Isolation and purification of ACE inhibitory peptidic fractions from freshwater fish muscle protein thermolysin hydrolysate

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Abstract— **"Haruan" (Channa striatus) is a freshwater fish popular among post-operative patients to induce wound healing in Malaysia. Hypertension is considered a risk factor for developing cardiovascular diseases and ACE is involved in increasing blood pressure. This study was conducted to evaluate the ACE inhibitory activity of haruan muscle protein hydrolysate. Myofibrillar protein was hydrolysed with thermolysin at 37° C for 2 h. The thermolysin catalysed hydrolysate with degree of hydrolysis of 89% was fractionated by ultrafiltration membranes size 10 and 3 KD molecular weight cut-off (MWCO), respectively. Peptides with MWCO < 3 KD exhibited high ACE inhibitory activity (IC_{50} = 0.033 mg/mL) and were separated into three fractions with gel chromatography on polyacrylamide Bio-Gel P-2 column. Fraction 2 with the ACE inhibitory activity of 67.23% was further separated into five (A-E) potent fractions with reversed-phased high performance liquid chromatography (RP-HPLC), which Fraction C with the lowest IC_{50} value of 10.32 µg/mL was lyophilized and characterized with HPLC mass spectrometry-Time Of Flight (LC-MS-TOF). Two peptides with the sequences of VPAAPPK and NGTWFEPP with IC_{50} value of 0.45 and 0.63 µM were identified for the most active fraction C, respectively. The high ACE inhibitory activity suggests that haruan muscle protein thermolysin hydrolysate could be used as functional ingredients for blood pressure reduction.**

Keywords— **Thermolysin hydrolysate, ACE-inhibitory activity, Mass Spectrometry.**

I. INTRODUCTION

Channa striatus, known as Haruan in Malaysia, is a freshwater fish with wound healing properties. Investigation of proximate composition and amino acid content revealed that *C. striatus* had higher amount of protein and essential amino acids in comparison with other freshwater fishes [1]. Correct combination of enzyme and protein results in high yield of the bioactive peptides. However, it was reported that thermolysin digests demonstrated the highest ACE-inhibitory activity form porcine myofibrillar protein [2] and casein and whey protein [3]. The purpose of this study was to isolate ACE-inhibitory peptides from enzymatic hydrolysates of *C. striatus* myofibrillar protein and identifiy the sequence using HPLC-ESI-micrOTOF-Q MS/MS techniques.

II.MATERILAS AND METHODS

A. Extraction and hydrolysis of myofibrillar protein

Myofibrillar protein was extracted from *C. striatus* meat [4] and was purified and suspended in 0.15 M KCl, 0.03 M Tris pH 7.6. Samples were hydrolyzed with 1:100, w/w thermolysin (pH 7.4; 37ºC) for 2 hr. The hydrolysate was centrifuged at 3000 x g for 20 min and the soluble aqueous fraction decanted and lyophilized.

B. Purification of hydrolysates

The thermolysin catalysed hydrolysate was 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. Amino Acids Analysis

Acid hydrolysis, performic acid and alkaline hydrolysis of *C. striatus* meat hydrolyates were performed using Waters AccQ.Tag amino acid analyzer (Waters Corporation, Irland).

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

The thermolysin catalysed hydrolysate of myofibrillar protein was fractionated by ultrafiltration, size exclusion chromatography (SEC) and reversed-phased high performance liquid chromatography (RP-HPLC). Three fractions were collected using SEC fractionation on polyacrylamide Bio-Gel P-2 column. F2 showed the highest inhibitory efficiency ratio (IER) being 190.345 %/(mg/ml). This fraction was collected and subjected to RP-HPLC C_{18} column for further peptide purification. Fig. 1 shows elution profile of peptide separation using RP-HPLC.

Fractions collected after RP-HPLC and ACE inhibitory activity were assayed. Fraction C showed the lowest IC_{50} value of 10.32 µg/mL and was collected and lyophilized for amino acid sequence identification with LC-MS-TOF.

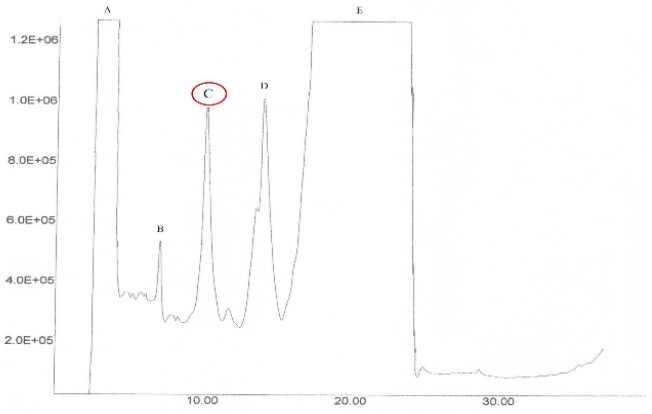

Fig. 1. Elution profile of peptide separation using RP-HPLC

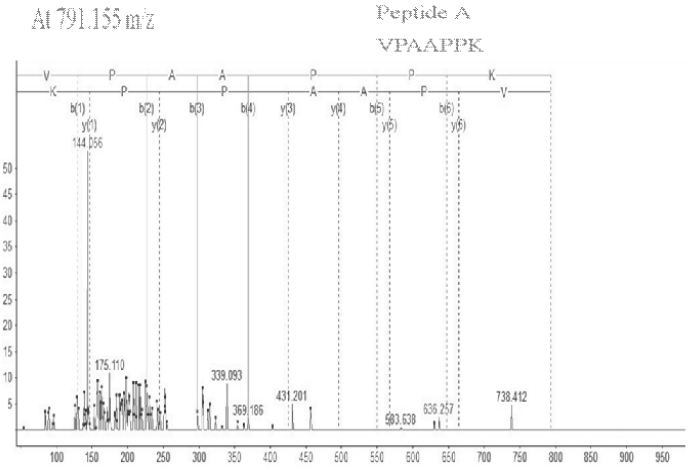

Fig. 2. ESI-TOFQ MS/MS of peak 1 and MS/MS ion sequencing at 791.155 m/z.

Total ion chromatogram (TIC) of the fraction C with illustrated two major peaks. The accurate mass data of the molecular ions of all MS/MS spectra of each peak were processed initially through the software Data Analysis 3.4 (Bruker Daltonics), and were searched for peptides with up to one miscleavage against Swiss Prot and mass spectrometry protein sequence database using Mascot (Matrix Science Ltd.) search engine.

Fig.2 shows the MS/MS spectrum of peak 1 at 791.155 m/z where the ion of 144.056 m/z was the most abundant. The MS/MS ion sequencing of this compound was performed using Mascot search engine and the sequence determined to be VPAAPPK with IC_{50} value of 0.45 µM. Inhibition activity of ACE is strongly related to the C-terminal tripeptide sequence of substrate and competitive inhibitors. However, the presence of aromatic (W, Y, F) and aliphatic (I, A, L, M) residues are preferred in the ultimate position to increase ACE inhibitory activity of peptides [5].

As results of this study indicate, thepotent heptapeptide of VPAAPPK, derived from thermolysin hydrolysis of C. striatus myofibrillar protein, has the sequence of Pro-Pro-Lys at the C-terminal. Presence of Pro-Pro-Lys at the C-terminal position has also been reported in the Myosin of rat, chicken, human and pork [2].

The MS/MS spectrum of the second most abundant component from fraction C (Peak 2) showed a molecular ion at 1085.841 m/z with a major ion at

867.991 m/z. The Mascot MS/MS ion sequence of NGTWFEPP was determined with IC_{50} value of 0.63 μM. Presence of two Pro residues at the C-terminal of

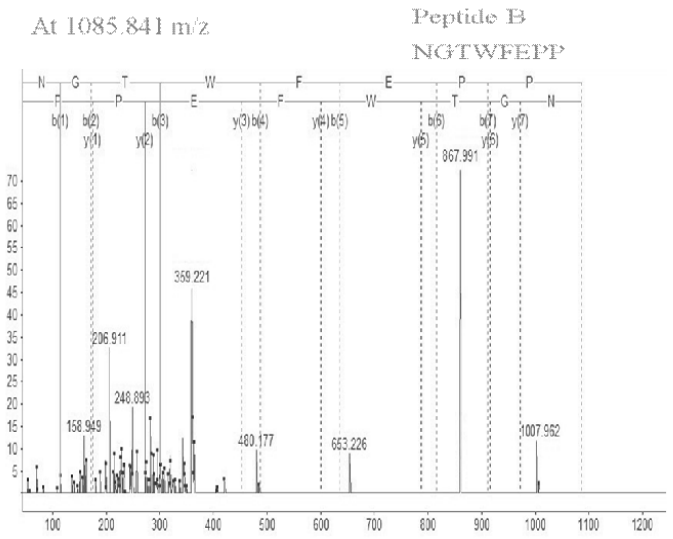

Fig. 3. ESI-TOFQ MS/MS of peak 2 and MS/MS ion sequencing at 1085.841 m/z.

The isolated octapeptide in this study is essential for binding to active sites of ACE. Also, several strong ACE inhibitors have a Pro residue in the C-terminal position. The presence of negatively charged amino acids such as glutamic and aspartic acid residue at the C-terminal may decrease the inhibition activity of peptide. However, in the present study, combination of Glu with two Pro residues in the C-terminal (-EPP) would cause an incorrect orientation of the peptide into active sites of ACE. In this sequence aromatic amino acids of Trp and Phe responsible for high ACE inhibitory activity were also observed, but not in the C-terminal.

The present results in Table 1 showed that after ultrafiltration and gel filtration treatment, the potent fraction F2 had different content of amino acids compared to its hydrolysate. The dominant amino acids in the potent gel F2 were glutamic and aspartic acid. The essential amino acid content of F2 fraction increased to nearly 54% in comparison with myofibrillar hydrolysate (MT) protein values of 45%, suggesting a controlled enzymatic hydrolysis resulted in a product with high nutritive quality source of protein without amino acids destruction. The contents

Table 1 Amino acid composition (%) of myofibrillar protein, hydrolysate and most active gel filtration fraction

Amino acids	Myofibrillar protein	MT	Gel F2
Asp	8.6 ±0.1	9.1 ±0.3	9.5±0.1
Ser	4.9 ±1.0	4.3 ±0.4	4.5±0.3
Glu	13.2 ±2.1	16.6 ±0.5	17.4±1.2
Gly	6.2 ±1.3	4.9 ±0.5	3.5±1.2
His*	2.3 ±0.3	2.4 ±0.2	2.0±2.1
Arg	5.4 ±0.4	6.6 ±0.1	2.1±0.5
Thr*	4.1 ±0.2	4.8 ±0.1	6.4±0.2
Ala	7.1 ±1.0	6.1 ±2.1	6.2±0.1
Pro	3.8 ±1.5	3.8 ±0.9	2.1±2.2
Tyr	2.1 ±1.2	3.6 ±2.1	5.9±1.3
Val *	4.9 ±0.3	4.8 ±2.2	6.4±0.4
Met*	3.0 ±0.5	3.2 ±2.3	3.4±0.3
Lys*	9.9 ±1.7	10.4 ±1.3	8.2±1.4
Ile*	4.9 ±0.9	4.5 ±0.1	6.8±0.4
Leu *	8.0 ±0.7	7.9 ±1.0	8.9±0.5
Phe*	4.7 ±0.1	3.4 ±1.0	6.5±1.2
Cys	1.5 ±1.0	0.4 ±0.2	0.5±2.3
Trp*	4.5 ±1.0	3.7 ±0.4	5.1±0.6

of aromatic amino acids (Trp, Phe, Tyr) in F2 were increased to 17.66 compared to that of MT (10.7%) hydrolysate. It was suggested that most naturaloccurring ACE inhibitory fractions contain hydrophobic (aromatic or branched chain) amino acids or proline residue in their peptide sequence.

IV. CONCLUSION

These findings confirmed that bioactive peptides can be released from Haruan meat protein hydrolysate. Two myofibrillar fragments of with sequence of VPAAPPK and NGTWFEPP with very high ACE inhibitory activity have been isolated and identified for the first time from Haruan fish meat. Haruan

freshwater fish with high essential amino acids, high protein content and wound healing properties have also showed remarkably high ACE inhibitory activities, believed can be used as antihypertention drug.

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REFERENCES

1. Ghassem M, Khoo T.C, Feni H.S et al. (2009) Proximate composition, fatty acid and amino acid profiles of selected Malaysian freshwater fish. Malay fish j 8:7-16
2. Arihara K, Nakashima Y, Mukai T et al. (2001) Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins. Meat Sci 57:319–324
3. Otte J, Shalaby S.M.A, Zakora M et al. (2007) Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. Int Dairy J 17:1460–1472
4. Hay J.D, Currie R.W, Wolf F.H et al. (1973) The effect of post mortem aging on chicken muscle fibrils. J food sci 38:981-990
5. 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.