ACE inhibitory peptides derived from sarcoplasmic enzymatic hydrolysate of freshwater fish haruan

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Abstract— **Hypertension is one of the major risk factor for development of cardiovascular diseases. Among the process related to hypertension, ACE plays a crucial role in the regulation of blood pressure. In this study, sarcoplasmic protein hydrolysates of freshwater fish haruan (*Channa striatus*), obtained by treatment with proteinase K, thermolysin and the mixture of enzymes, were analyzed for their ACE inhibitory activities. Hydrolysate generated with proteinase K, with degree of hydrolysis of 89% and molecular weight cut-off less than 3 KD, displayed the lowest IC_{50} value of 0.038 mg/mL. This hydrolysate was then fractionated by size exclusion chromatography on polyacrylamide Bio-Gel P-2 column into four fractions (P1–P4). P4 was found to display the highest ACE inhibitory activity (75.23%) and then fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) into seven fractions (A–G). The amino acid sequence of sub-fraction F with the strongest ACE inhibitory activity, (81%) was determined by ESI–MS/MS as YSMFPP with the IC_{50} value 1.8 μM. The ACE inhibitory activity of purified peptide did not change after in vitro digestion with gastrointestinal proteases. The results of this study presented a new ACE inhibitory peptide formed from sarcoplasmic protein hydrolysate of Haruan fish meat which could be used as health enhancing ingredient in the formulation of functional foods in order to prevent hypertension.**

Keywords— **Sarcoplasmic protein, Proteinase K, ACE-inhibitory activity**

I. INTRODUCTION

High blood pressure has been considered a risk factor for developing cardiovascular diseases. Synthetic hypotensive drugs, such as captopril, enalapril and lasinopril which are still the most broadly used method to prevent hypertension, are reported to have side effects. Therefore, an interest to search natural ACE inhibitors with minimum side effects as alternative approaches to synthetic drugs has been increased [1]. Recently, many ACE inhibitory peptides have been isolated from the enzymatic digestion of various food proteins [2]. The ACE inhibitory activity of these peptides is strongly influenced by the C-terminal tripeptide sequence of the substrate. It was suggested that the presence of aromatic and aliphatic residues in the ultimate position of the substrate or competitive inhibitor could increase ACE inhibitory activity of peptides [3]. In the present study the objective was to determine the antihypertensive activity of enzymatic hydrolysis of *C. striatus* sarcoplasmic protein and the sequences of the purified fractions with the highest ACE-inhibitory activity using HPLC-ESI micrOTOF-Q MS/MS techniques.

II.MATERILAS AND METHODS

A. Hydrolysis of sarcoplasmic protein

After extraction of sarcoplasmic protein from *C. striatus* meat, samples were hydrolyzed with 1:100, w/w thermolysin (pH 7.4; 37°C) (ST), proteinase K (pH 7.4; 37°C) (SP) and the mixture of enzymes (STP) for 30 min. The hydrolysate was centrifuged at 3000 x g for 20 min and the soluble aqueous fraction decanted and lyophilized.

B. Purification of hydrolysates

All sarcoplasmic catalysed hydrolysates were fractionated by ultrafiltration membranes size 10 and 3 KD molecular weight cut-off (MWCO), respectively. Peptides with MWCO < 3 KD were separated into fractions with gel chromatography on polyacrylamide Bio-Gel P-2 column. Fractions with high ACE inhibitory activity were further separated with the aim of reversed-phased high performance liquid chromatography (RP-HPLC) and lyophilized.

C. Effect of Gastrointestinal Protease on ACE Inhibitory Activity

Stability of sarcoplasmic protein hydrolysate solutions against *in vitro* gastrointestinal proteases were carried out in triplicate [4]. Purified hydrolysate solutions were incubated with pepsin (0.05% (w/v), pH 2) and trypsin (0.05% (w/v), pH 8) for 6 h at 37°C, and neutralized to pH 7 with 2 N NaOH. Neutralized suspensions were centrifuged (10,000 × g, 30 min) and the supernatant was assayed for ACE-inhibitory activity. The remaining neutralized suspension was further digested by a solution containing 0.025% (w/v) chymotrypsin and 0.025% (w/v) trypsin at 37°C for another 6 h. The enzymatic reaction was inactivated by boiling for 15 min and followed by centrifugation (10,000 × g, 30 min). The supernatant was used for determination of ACE-inhibitory activity.

D. Identification with LC-MS-TOF

Separation of the peak with the highest ACE-inhibitory activity from RP-HPLC fractionation was carried out using an Ultimate ® 3000 (Dionex, USA) HPLC system connected to Quadrupole micro- Time Of Flight (micrOTOF Q) (Bruker Daltonics, Germany), equipped with ESI interface. MS and Tandem MS experiments were controlled by micrOTOf Control software (Version 2.2, Bruker Daltonics) and HyStar (Version 3.2, Bruker Daltonics) software.

III. RESULTS AND DISCUSSION

Table 1 indicates IC_{50} value of protein hydrolysates with different molecular weight fractions with respect to the peptide content. Results indicated that by the increasing of cut-off MW, peptide content and ACE inhibition activity was increased. Peptide content of SP was higher than that of ST ($P< 0.05$). The untreated sarcoplasmic protein had higher IC_{50} value (1.52 mg/mL) than its hydrolysates. SP fraction (MWCO < 3 KDa) had lower IC_{50} value (0.038 mg/mL) than other sarcoplasmic hydrolysates. Based on these results, the concentration of the following study was based on SP hydrolysates.

Table 1 Protein solubility, peptide content and ACE inhibitory activity of sarcoplasmic protein hydrolysates

Sample	MWCO	Peptide content (mg/g)	IC_{50} (mg/mL)
S		16.9±2.01	1.521±2.1
ST	10,000	190.4±1.05	0.181±1.13
	3,000	220.2±0.06	0.137±0.05
SP	10,000	210.4±0.05	0.087±0.04
	3,000	230.1±1.12	0.038±0.73
STP	10,000	250.4±0.01	0.195±0.52
	3,000	284.3±0.05	0.157±0.04

Four major absorbance peaks were observed at 280 nm when SP hydrolysate was fractionated on Bio-Gel P-2. All fractions were also collected and assayed for ACE inhibitory activity. Fraction P4 with lower peptide content indicated ACE inhibition activity of 75.23% and IER of 313.46 %/mg/mL, which was significantly higher than those of other fractions obtained from gel-filtration chromatography of SP hydrolysate ($P< 0.05$).

Fraction P4 was further separated by RP-HPLC column (C18) with linear gradient of 0-65% acetonitrile. Fig. 1 shows the elution profile of peptide separation of P4 into seven potent fractions and assayed for inhibitory activity.

Table 2 ACE inhibitory activity of fractions

Samples	Fractions	ACE inhibitory activity (%)
SP hydrolysate	A	24
	B	18
	C	ND
	D	28
	E	ND
	F	78
	G	10

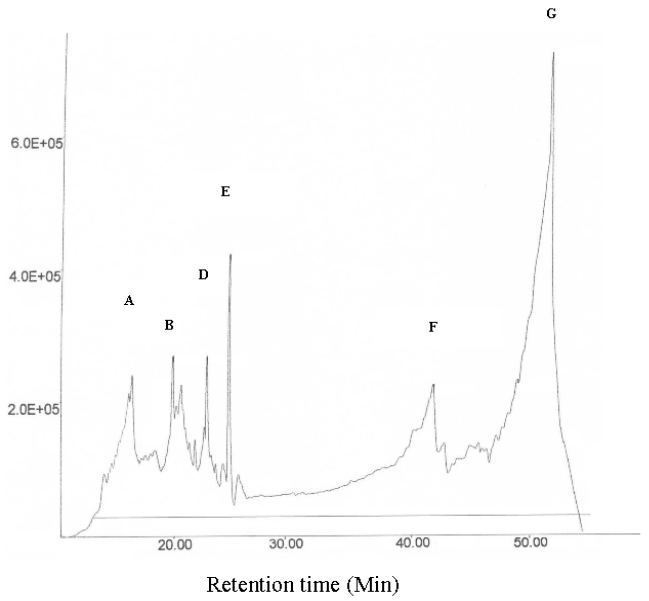

Fig. 1.RP-HPLC elution profile pattern on C_{18} column of fraction P4 obtained from gel chromatography of SP hydrolysate

Table 2 shows the ACE inhibitory activity of these fractions. Fraction F showed the highest ACE inhibitory activity of 78% in comparison to other fractions. Active fractions obtained from RP-HPLC of separation of SP hydrolysates were collected and lyophilized for measurement of in vitro stability and amino acid sequence with LC-MS-TOF.

Previous reports have suggested that some ACE-inhibitory peptides failed to show potent hypotensive activity in vivo although they exhibited powerful ACE-inhibitory activity in vitro [5]. There are two possibilities; one is that the peptides may be susceptible to hydrolysis by ACE under physiological conditions; two, the peptides may be destroyed by gastric enzymes [4]. Therefore, prior to further processing, it is necessary to determine the susceptibility of the active protein hydrolysate to simulate gastric digestion. Simulated gastrointestinal tract stability studies showed that the ACE inhibitory activity of the fraction F was not affected by *in vitro* incubation with gastrointestinal proteases and was measured as 61.5, 61.1 and 60.8% when digested with pepsin, trypsin and mixture of trypsin and chymotrypsin compared to the control value of 62.1% (Table 3).

Table 3 ACE inhibitory activity of hydrolysates after digestion by gastrointestinal proteases

Enzymes	Fraction F(%)
Control	62.1±4.3
pepsin	61.5±2.2
trypsin	61.1±4.5
trypsin + chymotrypsin	60.8±1.6

In order to identify the isolated peptides, fraction F was subjected to LC- MS/MS analysis. Two major peaks were detected in the total ion chromatograph (TIC) profile of isolated peptides (Fig. 2). Based on their molecular mass information and MS/MS fragmentation of MASCOT ion search, major peptides of fraction F were tentatively identified and listed in Table 4. The sequence of peak 1 of TIC profile of fraction F with the highest score (Ion score, 44) was identified as YSMFPP with the IC_{50} value of 1.8 μM. The tandem mass spectrometry of this peak was illustrated in figure 4.15 at molecular ion of 871.102 m/z which contained a major ion at 298.333 m/z. The hexapeptide isolated in the present study also contained one aromatic Phe residue with two proline residues at the C-terminal, responsible for its high ACE inhibition activity. Moreover, instead of branched amino acid residue such as Val or Ile at the N-terminal, it contained a Tyr residue.

The sequence of the second peak was identified as YLFLSFWP with the IC_{50} value of 2.2 μM. This peptide also contained a Tyr residue at the N-terminal and –FWP at the penultimate position of C-terminal sequence.

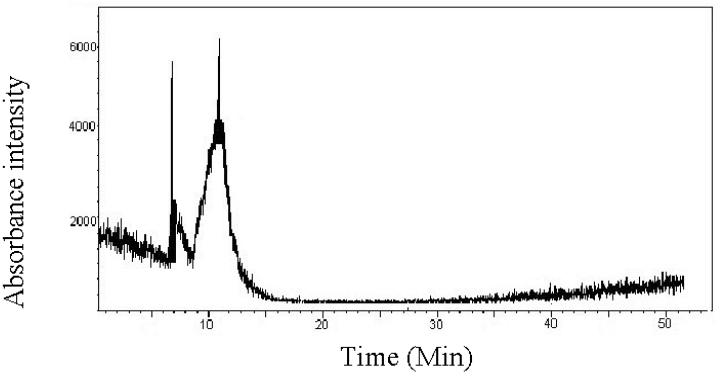

Fig. 2. TIC of fraction F obtained from RP-HPLC.

Table 4 Peptides matching fraction F according to a data base Mascot MS/MS Ion Search

M_{exp}	Delta	Sequence	Ion Score
900.358	0.15	P.YSMFPP.K	44
1078.343	-0.13	R.YLFLSFWP.L	39
856.425	-0.06	K.EIGPTWP.D	31
824.479	0.08	R.ESGPPGPY.G	14
1236.721	0.26	K.YGKGNYFPP.D	5

However, the ACE inhibitory activity of the second peptide was less than the first one. The fact that there are two active enzymatic sites in ACE may explain its wide substrate or inhibitor specificity. Difference in substrate preparation and methods of purification could result in different peptide sequences [4].

IV. CONCLUSION

These peptides with very high ACE inhibitory activity have not been identified yet as ACE inhibitory peptides and thus the results of this study presented new highly ACE inhibitory peptides formed by proteinase K- catalysed hydrolysis of sarcoplasmic proteins of Haruan fish meat.

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REFERENCES

1. Hernández-Ledesma B, Recio I, Ramos M et al. (2002) Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. Int Dairy J 12:805–12
2. Curis J.M, Dennes D, Waddell D.S et al. (2002) Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC-MS/MS. J Agri food chem 50:3919–25
3. Murray B.A, FitzGerald R.J (2007) Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. Curr Pharm Des 13:773-791.
4. Wu J, Rotimi E.A, Muir A.D (2008). Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal. Food Chem 111:942–950
5. Fujita H, Yokoyama K Yoshikawa M (2000) Classification and antihypertensive activity of angiotensin I–converting enzyme inhibitory peptides derived from food proteins. J Food Sci 65:564-569