ACE-INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATE FROM THE SKIN OF STRIPED CATFISH (*Pangasius hypophthalmus*)

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**ABSTRACT**

There has recently been an increasing demand to produce protein hydrolysates containing peptides with specific biological properties, which could be marketed as functional food ingredients. The objective of this study was to evaluate the in vitro angiotensin converting enzyme inhibitory activity of striped catfish skin hydrolysates and its corresponding fractionates. The striped catfish skin from fillet processing was extracted in an autoclave at 121°C for 30 minutes to obtain an extracted protein. Then it was further hydrolysed with Alcalase with the enzyme to substrate ratio of 20 units/gram protein at 50°C, pH 8 for 7h to obtain protein hydrolysate. The degree of hydrolysis (DH) increased with the increase of hydrolysis time and reached the highest DH of 91.9% after 7h hydrolysis. The 5-h hydrolysate (DH = 60.8%) exhibited the highest ACE-inhibitory activity (IC₅₀ = 831 µg/ml). Therefore, the 5-h hydrolysate sample was used as material for studying enrichment of ACE-inhibitory peptides by ultrafiltration using three different molecular weight cut-off membranes (10, 5, and 1 kDa). Six sample fractions obtained during ultrafiltration process (permeate and retentate) were tested for angiotensin converting enzyme inhibition activity. Permeate of 1 kDa membrane showed the highest activity. The obtained hydrolysates were fractioned using Sephadex™ G-15. Based on gel filtration chromatography results, angiotensin converting enzyme inhibitory peptides had molecular weight ranging of 307 Da to 429 Da. Our findings revealed the potential of using catfish skin as a promising material for retrieving angiotensin converting enzyme inhibitory substances.

Keywords: Alcalase, ACE-inhibitory activity, hydrolysate, ultrafiltration, *Pangasius hypophthalmus*

**I. INTRODUCTION**

High blood pressure is a major risk factor associated with cardiovascular disease, the biggest cause of casualty. Hypertension is commonly treated with antihypertensive or blood pressure lowering drugs, such as captopril, benazepril, enalapril. These drugs are angiotensin I converting enzyme (ACE) inhibitors. ACE (EC 3.4.15.1) is a zinc-metallopeptidase that needs zinc and chloride ions for its activity. In the renin-angiotensin system (RAS), ACE plays a crucial role in the regulation of blood pressure as well as cardiovascular function (Li *et al*., 2004). Within the enzyme cascade of the RAS, ACE converts the inactive angiotensin I by cleaving dipeptide from the C-terminus into the potent vasoconstricting angiotensin II. This potent vasoconstrictor is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure. As many synthetic drugs like ACE inhibitors have side effects, peptides from food sources provide an attractive alternative (Howell and Kasase, 2010). Recent researches have reported discoveries of peptides, which are isolated and characterized from a number of fish skin by-products such as Nile tilapia skin (Vo *et al*., 2011), Pacific cod skin (Ngo *et al*., 2011), Atlantic salmon skin (Gu *et al*., 2011), Skate skin (Lee *et al*., 2011), Pangasius catfish (Mahmoodani *et al*., 2014).
that inhibited ACE and can be used as nutraceuticals and functional food ingredients. A group of peptides from sardine (Fujita, 2001) could decrease blood pressure and approved products containing these components can claim that the product is suitable for individuals with slightly elevated blood pressure. A commercial product from sardine peptides that lowers blood pressure was approved by food for specified health uses (FOSHU), an official functional food approved by the consumer affairs agency of Japan (Shimizu, T, 2003). Striped catfish (Pangasius hypophthalmus) is a large freshwater fish. It is an important species in freshwater aquaculture in Vietnam, Thailand, Malaysia, Indonesia and China. The fillet processing generates considerable quantities of by-products, including abdominal organs, head, bone and skin, that in total represent about 65% of the fish by weight (Thuy et al., 2007). The objective of this study was to investigate ACE inhibitory activity of protein hydrolysate from striped catfish skin by-products by enzymatic hydrolysis using Alcalase.

II. MATERIALS AND METHODS

1. Materials

Catfish skins were obtained from a striped catfish processing plant (Dong Thap, Vietnam), the skins were frozen and stored at -20°C before use. Alcalase from Bacillus licheniformis 2.4 L, o-phthalaldehyde, DL-dithiothreitol, ACE from rabbit lung and other chemicals were purchased from Sigma-Aldrich Chemical Company. Polysulphone hollow fiber membranes with 10, 5, and 1 kDa MWCOs (diameter = 1, 1, and 0.5 mm; area = 0.01, 0.01, and 0.014 m²) were purchased from GE Healthcare Bio-Science Ltd. (Bangkok, Thailand).

2. Methods

2.1 Extraction of protein from striped catfish skin

The clean skins were added with distilled water (1:2, w/v) and the protein was extracted using an autoclave at 121°C for 30 min. After extraction, the extracted protein solution was filtered through a metal sieve to remove skin residues. Extracted protein solution was centrifuged at 3,000g for 20 min at 25°C to remove insoluble residues and used as a substrate for enzyme hydrolysis. Protein content in the skin and the extracted protein solution were determined by Kjeldahl method (AOAC, 1999).

2.2 Enzymatic hydrolysis of extracted protein solution

The extracted protein solution was diluted to obtain a protein concentration of 1% (w/v) by 0.1 M sodium phosphate buffer, pH 8.0. The protein solution was hydrolysed by 20 units/g protein of Alcalase 2.4 L at pH 8.0 and 50°C in a 4-L reactor for 6h. The pH of the mixture was measured by a pH meter (Eutech, Cyber Scan pH 110, Singapore) and manually adjusted to pH 8.0 during the hydrolysis by 6N NaOH and 6N HCl. Aliquots of hydrolysate were collected every 60 mins during the hydrolysis. The sample aliquots were heated in boiling water (95°C) for 10 mins to inactivate Alcalase. They were kept in plastic bottles at -20°C for analyses.

The degree of hydrolysis (DH) of the sample was determined by measure the available cleaved peptides bonds upon hydrolysis, using the o-phthalaldehyde (OPA) method as described by Hue et al. (2013).

2.3 Enrichment of ACE-inhibitory peptides derived from hydrolysate of striped catfish skin by ultrafiltration

The protein hydrolysate was separated using three different MWCO membranes (10, 5, and 1 kDa). The operating condition in batch mode was transmembrane pressure (TMP) of 1.5 bars, and cross flow velocity (CFV) of 1.5 m/s. The ACE-inhibitory activity of the feed and permeate were analyzed.
2.4. Angiotensin-I converting enzyme inhibitory activity of protein hydrolysates from striped catfish skin

The inhibition of ACE activity was determined by the method of Cushman and Cheung (1971) described by Lee et al. (2010) with some modifications. The reaction mixture contained 8.3 mM Hippuryl-L-Histidyl-L-Leucine (Hip-His-Leu) in 0.5M NaCl and 5 mU ACE in 50 mM sodium borate buffer (pH 8.3). A sample (50 μl) was added to above reaction mixture (50 μl) and mixed with 8.3 mM HHL (150 μl) containing 0.5 M NaCl. After incubation at 37°C for 1 h, the further reaction was stopped by the addition of 0.1M HCl (250 μl). The resulting hippuric acid was extracted by the addition of 1.5 ml of ethyl acetate. After centrifugation (800 x g, 15 mins), 1 ml of the upper layer was transferred into a glass tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was redissolved in 3 ml of distilled water, and absorbance was measured at 228 nm using a spectrophotometer (GENESYS 10S UV-VIS Thermo Scientific, Tokyo, Japan). The control and blank were prepared in the same manner, except that 50 μl of buffer was used instead of the sample. The ACE inhibitory activity was expressed as IC50 value (μg/ml). The IC50 value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. The percentage of inhibition level was calculated as follows:

\[
\text{Inhibition level (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}} - A_{\text{Blank}}} \times 100
\]

Where \(A_{\text{Control}}\) is the absorbance of control
\(A_{\text{Sample}}\) is the absorbance of the sample
\(A_{\text{Blank}}\) is the absorbance of the blank

2.5. Fractionation of ACE-inhibitory peptides from hydrolysate

The obtained hydrolysate from UF with the highest ACE-inhibitory activity was used for fractionation. It was dried using freeze dryer (Flexi Dry, Dura Dry, NY, USA). The hydrolysate was fractioned using Sephadex™ G-15. The elution was carried out with 50 mM sodium phosphate buffer pH 7.0 at a flow rate of 0.3 ml/min. The 3 ml fractions were collected and their absorbance was read at 220 and 280 nm. A standard distribution was determined by chromatographing independently using the following standards: Reduced glutathione (429 Da), Hip-His-Leu (307 Da), and Tyrosine (181.91 Da). The fractions of Sephadex™ G-15 column were determined for their ACE inhibitory activity. All fractions were determined soluble protein content by Lowry method (Lowry et al., 1951).

2.6. Statistical analysis

All experiments were carried out in triplicate. Analysis of variance was performed. Mean comparisons were run by Duncan’s multiple range tests. Analysis was performed using an SPSS package.

III. RESULTS AND DISCUSSION

1. Effect of hydrolysis time on degree of hydrolysis (DH)

The DH is generally used as a proteolysis monitoring parameter, and it is the most widely used indicator for comparison among different protein hydrolysates (Guérard et al., 2002). There was a sharp increase of DH in the first 30 min (DH = 28%) and it increased slightly during 30 to 180 min hydrolysis stage. From 180 min onwards, the DH rose dramatically and reached a peak of 91.9% at the end of the period (Figure 1). High value of DH resulted from the increase of short peptides. These results indicated that rapid cleavage of peptides from the extracted protein solution by Alcalase occurred after 3 h.
2. Effect of hydrolysis time on ACE inhibitory activity of hydrolysate

ACE inhibitory activity of hydrolysate with different hydrolysis time expressed as IC$_{50}$ is shown in Figure 2. IC$_{50}$ value of hydrolysate decreased as hydrolysis time increased ($p < 0.05$). ACE inhibitory activity of extracted protein (IC$_{50}$ value of 1,556 ± 16.61 µg/ml) increased after hydrolysis (IC$_{50}$ value ranging from 1,233 ± 29.31 µg/ml to 831 ± 33.39 µg/ml). It was suggested that peptides with ACE inhibitory activity could be generated during hydrolysis. The ACE inhibitory activity appeared to increase as hydrolysis time increase because the lengths of peptides were shortened and increased ACE inhibitory activity (Je et al., 2004). The highest ACE inhibitory activity of striped catfish skin protein hydrolysate (IC$_{50}$ value of 831 ± 33.39 µg/ml) was found at hydrolysis time of 5 h. The highest ACE inhibitory activity of skin hydrolysate in the present study was almost similar with that of blacktip shark gelatin (0.94 - 1.77mg/ml) (Kittiphattanabawon et al., 2013), salmon skin gelatin (1.17 mg/ml) (Gu et al., 2011), and skate skin gelatin (1.89 mg/ml) (Lee et al., 2011). Enzyme hydrolysis was performed in order to achieve the desired degree of hydrolysis to obtain biologically active peptides. From previous studies, ACE inhibitory activity of peptides increased with prolonged incubation with enzyme. However, longer hydrolysis time led to the peptides lost their ability to inhibit ACE (Wu et al., 2008; Xu et al., 2014). The structure of amino acid for interactions between the substrate and the active site of ACE affected ACE inhibitory activity (Ondetti et al., 1977). Cushman and Cheung (1971) reported that peptides containing aromatic at the C-terminal end and the branch-chain aliphatic amino acid at the N-terminal were effective for high ACE inhibitory activity because of the interaction between these amino acids at the active site of ACE.
3. Effect of different MWCO membranes on ACE-inhibitory activity of peptides

Permeate of MWCO 1 kDa membrane showed the highest ACE inhibitory activity. The results indicated that molecular weight of most ACE inhibitory peptides, which was produced and separated from the hydrolysate, was smaller than 1 kDa. This result was in accordance with Je et al. (2004), who reported that Alaska pollack frame protein hydrolysate that having a molecular mass below 1 kDa showed the highest ACE inhibitory activity.

Figure 3 shows filtration time versus ACE inhibitory activity of peptides in permeation and retention during ultrafiltration of protein hydrolysate. In general, the ACE inhibitory activity of peptides in permeate and retention fell steadily when the operating time increase (IC$_{50}$ value increased steadily). The ACE inhibitory activity of peptides in permeates was always higher than that in the retentate because low molecular weight of peptides in permeates exhibited high ACE inhibitory activity. The ACE inhibitory activity (IC$_{50}$ average value) of permeates of MWCO 10, 5, and 1 kDa membranes were 159.7, 125.0, and 8.3 µg/ml, respectively.

Figure 3. ACE inhibitory activity of peptides in permeate and retentate during ultrafiltration of 5-h hydrolysate in batch mode (TMP = 1.5 bars, CFV = 1.5 m/s, temperature = 50°C)

10 kDa MWCO (A), 5 kDa MWCO (B), and 1 kDa MWCO (C) membranes. The lower IC$_{50}$ value represents the higher ACE inhibitory activity.
4. Fractionation of ACE-inhibitory peptides from hydrolysate

The chromatogram of hydrolysate subjected to Sephadex™ G-15 column is shown in Figure 4. Amarowicz and Shahidi (1997) reported that the optical density at 220 nm ($A_{220}$) indicates the peptide bonds and the optical density at 280 nm ($A_{280}$) represents peptides, proteins or amino acids with aromatic rings. Figure 4 shows the chromatogram of the hydrolysate from permeates of UF 1 kDa MWCO membrane which was fractionated using Sephadex™ G-15 gel filtration chromatography. A peak of $A_{220}$ was observed in fraction 4, reflecting the presence of peptides bonds and a distinct peak of $A_{280}$ was found in the same fraction indicated the presence of peptides containing aromatic amino acids. The highest ACE inhibitory activity was obtained at fractions 15 to 18 that having molecular weights 307 Da to 429 Da. Similar findings were also observed from previous works by Je et al. (2004); Mahmoodani et al. (2014); Raghavan and Kristinsson (2009), who reported that peptides with molecular masses below 1 kDa showed the highest ACE inhibitory activity. The peaked fractions showed the highest ACE inhibitory activity ($IC_{50}$ value ranging from 1.22 to 5.88 µg/ml) (Table 1), which ranged from 141.45 to 681.72 fold higher than hydrolysate ($IC_{50}$ value 831.7 µg/ml). Fractions 15-18 showing the highest ACE inhibitory activity. The result suggests that peptides without or low ACE inhibitory activity was removed during fractionation while peptides with high ACE inhibitory activity were concentrated.

![Figure 4. Elution profile of striped catfish skin hydrolysate (from UF 1 kDa MWCO membrane) separated by size exclusion chromatography on Sephadex™ G-15](image)

Reduced glutathione (MW = 429 Da), Hip-His-Leu (MW = 307 Da), Tyrosine (MW = 181.91 Da), were used to calibrate the standard molecular weights.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>ACE inhibitory activity ($IC_{50}$)</th>
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<tbody>
<tr>
<td>15</td>
<td>4.98 ± 0.03 µg/ml</td>
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<tr>
<td>16</td>
<td>1.22 ± 0.01 µg/ml</td>
</tr>
<tr>
<td>22</td>
<td>5.88 ± 0.06 µg/ml</td>
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IV. CONCLUSION

This study found that the protein hydrolysate from striped catfish skin exhibited strong ACE-inhibitory activity. The ultrafiltration usage of 1 kDa was successful for separation ACE inhibitory activity peptides since ultrafiltration of the hydrolysate resulted in a significant increase its ACE inhibitory activity in the permeate fractions ($IC_{50} = 8.3 \mu g/ml$). It was concluded that peptides receiving from alcalase hydrolysis of striped catfish skin could be utilized as a part of functional food or ingredients of a formulated drug in order to control high blood pressure.

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