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# Antihypertensive effect of angiotensin-converting enzyme inhibitory peptides from silver carp (*Hypophthalmichthys molitrix*) hydrolysate in spontaneously hypertensive rats

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**Abstract**

In order to utilize silver carp (*Hypophthalmichthys molitrix*), which is abundant and very cheap fresh-water fish in China, defatted silver carp muscle was hydrolysed by compound protease to achieve angiotensin-converting enzyme (ACE) inhibitory peptides. The protein hydrolysates were fractionated into low and high molecular weight fractions using an ultrafiltration (UF) membrane system. The ACE-inhibitory activity tests showed that the low molecular weight fraction was more effective. Most of the peptides in low molecular weight fraction ranged from 600 to 1600 Da. Anti-hypertensive effects of silver carp ACE-inhibitory peptides on spontaneously hypertensive rats (SHR) following oral administration was determined as the tail systolic blood pressure significantly decreased after peptides ingestion.

***Key words:*** Silver carp, *Hypophthalmichthys molitrix*, ACE-inhibitory peptide, antihypertensive effect.

## Introduction

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is one of the main regulators of blood pressure through its action on two body systems, renin-angiotensin system and kinin-kalicrein system. By inhibiting this enzyme, bioactive peptides have been shown to lower blood pressure in animal and clinical studies. Milk from different species is reported to be the main source of ACE-inhibitory peptides [1-4]. Other animal protein sources of these peptides are muscle [5], ovalbumin [6], blood [7], and fish proteins[8]. Plant protein sources include, among others, pea [9], garlic [10], rice [11], soybean [12], wheat [13], and Amaranth proteins [14]. However, some studies indicate that ACE-inhibitory peptides derived from sardine, crab and other aquatic animals have stronger ACE inhibitory activities than those from soybean or casein [15].

Silver carp (*Hypophthalmichthys molitrix*) is reported to be one of the highest annual harvest fresh-water fish in China, with annual harvests of 3.5 million metric tons in 2005 [16], due to its fast growth rate, easy cultivation, high feed efficiency ratio as well as high nutritional value [17]. However, silver carp is not used for direct cooking normally because of its strong earthy or musty taste and odour. Only small part are used for process of fish gruel, pellet or sausages, because of its poor gel-forming ability [18-20]. Furthermore, silver carp contains many intramuscular small bones. Therefore, the consumption of silver carp has been limited and the price of this fish is low. Converting this unexploited resource to a high value healthy food would go a long way to feeding an increasing population. Recently, antioxidative peptides from silver carp enzymatic hydrolysate was reported [21, 22]. However, little information about ACE-inhibitory peptides from silver carp is available.

In a previous study, we optimized the enzymatic hydrolysis preparation of ACE-inhibitory peptides from silver carp. In the present study, the objective was to investigate antihypertensive action of the ACE-inhibitory peptides by oral administration in spontaneously hypertensive rats (SHR).

## Materials and Methods

***Materials:*** ACE (EC 3.4.15.1) from rabbit lung, hippuryl-histidyl-leucine (HHL) as a substrate peptide of ACE, was purchased from Sigma Chemical Co. (St. Louis, MO). Spontaneously hypertensive rats (SHR) were provided by Shanghai Hypertensive Institute, China. The compound protease (120 U.mg$^{-1}$) was purchased from Shanghai Chemical Reagent Co. Ltd, China. All other reagents used in this study were analytical grade chemicals.

Male spontaneously hypertensive rats (SHR) weighing 250 to 300 g were 14 to 15 weeks old. Before the experiment, the animals were kept in a temperature-controlled room with a light/dark (14 and 10 h) cycle for 1 month.

***Preparation of ACE-inhibitory peptides from silver carp:*** The fish were split, gutted, washed with tap water, and filleted. The fillets were defatted with petroleum ether at 50ºC by reflux extraction. Then 50 g fillets were cut into slices, and 125 ml distilled water was added. The mixture was homogenated and boiled for 10 min to inactive the inner protease. The silver carp protein was digested by compound protease with 25 U.ml$^{-1}$ at 60ºC for 2 h. The pH of the reaction mixture was maintained at pH 7.0 by addition of either 1 M NaOH or HCl. The reaction was stopped by heating in a boiling water bath for 5 min to inactivate the enzyme. The hydrolysate was centrifuged (4,000×*g*, 4ºC, 10 min) to obtain the supernatant. The hydrolysate was fractionated into two ranges of molecular weight (>3 kDa, <3 kDa) using an ultrafiltration (UF)

membrane system. The low molecular weight fraction was chosen to test its antihypertensive activities.

***Measurement of ACE-inhibitory activity:*** ACE-inhibitory activity was assayed according to the method of Lieberman, with some modifications [23]. In short, hippuric acid released from hippuryl-L-histidyl-L-leucine by ACE was extracted with ethyl acetate, and the concentration was measured by HPLC. The ACE inhibitory activity of each substance was measured at the concentration of 0.5 mg.ml$^{-1}$.

***Molecular weight distribution of the silver carp peptides:*** High performance gel filtration chromatography was performed with a Shimadzu LC-10A system (Shimadzu Co., Kyoto, Japan) under the following conditions: column, Bio-Sil SEC 25025 (7.8 mm × 30 cm); mobile phase, 0.15 M NaCl-containing 0.05 M phosphate buffer (pH 6.8); flow rate, 0.6 ml.min$^{-1}$; column oven temperature, 25ºC; ultra violet detection at 220 nm. The molecular weight (M.W.) distribution was determined from the calibration curve plotted from the correlation between peptide and the MW-protein markers (albumin, M.W. 44,000; myoglobin, M.W. 17,000; cytochrome C, 12,500; vitamin $B_{12}$, M.W. 1350) and the elution time.

***Antihypertensive activities of silver carp peptides in SHR:*** The SHR were randomly divided into two groups: the control group and the ACE-inhibitory peptide group. For the oral administration, 7 rats were used in each group. The substances were administered at a volume of 3.0 g.kg$^{-1}$. Tail systolic blood pressure was measured at 2-h intervals (0, 2, 4, 6, 8 and 10 h) after the administration.

***Statistical analysis:*** Values are the means ± standard deviation (SD). Paired-*t* test and one-way analysis of variance followed by Dunnett's multiple comparison test were used to analyze the hypertensive effects by intravenous and oral administration, respectively. Values of $p<0.05$ were considered to indicate statistical significance.

## Results and Discussion

***The ACE-inhibitory activities and molecular weight distribution:*** Silver carp protein was hydrolyzed with compound protease. The hydrolysate obtained was fractionated by ultrafiltration into Fraction-I (>3 kDa) and Fraction-II (<3 kDa). The ACE-inhibitory activities of the two fractions were 39.02% and 88.08%, respectively. It obviously varied with the molecular weight distribution, and the Fraction-II with M.W. below 3 kDa showed the most potent ACE inhibitory activity. Most of reported ACE-inhibitory peptides belonged to low molecular weight peptides. Such as Jung [24] revealed that peptides from yellowfin frame protein were fractionated into two fractions of high and low molecular weight by ultrafiltration. The low molecular weight fraction had more potent ACE-inhibitory activity than that of the high molecular weight. Matsui *et al.* [25] also reported that the ACE-inhibitory activity markedly increased with increasing proteolysis by alkaline protease hydrolysate of sardine muscle. We also determined the molecular weight distribution of the low molecular weight fraction of silver carp hydrolysate, shown in Fig. 1. Most of the M.W. in Fraction-II was focused on 600 to 1600 Da.

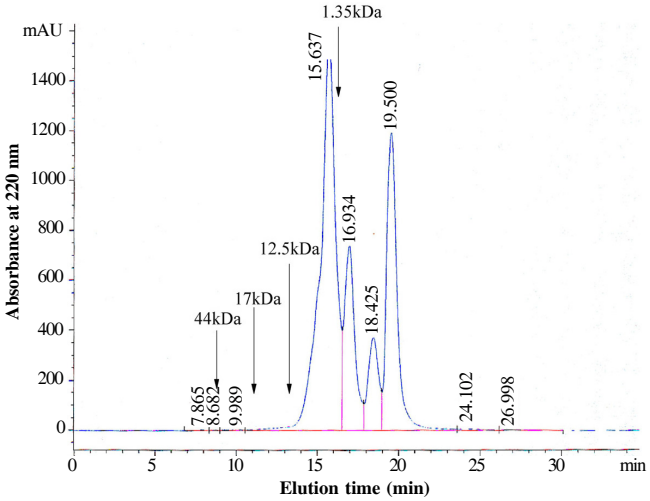


***Figure 1.*** The molecular weight distribution of the low M.W. fraction of silver carp hydrolysate.

***Antihypertensive activities of silver carp peptides in SHR:*** Antihypertensive activity of the silver carp peptides was evaluated by measuring the change of tail systolic blood pressure (SBP) at 2, 4, 6, 8 and 10 h after oral administration of 3.0 g.kg$^{-1}$ of body weight. There was no change in SBP in the control group over the investigation period of 10 h. As shown in Fig. 2 and Table 1, a significantly recorded SBP reduction of 18, 26.7 and 28.7 mmHg ($p < 0.05$) at 2, 4 and 6 h after administration were observed, and the activity was maintained for 4 h, but 8 h after administration, the SBP was increased and was not significantly different compared to control. Recently, many ACE-inhibitory peptides were isolated from food proteins and their antihypertensive effect tested in SHR. Fujita and Yoshikawa [26] reported that the antihypertensive effect showed maximal effect at 6 h after oral administration of a prodrug type ACE inhibitory peptide in SHR. Because of some synthetic antihypertensive drugs are known to produce side effects such as an abnormal elevation of the blood pressure after administration, the result of this study suggests that ACE inhibitory peptide derived from silver carp protein could be utilized to develop nutraceuticals and pharmaceuticals. In addition, it is expected that

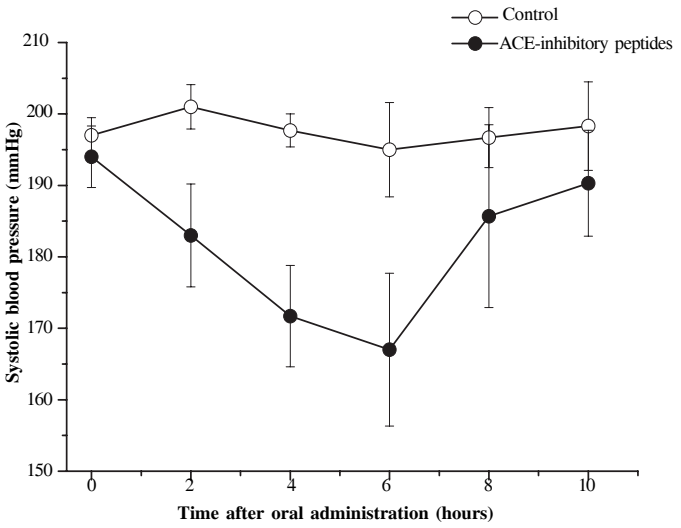


***Figure 2.*** Antihypertensive activities of silver carp peptides in spontaneously hypertensive rats (SHR). Values are the means ± standard deviation (SD).

**Table 1.** Antihypertensive effect of the silver carp ACE-inhibitory peptides.

| Hours after administration | Means difference in sample and control | Standard Deviation | Significant level |
|---|---|---|---|
| 2 | -18.0 | 7.26 | 0.030[a] |
| 4 | -26.7 | 7.06 | 0.004[a] |
| 6 | -28.7 | 0.78 | 0.022[a] |
| 8 | -11.2 | 12.89 | 0.305 |
| 10 | -8.6 | 7.44 | 0.215 |

[a] significant at $p < 0.05$. The oral administration, 7 SHR was used in each group and the substances were administered of 3.0 g.kg$^{-1}$ body weight. Tail systolic blood pressure (SBP) was measured at 2-h intervals (0, 2, 4, 6, 8 and 10 h) after the administration. The difference of SBP in sample and control group was statistical analyzed.

this will contribute developing interest in basic research and potential applications of bioactive peptides.

## Conclusions

The present results show that the compound protease hydrolysate of silver carp protein possess ACE-inhibitory activity and low molecular weight fraction (<3 kDa) of the hydrolysate had higher activity. The molecular weight distribution of the <3 kDa fraction showed that most of them were ranged from 600 to 1600 Da. Antihypertensive effect in spontaneously hypertensive rats also revealed that oral administration of the ACE-inhibitory peptides from silver carp can decrease systolic blood pressure significantly ($p < 0.05$). We suggested that the ACE-inhibitory peptides derived from silver carp protein could be utilized to develop nutraceuticals and pharmaceuticals.


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