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Angiotensin I-converting enzyme inhibitory activity of shrimp meat hydrolysate by endogenous enzymes from shrimp head and its antihypertensive effects on spontaneously hypertensive rats

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Abstract: Hydolysate of shrimp meat hydrolyzed by the endogenous enzymes of the head of *Penaeus vannamei*, with angiotensin I-converting enzyme (ACE) inhibitory activity of 41.9% (2 mg · mL^{-1}), was prepared under the conditions of pH 7.35, temperature 57.2 ℃, hydrolysis time 4 hours, shrimp head/shrimp meat 1∶1, and substrate concentration 20%. The hydrolysate had a broad molecular weight distribution (between 7.4×10^4 and 29.7). Fractions with molecular weight under 2.7×10^3 covered 83.1% of the total hydrolysate. Fractions with molecular weight between 959-338 exerted an inhibitory activity of IC_{50} 0.32 mg · mL^{-1}. Phenylalanine, hydrophobic (Leucine, Isoleucine and Valine) and aromatic(Proline, Tyrosine and Tryptophan) amino acids covered 42.33% of the total amino acids of Peak C. Results of the experiments *in vivo* showed that the hydrolysate exerted a significant antihypertensive activity in spontaneously hypertensive rats after oral administration.

Key words: *Penaeus vannamei*; endogenous enzymes; angiotensin I-converting enzyme inhibitory activity; antihypertensive effects
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1 Introduction

Hypertension was identified as a cardiovascular risk factor in the late 1950s and still remains a public health issue[1]. It is one of the most common lifestyle-related diseases, and has become a significant problem in recent years. The renin-angiotensin system plays an important role in the regulation of blood pressure[2]. Angiotensin I-converting enzyme (EC 3.4.15.1; ACE) in the the renin-angiotensin system plays an important physiological role in regulating blood pressure, which participates in the regulation of blood pressure by virtue of two different reactions, i.e. conversion of inactive peptide angiotensin I into powerful vasoconstrictor angiotensin II and inactivation of vasodilator peptide bradykinin[3]. Therefore, it is capable of suppressing the elevation of blood pressure by inhibiting the catalytic action of ACE.

Some synthetic inhibitors of ACE, such as Captopril and Enalapril, have been proved to be useful as antihypertensive drugs. However some undesirable side effects may occur such as cough, loss of taste, renal impairment and angioneurotic oedema[4]. It was found that some ACE inhibitory peptides could be produced by enzymatic digestion of various food proteins. These peptides are less potent than synthetic ones, but they do not exhibit side

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effects. Daily use of food that contains some peptides with potent ACE-inhibitory activity could be effective for maintaining blood pressure at a healthy level. The objective of this study was to prepare hydrolysate with ACE inhibitory activity by utilizing the endogenous enzymes of the head of *Penaeus vannamei* to hydrolyze its own meat. Moreover, we also determined the molecular weight distribution of the hydrolysate and assessed the antihypertensive action of the hydrolysate by oral administration to spontaneously hypertensive rats.

2 Materials & methods

2.1 Materials

Fresh shrimp meat and shrimp head of *Penaeus vannamei* used as raw materials were obtained from Zhanjiang Guolian Fisheries Ltd., Guangdong Province, China. Clean sanitized containers were provided to pickers for the package of shrimp meat and shrimp head, which was on ice and immediately transported to the laboratory.

Crude ACE was prepared with pig lung in the laboratory using the method of Hayakari[5]. Its substrate peptide (Hippuryl-Histidyl- Leucine) was purchased from Peptide Co., Japan. Standard peptides, Bovine serum albumin (MW = 67 000 U), Aprotinin (MW = 6 500 U), Bacitracin (MW = 1 450 U) and L-Arginine (MW = 174.2 U) were purchased from Sino-America Biotechnology Company, Beijing.

2.2 Preparation of hydrolysate with ACE inhibitory activity

Fresh shrimp head and meat were mixed (1∶1) (w∶w) and then ground in a blender with distilled water added. The homogenized shrimp was poured into a reaction vessel. The pH was adjusted to 7.35 and the final shrimp concentration was adjusted to 20% (W/V). The system was incubated in a water bath of 57.2 ℃. The mixture was vigorously stirred during reaction using a magnetic stirrer. The reaction was terminated after a specified time and the reaction system was heated at 100 ℃ in a water bath for 10 min to inactivate the enzymes. The resulting solution was centrifuged at 1.8×10^3 g for 10 min and the supernatant was collected for further analysis.

2.3 Determination of ACE inhibitory activity *in vitro*

The ACE inhibitory activity was measured by the method of Cushman[6]. The inhibitory activity was calculated using the following equation:

$$\text{ACE inhibitory activity}(\%) = \frac{A-B}{A-C} \times 100$$

Where A is the absorbance of a solution containing ACE at 228 nm, but without enzymatic hydrolysate sample and ACE substrate solution, B is the absorbance of a mixture solution with ACE, ACE substrate solution and sample at 228 nm, and C the absorbance of the ACE substrate solution without ACE and sample at 228 nm. The ACE inhibitory activity was the mean value of three times. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

2.4 Estimation of degree of hydrolysis

The degree of hydrolysis (DH) was determined using the method described by Faswal[7] that was defined as followed:

$$DH(\%) = \frac{\text{Number of peptide bonds cleved by enzyme}}{\text{Number of peptdie bonds in the sample}} \times 100 = \frac{D'-D_0}{D_{max}-D_0} \times 100$$

Where D_{max} is the total amount of amino acid nitrogen of the sample, D' is the amount of amino acid nitrogen in the hydrolysate, and D_0 the amount of free amino acid nitrogen in the sample.

2.5 Molecular weight distribution of the hydrolysate

The hydrolysate was prepared from shrimp protein according to Materials & methods 2.2. Molecular weight distribution of the hydrolysate was

determined by column gel chromatogram. In the first, the standard peptides (2.5 mg · mL^{-1}) were separated on a column (1.6 cm × 68 cm) packed with Sephadex G-25 using distilled water for elution at a flow rate of 0.4 mL · min^{-1}. A regressive equation between the elution volume and the logarithm of the molecular weight of the standard peptides was obtained. Hydrolysate was separated under the same condition. The fractions were pooled according to the absorbance at 280 nm and lyophilized immediately. Their ACE inhibitory activities and their amino acid profiles of the lyophilizate were determined.

2.6 Amino acid profiles [8]

The amino acid profiles for both essential and non-essential amino acids were estimated by using a high speed amino acid analyzer (L-8500 A, HITACHI). In a test tube 0.1 g sample was taken and 10 mL of 6 mol · L^{-1} HCl were added in a test tube. The test tube was evacuated with nitrogen, sealed and placed in the oven at 110 ℃ for 22 h, then cooled at room temperature. The formed hydrolysate was evaporated under vacuum at 60 ℃ to dryness to remove HCl. The hydrolysate was then dissolved in 5 mL 0.02 mol · L^{-1} HCl, centrifuged at 1 000 r · min^{-1} and filtered to remove the visible sediments. A known volume (20 μL) of the supernatant was injected into an amino acid analyzer to estimate the amino acid profile of each sample. For determination of Tryptophan, the sample (weight equivalent to about 2 mg Trp) was treated with 4.2 mol · L^{-1} NaOH (100 mL) and 0.3 mL triglycerin and placed in the oven at 110 ℃ for 24 h. 7 mL 6 mol · L^{-1} HCl was then added to the mixture and the pH was adjusted to 4.5 using pH 4.2 citric acid buffer solution. Tryptophan content was determined by colorimetric analysis at 400 nm under the condition of pH 5.0 – 5.5, columniation temperature 55 ℃, reactor temperature 100 ℃, and reaction time 10 – 15 min.

2.7 Animals and measurement of blood pressure [9]

25 spontaneously hypertensive rats (SHR, 18-week-old, male, 300 – 330 g BW) with tail systolic blood pressure (SBP) over 150 mmHg were obtained from Beijing Vitalriver Laboratory Animal Ltd. Co. The animals were housed at a constant temperature of 22 – 24 ℃ under a 12 h/12 h light-dark cycle with free access to feed and tap water. They were divided into trial groups (three groups), drug group (Captopril) and control group, 5 rats each group. The hydrolysate was filtered by an ultrafilter with a cut-off molecular weight of 6 000 U and then lyophilized. The lyophilized hydrolysate of shrimp was dissolved in 0.9% saline and injected orally at a dosage of 1.5 g · kg^{-1}, 1.0 g · kg^{-1} and 0.5 g · kg^{-1} body weight (BW) using a metal gastric zoned in trial SHR. Control rats were administrated with the same volume of saline. The lowering efficacy of hydrolysate on systolic blood pressure (SBP) was compared with that of 0.9% saline as control. Drug group were injected at a dose of 2.5 mg · kg^{-1} BW. 5 Wistar rats with normal blood pressure were also injected orally the hydrolysate dissolved in 0.9% saline at the same dose as the trial ones. Following oral administration of sample, SBP was measured by tail-cuff method with a blood pressure system (PF5001, Sweden) after warming up SHR in a chamber maintained at 40 ℃ for 10 min.

3 Results and discussion

3.1 Hydrolysate from shrimp protein with ACE inhibitory properties

The naturally occurring peptides with ACE inhibitory activity were first obtained from snake venom. Afterward, many other ACE inhibitory peptides had been discovered from enzymatic hydrolysate of different food proteins. Most of them were derived from food proteins by enzymatic hydrolysis. However, the use of commercial enzymes increased the cost. In the present study we found the hydrolysate prepared from shrimp protein by its endogenous enzymes also exerted inhibitory activity for ACE.

Fig. 1 showed the ACE inhibitory activity of

hydrolysate during hydrolysis under the conditiosn of pH 7.35, temperature 57.2 ℃, shrimp head/shrimp meat 1 ∶ 1 (W/W) and substrate concentration 20% (W/V)[10]. 0 – 4 h incubation showed an increasing ACE inhibitory activity, but it came to be degressive after 4 h incubation. There are many possible explanations for the progress curve obtained. In enzymatic hydrolysis of shrimp head and meat, downward curvature of the progress may be explained by the combination of these factors since shrimp head and meat contain both soluble and insoluble proteins. As stated before, many peptides exhibit ACE inhibitory activity. The combination of all these peptides determines the ACE inhibitory activity of the hydrolysate. During hydrolysis, ACE inhibitory peptides are continuously formed and degraded again. Maximum ACE inhibition by the hydrolysate is a result of an optimum between these two processes. The homogenized shrimp mixture also showed 25.9% ACE inhibitory activity. It was mainly due to the peptides existing naturally in its body. The ACE inhibitory activity increased in the first 4 hours. 4 h incubation resulted in a higher inhibition activity of 41.9% compared to those of other incubation times. The DH was 61.9% and peptide concentration was 24.9 mg · mL^{-1}.

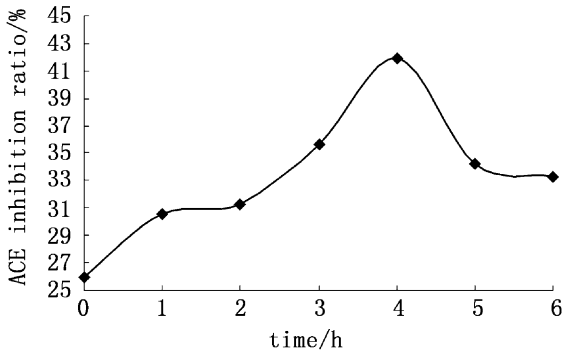

Fig. 1　ACE inhibitory activity during hydrolysis (2 mg · mL^{-1})

3.2　Molecular weight distribution of hydrolysate and its ACE inhibitory activity

Fig. 2 was the Sephadex G-25 gel column chromatogram of four standard peptides and the hydrolysate. A regressive equation (Equation 1) between the elution volume (Y) and the logarithm of the molecular weight of the standard peptides (X) was obtained (Fig. 2-a).

Equation 1: $Y = -31.799X + 214.83$ ($R^2 = 0.9877$)

Where Y is the elution volume, X is the logarithm of the molecular weight of the standard peptides.

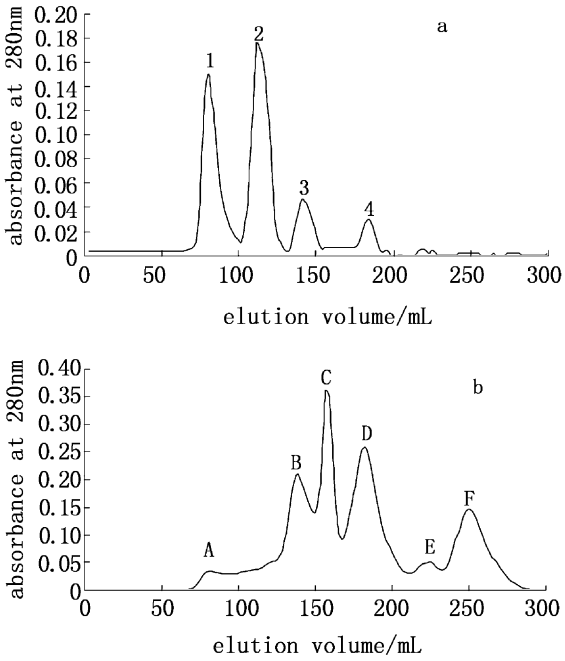

Fig. 2　Sephadex G-25 gel column chromatogram of standard peptides (a) and hydrolysate (b)

1. bovine serum albumin (MW = 67 000 U); 2. aprotinin (MW = 6 500 U); 3. bacitracin (MW = 1 450 U); 4. L-arginine (MW = 174.2 U)

Gel filtration of the hydrolysate on Sephadex G-25 chromatogram yielded six absorbance peaks (Fig. 2-b). They were collected and determined for the ACE inhibitory activity. The molecular weights were calculated with Equation 1. As shown in Tab. 1, it showed that the hydrolysate had a broad molecular weight distribution (between 7.4×10^4 and 29.7). The gel filtration of the hydrolysate on Sephadex G-25 showed that it has a low molecular

weight. Fractions of Peak B, C and D with molecular weight under 2.7×10^3 covered 83.1% of the total hydrolysate. The molecular weight of Peak E and Peak F was much lower, it may be some other organic substances. Peak A, B, C and D exerted ACE inhibitory properties. Peak C with molecular weight between 959 – 338 exerted the most potent inhibitory activity. Its IC_{50} was 0.32 mg · mL^{-1}. Oshima[11] suggested that ACE inhibitors derived from food proteins were usually small peptides with molecular weight less than 1 500. Most of the ACE inhibitory peptides were short peptides with low molecular weight.

Tab.1 Distribution of MW, containing ratio and IC_{50} of the hydrolysate

peak number	molecular weight	percentage (%)	IC_{50} (mg · mL^{-1})
A	$7.4 \times 10^4 - 2.6 \times 10^4$	3.9	5.17
B	$2.7 \times 10^3 - 1.1 \times 10^3$	19.1	2.11
C	959 – 338	26.5	0.32
D	284 – 30	37.5	2.04

3.3 Amino acid profiles of the collected fractions

ACE appears to prefer substrate or competitive inhibitors containing hydrophobic (aromatic or branched-side chains) amino acid residues at each of the three C-terminal positions. Many studies have shown that tripeptides or dipeptides with high potent inhibitory activity have Tryptophan, Phenylalanine, Tyrosine, or Proline at their C-terminal, and branched aliphatic amino acid at the N-terminal [12].

The amino acid composition of Peak B, Peak C and Peak D were showed in Tab. 2. The percentage of Phenylalanine, hydrophobic (leucine, isoleucine and valine) and aromatic (proline, tyrosine and tryptophan) amino acids was 32.49, 42.33, 30.14 respectively. It indicated that peptides containing these amino acids in the fractions exerted a potent ACE inhibitory activity.

3.4 Antihypertensive activity of hydro-lysate in SHR

Susceptibility to absorption as well as resistance to digestion by gastrointestinal proteases is essential for the antihypertensive effect of ACE inhibitory peptides *in vivo*. Although a number of ACE inhibitory peptides have been isolated from various food proteins, some peptides with potent ACE inhibitory activity *in vitro* or intravenously are inactive orally. For the practical purpose of utilizing food materials as a physiological modulator, it is necessary to confirm the antihypertensive effect of the orally administrated hydrolysate on SHR[13]. The blood pressure of SHR was measured at 0, 2, 4, 6, 8 and 24 h after oral administration of the hydrolysate. As shown in Fig. 3, the administration of the hydrlysate at a dose of 1.5 g · kg^{-1} BW significantly lowered the blood pressure of SHR, a recorded SBP reduction of 22.5 mmHg ($P < 0.01$) at 4 h and 15 mmHg ($P < 0.01$) at 6 h after administration was observed. A recorded SBP reduction of 13 mmHg ($P < 0.01$) at 4 h and 18 mmHg ($P < 0.01$) at 6 h after administration at a dose of 1.0 g · kg^{-1} BW was observed. While 10 mmHg ($P < 0.05$) and 15 mmHg ($P < 0.01$) for a dose of 0.5 g · kg^{-1} BW. The hypotensive effect for all doses continued for at least 4 h, whereas no decrease in blood pressure was observed in control SHR fed no shrimp hydrolysate.

Tab.2 Amino acids composition of peaks of Sephadex gel filtration chromatogram

mg · mL^{-1}

amino acid	B	C	D
asparagine	0.643	0.240	0.367
threonine	1.12	0.355	0.203
serine	0.449	0.114	0.143
glutamic acid	0.834	0.234	0.355
proline	1.93	<0.08	<0.08
glycine	2.93	1.11	0.414
alanine	3.79	0.655	0.245
cystine	0.17	0.17	0.05
valine	1.66	0.437	0.334
methionine	0.84	0.502	0.280
isoleucine	1.28	0.315	0.209
leucine	2.24	0.414	0.191
tyrosine	0.241	0.483	0.124
phenylalanine	0.318	1.05	0.181
lysine	3.00	0.124	0.174
histidine	0.363	<0.04	<0.05
arginine	1.80	0.173	0.177
tryptophan	<0.002	<0.002	<0.002

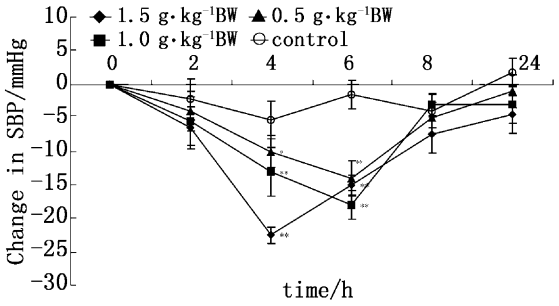

Fig. 3　Antihypertensive activity after oral administration in SHR

Changes of SBP from time zero were expressed with mean ± SEM. *, ** indicate significant differences against control (*, $P < 0.05$; **, $P < 0.01$).

The administration of Captoril, a synthetic ACE inhibitor, also caused a great decrease in blood pressure, 8 mmHg ($P < 0.05$), 13 mmHg ($P < 0.01$), 10 mmHg ($P < 0.05$) at 2 h, 4 h, 6 h after administration, respectively (Fig. 4). However, it did not exert any hypotensive effect in Wistar rats with normal blood pressure at the same dose as the group at a dose of 1.0 g · kg^{-1} BW. (Fig. 4).

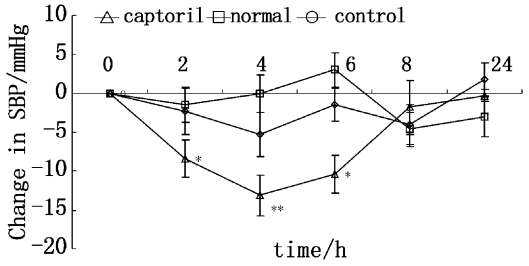

Fig. 4　Changes in SBP levels in SHR and Wistar rats after administering samples.

Changes of SBP from time zero were expressed with mean ± SEM. *, ** indicate significant differences against control. (*, $P < 0.05$; **, $P < 0.01$)

Results of the present study confirmed that the hydrolysate of *Penaeus vannamei* showed antihypertensive activities in SHR after oral administration.

4　Conclusion

The hydrolysate of shrimp meat hydrolyzed by endogenous enzymes of the head of *Penaeus vannamei* exerted a strong activity for ACE. The conditions for preparation the hydrolysate were pH 7.35, temperature 57.2 ℃, hydrolysis time 4 hours, shrimp head/shrimp meat 1 : 1, and substrate concentration 20%, under which the hydrolysate with ACE inhibitory activity being 41.9% (2 mg · mL^{-1}) was obtained. The hydrolysate had a broad molecular weight distribution (between 7.4×10^4 and 29.7). Fractions with molecular weight under 2.7×10^3 covered 83.1% of the total hydrolysate. Fractions of Peak C with molecular weight between 959 – 338 exerted an inhibition activity of 0.32 mg · mL^{-1} IC_{50}. Phenylalanine, hydrophobic (leucine, isoleucine and valine) and aromatic (proline, tyrosine and tryptophan) amino acids covered 42.33% of the total amino acids of the most active peak. It showed a clear antihypertensive effect in spontaneously hypertensive rats. The administration of the hydrlysate at a dose of 1.5 g · kg^{-1}) BW significantly lowered the blood pressure of SHR, a recorded SBP reduction of 22.5 mmHg ($P < 0.01$) at 4 h after administration was observed, and 15 mmHg ($P < 0.01$) at 6 h. A recorded SBP reduction of 13 mmHg ($P < 0.01$) at 4 h and 18 mmHg ($P < 0.01$) at 6 h after administration at a dose of 1.0 g · kg^{-1} BW was observed. While 10 mmHg ($P < 0.05$) and 15 mmHg ($P < 0.01$) for a dose of 0.5 g · kg^{-1}BW. The hypotensive effect for all doses continued for at least 4 h, whereas no decrease in blood pressure was observed in control SHR fed no shrimp hydrolysate.

The present study indicated that hydrolysate of shrimp meat hydrolyzed by endogenous enzymes of the head of *Penaeus vannamei* could serve as a protein source of antihypertensive peptides. Further work is needed to be carried out to isolate and characterize the antihypertensive peptides from the hydrolysate. The result of this study suggests that ACE inhibitory hydrolysate derived from *Penaeus vannamei* protein could be utilized to develop nutraceuticals and pharmaceuticals.

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虾头内源酶酶解虾肉产物的ACE抑制活性及其降低原发性高血压老鼠血压的效果

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摘要：在pH 7.35、温度57.2 ℃、水解时间4 h、虾肉/虾头为1∶1，底物浓度为20%（W/V）的条件下，利用虾头的内源酶酶解虾肉（南美白对虾），制备了具有血管紧张素转化酶（ACE）抑制活性的酶解产物，其对ACE的抑制率为41.9%（2 mg·mL^{-1}）。通过凝胶过滤层析测定出酶解产物的分子量分布较广，在7.4×10^4至29.7之间。分子量在2.7×10^3以下的凝胶层析组分占酶解产物氨基酸总量的83.1%。收集的高活性组分分子量在959～338之间，其IC_{50}为0.32 mg·mL^{-1}苯丙氨酸、疏水氨基酸（亮氨酸、异亮氨酸和缬氨酸）和芳香族氨基酸（脯氨酸、酪氨酸和色氨酸）占氨基酸总量的42.33%。体内试验的结果显示，南美白对虾的虾肉虾头内源酶酶解产物经灌喂原发性高血压老鼠后显示较强的降血压活性。

关键词：南美白对虾；内源酶；血管紧张素转化酶抑制活性；抗高血压作用
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