH or its fractions from gel filtration in saline by gastric incubation (8 rats/group). Control group rats were administered the same amount of saline. Systolic blood pressure (SBP) was measured 0, 2, 4, 6, 8 and 10 hr thereafter. Long-term oral administration for SHR was as follows: commercial diets mixed with 0.25, 1.25 or 2.50% (w/w) lyophilized OAH were fed to SHR from age 9 weeks to age 17 weeks. The SBP of SHR was measured once a week.

V. *Characterization of the Inhibition of ACE*

ACE inhibitory activity was measured spectrophotometrically using hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as substrate, as described by Cushman and Cheung[16]. Fifty microliter of sample solution and 100 μL of 2.5 mU ACE solution were added to 100 μL of 12.5 mM Hip-His-Leu solution in 1.0 M NaCl-borate, pH 8.3. After incubation at 37°C for 1 hr, the reaction was stopped by adding 250 μL of 0.5 N HCl. The liberated hippuric acid was extracted with 1.5 mL of ethyl acetate, and the absorbance at 228 nm was measured to determine the ACE inhibitory activity. The inhibition rate (%) is expressed as {(Ec-Es)/(Ec-Eb)} × 100, where Es is the absorbance when the sample is added to the reaction mixture; Ec is the absorbance with buffer (instead of the sample) added, and Eb is the absorbance where the stop solution was added before the reaction started. The IC_{50} value is the sample concentration associated with 50% inhibition on ACE.

VI. *Measurement of Blood Pressure*

The systolic blood pressures (SBP) of five rats were measured by the tail-cuff method using an indirect blood pressure meter (BP-98A, Softron Co. Ltd., Japan). The rats were warmed in a 39-40°C box for 10 min before their blood pressure was measured. Five readings were recorded and the average SBP calculated. The significance of the differences between the SBP before and after the OAH administration was analyzed using the paired t-test.

RESULTS AND DISCUSSION

I. *ACE Inhibitory Action from OAH*

Intact tuna cooking juice or its protein fraction did not suppress the action of ACE. However, the hydrolysate by a commercial protease, Orientase from *Bacillus subtilis*, inhibited ACE, and the IC_{50} was 5.85 mg protein/mL (Table 1). The IC_{50} value of OAH was slightly higher than that of sardine muscle hydrolysate (3.15 mg protein /mL)[17]. Inhibition of ACE activity results in the decrease of blood pressure. Pharmatherapeutic efforts to reduce the increase in blood pressure in hypertensives have been undertaken by suppressing the production of active angiotensin II precursor, or by inhibiting the catalytic activity of ACE[18]. The intake of sardine muscle hydrolysate and casein hydrolysate for a 4-week protocol could induce potent anti-hypertensive effects in mild hypertensives[3,19,20]. Thus, the intake of protein hydrolysate OAH with ACE inhibitory activity seems to be of great potential for regulating blood pressure.

II. *Anti-hypertensive Activity of OAH in SHR*

Rats, age of 9 weeks, were given 0.25 g OAH per kg of body weight by gastric incubation. The mean SBP

Table 1. Change in inhibition of ACE in tuna cooking juice samples following various treatments

Treatment	IC_{50} value	
	(mg/mL)	(mg protein/mL)
Intact (tuna cooking juice)	NI	NI
TCA	NI	NI
Orientase hydrolysis & lyophilization	46.89	5.85

Intact tuna cooking juice was hydrolyzed with 0.4 weight % of orientase for 3 hr, and subsequently lyophilized. Trichloroacetic acid (TCA, 10%) treatment was performed to determine the protein fraction of the tuna cooking juice. NI: no inhibition.

was 172 ± 3.5 mmHg before OAH administration and was considerably reduced by 18 ± 2 mmHg 2 hr after administration (Figure 1). SBP then returned to the initial level at 4 hr after administration. In contrast, the SBP of rats who had been given saline or tuna cooking juice did not change significantly during the period of 24 hr. Also as shown in Figure 1, OAH significantly lowered SBP in SHR between 2 hr and 4 hr after 0.5g OAH /kg body weight was orally administered. A higher dose (1.0 g/kg body weight) was resulted in a stronger anti-hypertensive effect and a reduction in SBP by 30 mmHg 2 hr after administration was observed; this effect was maintained for 8 hr. OAH (0.25, 0.5, 1.0 g/kg body weight) yielded, in a dose-dependent manner, a lower blood pressure than control (saline) did.

This study demonstrated for the first time that a single oral administration of the ACE-inhibiting hydrolysate from tuna cooking juice could persistently lower, but did not normalize, the SBP in SHR. This finding is similar to that obtained in other studies on SHR treated with several other ACE-inhibiting hydrolysates[21,22]. In the present study, tuna cooking juice did not lower the blood pressure in SHR at all. However, protelyotic hydrolysis by orientase resulted in a boost of anti-hypertensive effect. OAH exhibited a more potent anti-hypertensive effect (1 g/kg dose lowered SBP by 30 ± 2.7 mmHg after 2hr) than the royal protein hydrolysate (1g/kg dose lowered SBP by 22 ± 3.6 mmHg after 2hr) or α-zein hydrolysate (2 g/kg dose lowered SBP by 25 mmHg after 3 hr) did[23,24]. Hydrolysed proteins in tuna cooking juice might thus server as a good source of inhibitors of ACE for regulating blood pressure.

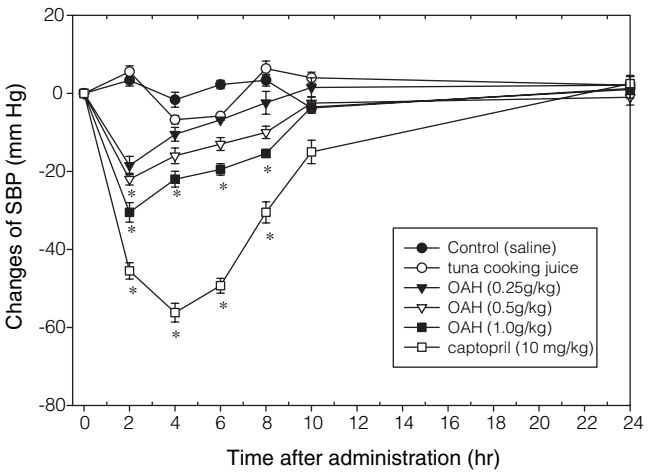

Figure 1. Changes in SBP after single oral administration of tuna cooking juice hydrolysate or Captopril (10 mg/kg) to SHR. *Outcomes were significantly different from those of controls; $p < 0.05$.

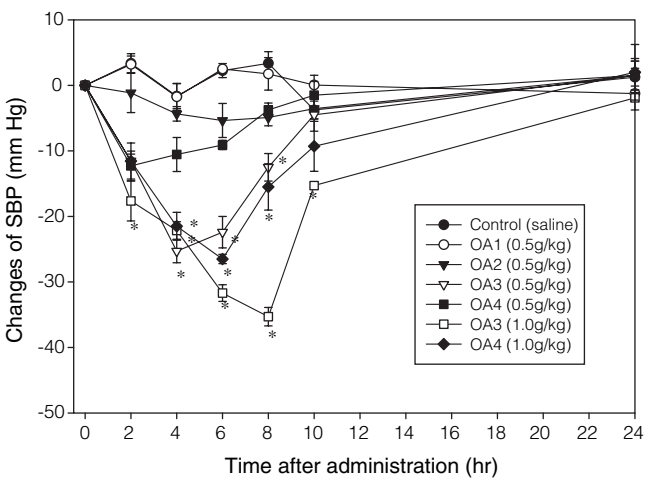

Figure 2. Changes in SBP after single oral administration of tuna cooking juice hydrolysate fractionated by gel filtration to SHR. *Outcomes were significantly different from those of controls; $p < 0.05$.

In order to compare the anti-hypertensive activity of OAH with commercial drugs, Captopril was used as a reference drug for the reducing the SBP on SHR. As shown in Figure 1, SBP significantly dropped in the period of 2 to 8 hr after Captopril (10 mg/kg body weight) was orally administered. Captopril was clearly shown to affect the hypertension rapidly and for a prolonged period. Obviously, OAH did not lower blood pressure as readily as Captopril. Nevertheless, Captopril does not negate the application of cooking juice hydrolysates in preventing hypertension. OAH, unlike Captopril, is expected to have no undesirable side effects.

III. *Anti-hypertensive Activity of Fractions from OAH in SHR*

Gel filtration was used to fractionate the OAH. The changes in SBP for 24 hr after the administration of various fractions of OAH to SHR were shown in Figure 2. Of these fractions, OA3 inhibited ACE most remarkably reducing the blood pressure by 25 ± 2.7 mmHg when orally administered 0.5 g/kg body weight. Reduction effect lasted for 8 hr, though the reduced SBP gradually returned to the initial level. The next strongest inhibitor was the OA4 fraction, which reduced blood pressure by 12 ± 2.2 mmHg in SHR 2 hr after orally administered 0.5 g/kg body weight. In contrast, the SBP of rats given 0.5 g/kg body weight of OA2 fraction reduced only slightly; it did not change significantly over the 24 hr period of observation. The rats administered the OA1 fraction were similar to the control group. Higher doses of OA3 and OA4 fractions (1.0 g/kg) reduced SBP by 35 mmHg and 26 mmHg, respectively as compared to the control. The reduction effect from OA4 held only for 10 hr; on the other hand, that from OA3 lasted for more than 10 hr. The results indicate that OA3 and OA4 fractions had stronger and lasting effects on the blood pressure of SHR. From our previous study[14], OA3 and OA4 with MW ranging from 0.204 to 1.06 kDa, exhibited ACE-inhibiting effect with IC_{50} value of 0.21 mg and 0.52 mg protein/mL, respectively. On the contrary, OA1 (MW > 2573)

and OA2 (926 > MW > 565) had no effect on ACE. Inhibition of ACE activity shall results the lowering of blood pressure. Fractioned OAH exerted an anti-hypertensive effect by inhibiting the ACE activity. Moreover, fragments generated herein were composed of peptides from 0.204 to 1.06 kDa, which were responsible for lowering the blood pressure. This finding was consistent with that of another report on anti-hypertensive peptides with small MW (usually 2 to 14 amino acids residues)[25].

IV. *Effect of Long Term Feeding on Hypertension*

The long term effects during eight weeks of treatment on a diet that including OAH were illustrated in Figure 3. The standard commercial diets were minced with 0.25%, 1.25% or 2.5% (w/w) OAH. The SHR were first fed the hydrolysate-containing diet at age of 9 weeks. The SBP in all the control rats gradually elevated in the course of the experiment, reaching about 216 ± 3.2 mmHg at age of 17 weeks. At age of 12 weeks, the SBP of rats fed a OAH-containing diet tended to be lower, in a dose-dependent manner, than that of the control group. When the rats were 15 weeks old, SBP was significantly lower in the 2.50% (w/w) OAH group than that in the control group. At the eighth week, the blood pressures were, respectively, 3.2% and 6.5 % lower in the groups to which the OAH 1.25 and 2.5% had been administered than those in the control group. The level of SBP typically dropped from the third week of the experiment in OAH-fed rats rather than the controls. The increases in blood pressures were reduced in a dose-dependent manner from the sixth to eighth weeks of the experiment. Moreover, SBP of SHR fed diets containing 2.5, 1.25 and 0.25% OAH dropped, then increased to and stayed at 200, 205 and 215 mmHg, respectively. These findings imply that OAH participates in suppressing or delaying the development of hypertension.

However, the SBP value were higher in the OAH groups than in the normotensive rats reported in earlier studies, 200 mmHg at 17 weeks old in the group at the highest dose administered herein versus 150 mmHg in the normotensive rats, according to Rizzoni *et al.*[26]. This finding indicates that OAH can moderately suppress the increase of blood pressure though the SBP in SHR will continue to increase until 20 weeks old. This characteristic of OAH is potentially important in its use as a physiologically functional food since excessive reduction in blood pressure has been shown to induce myocardial infarction and to affect survival rate[27-29]. These results indicate that OAH may be useful in regulating blood pressure.

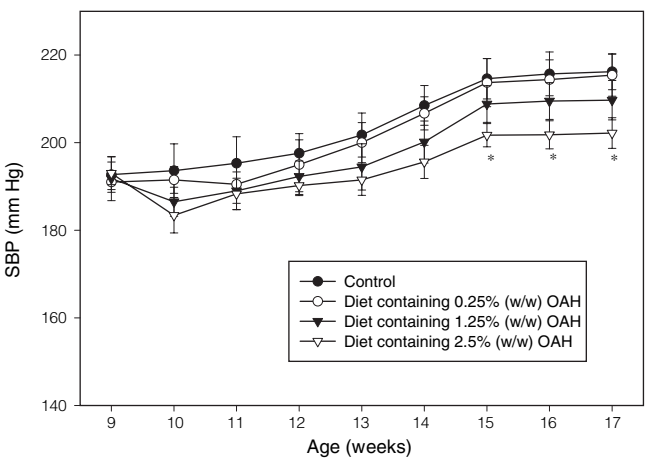

Figure 3. Changes in SBP of SHR fed on a diet that includes tuna cooking juice hydrolysate. *Outcomes were significantly different from those of controls; $p < 0.05$.

CONCLUSIONS

In conclusion, hydrolysate and its fractions obtained by hydrolyzing the tuna cooking juice have been shown inhibit ACE action in vitro and to exert anti-hypertensive effect by oral administration. They actually prevent the development of hypertension in SHR. These results suggest that the increase in blood pressure can be partially inhibited by taking hydrolysate derived from the tuna cooking juice. Finally, such hydrolysates containing oligopeptides are expected to be usable in food to control the blood pressure in patients with essential hypertension. However, further studies should be undertaken to examine the amino acid sequences, active mechanisms, and the side effects of using hydrolysates.

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