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Abstract. Hypertension is a major cause of mortality in the developing country and affects up to 30% of adult population in the world. The angiotensin-I converting enzyme (ACE) is a key therapeutic target when combating hypertension and it has been studied extensively. The aim of this study was to efficiently screen the ACE inhibitory peptide from soft-shelled turtle yolk (TY) by using the chymotrypsin enzyme. The TY proteins were digested followed by ultrafiltration (MWCO 3 kDa). The resulting hydrolysate was fractionated using reversed phase-high performance liquid chromatography (RP-HPLC) and offline strong cation exchange chromatography (SCX). The inhibitory activities of each fraction were measured using an in vitro ACE inhibitory assay. The peptides in the most active fractions of both RP and SCX separations were characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database-assisted peptide sequencing. The result showed that KF-11 and KY-10 were simultaneously identified from the best ACE inhibitory RP and SCX fractions. The identities and ACE-inhibitory activities of KF-11 and KY-10 were further confirmed using synthetic peptides.

1. Introduction

The prevalence of hypertension is commonly caused by behavioral risk factors such as ageing and population growth, which is dramatically emerging in upper-middle income countries in adults aged >25 years. Globally, hypertension has reached 9.4 million deaths every year [1]. Hypertension, also known as high blood pressure, is one of the current global health burdens followed by metabolic disorders that increase the risk of morbidity and mortality [2]. The common examination routine for a person suffering from hypertension is measuring their systolic blood pressure (SBP) and diastolic blood pressure (DBP); it is positive if it is more than 140 mmHg and 90 mmHg, respectively. In order to mitigate the aberrations and to restore normal physiological function, the functional molecules derived from food have been widely pursued.

Bioactive peptides have now been isolated and characterized in many natural and processed foods. They are inactive in the original protein and can be released in an active form through intestinal digestion or hydrolysis by protease [3]. Bioactive peptides may function as potential physiological modulators in the process of metabolism during intestinal digestion, and they are liberated depending

on their structure, composition, and amino acid sequence. Some of these bioactive peptides have been identified to possess nutraceutical potentials that are beneficial for the promotion of human health [4]. The peptides IVRDPNGMGAW were obtained from soft shelled turtle egg white and revealed potent ACE inhibitory activities with an IC_{50} value of 4.39 μM. The peptide was identified as a competitive inhibitor [5]. On the other hand, the peptides AKLPSW obtained from soft shelled turtle yolk hydrolyzed by thermolysin also revealed potent ACE inhibitory activity with an IC_{50} value of 15.3 μM [6]. Angiotensin I converting enzyme (ACE; dipeptidyl carboxypeptidase, EC3.4.15.1) plays an important role in regulating blood pressure. This enzyme can hydrolyze angiotensin I related to the potent vasoconstrictor angiotensin II and inactivate the potent vasodilator bradykinin. The inhibition of ACE activity is thus considered to be a pivotal therapeutic approach for treating hypertension [7]. Currently available ACE-inhibitors such as captopril, enalapril, lisinopril and ramipril are used in the treatment of hypertension in humans. However, the synthetic drugs have several side effects, such as a dry cough, skin rashes and angioneurotic edema. Thus, developing safe and natural ACE-inhibitors is necessary for the future treatment and prevention of hypertension [8]. Soft shelled turtle eggs have been investigated as potential ACE inhibitory peptides due to their existence as having been widely used as tonic food for a long time in China. The low molecular weight peptides were collected using 3 kDa molecular weight cut-off ultrafiltration and the peptides were fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). The resulting fractions were screened in vitro for ACEI activities.

2. Materials and method

2.1. Sample preparation

The soft shelled turtle shells were separated between the egg white and the yolk. The yolk was collected and then lyophilized for further analysis. The lyophilized yolk was defatted using hexane (1/9; w/v). The defatted yolk (10 mg) was dissolved in 1 ml of 50 mM ammonium bicarbonate at pH 8.5 and vortexed to mix the solution. Subsequently, the turtle yolk was digested by chymotrypsin enzyme, with an enzyme to protein ratio of 1:50 (w/w) using temperatures of 37 ℃ for 16 h. The reaction was stopped by centrifugation 14000 rpm at 4 ℃ for 15 min using centrifuge (Hitachi Koki Co., Japan) in an ultra-filtration membrane (3 kDa MWCO). The filtrate (< 3 kDa) was transferred to a fresh tube, lyophilized and kept at -20 °C for further analysis.

2.2 Fractionation of the soft shelled turtle yolk

Twenty μl of the hydrolysate was separated using reverse phase high performance liquid chromatography (RP-HPLC) (Hitachi Chromaster, Tokyo, Japan) with a C18 column (4.6 mm x 250 mm; particle size 5 μm, Thermo Scientific Inc., USA). The gradient was arranged as follows: 0-50 min gradient from 0% B to 25% B; 50-53 min gradient from 25% B to 80% B; 53-58 min isocratic elution with 80% B; 58-60 min gradient from 80% B to 0% B at a constant flow rate of 1 ml/min using UV absorbance at 214 nm.

The hydrolysate of turtle yolk passed through at the 3 kDa MWCO ultrafiltration membrane, and was lyophilized and dissolved in 5% ACN and 0.2% FA respectively. The mobile phase was composed of solution A (5% ACN and 0.2% FA in deionized water) and solution B (5% ACN, 0.2% FA and 0.5 M NaCl in deionized water). The hydrolysate was separated into 10 fractions according to the gradient as follows: 10% B, 20% B, 30%B, 40% B, 60% B, 80% B and 100% B. The separation was arranged at the constant flow rate of 20 μl/min. The resulting fractions were collected, lyophilized and kept at -20ºC for the ACE inhibitory assay. The peptides in the fraction with the best inhibition were sequenced using liquid chromatography-tandem mass spectrometry (LCMS/MS) and database searches.

2.3 ACE inhibitory assay

The sample solution containing 30 μl of 2.5 mM hippuryl-L-histidyl-L-leucine (HHL) as a substrate and 10 μl of inhibitor in 200 mM borate buffer containing 300 mM NaCl (adjusted to pH 8.3) was pre-

incubated at 37 ºC for 5 min. The control solution was prepared using the same buffer without inhibitor. Subsequently, 20 µl of 0.05 mU/µL ACE in 200 mM borate buffer was added to the sample solution and control solution. The reaction was incubated statically at 37 ºC for 30 min and then shaken in a thermostatically controlled shaker incubator (200 rpm) at 37ºC for 30 min. The reaction was stopped by adding 1 M HCl (60 µl). HHL and its hydrolyzed product hippuric acid (HA) were analyzed using an HPLC equipped with a C18 column (4.6 mm x 250 mm; particle size 5 µm, Thermo Scientific Inc., USA). The mixtures were separated using an isocratic elution composed of 79% mobile phase A (5% ACN and 0.1% TFA in deionized water) and 21% mobile phase B (95% ACN and 0.1% TFA in deionized water) at constant flow rate of 1 ml/min for 15 min. The resulting HA was detected using a UV detector at 228 nm. The ACE inhibition (%) was determined according to the following equation:

$$\text{ACE Inhibition (\%)} = [1-(\Delta A \text{ inhibitor})/(\Delta A \text{ control})] \times 100$$

Where ΔA inhibitor and ΔA ontrol were the peak areas in the samples with or without inhibitor, respectively. IC_{50} is defined as the required concentration for 50% inhibition of ACE's activity. The IC_{50} value was determined using a linear regression of ACE inhibition results (%) from six samples in the presence of inhibitory peptide at various concentrations.

3. Results and discussion

3.1. ACE inhibitory activity

The soft shelled turtle yolk was digested using chymotrypsin. Proteolytic hydrolysis is usually used to get the bioactive peptide. The measurement of the ACE inhibitory activities was analyzed using the ultrafiltrated fraction of 3 kDa MWCO due to the low MW peptide being more effective at ACEI than the high MW peptides. The percentage of the ACE inhibition of the hydrolysates was observed to be 86.39% at the peptide concentration of 1 mg/ml. The determination of the IC_{50} value of ACEI from chymotrypsin hydrolysate was calculated using an interpolation of the ACE activity (%) from seven different concentrations of inhibitory samples. The IC_{50} value of chymotrypsin hydrolysate indicated as a potential ACE inhibitory peptide with an IC_{50} of 29.66 µg/ml as seen in Figure 1.

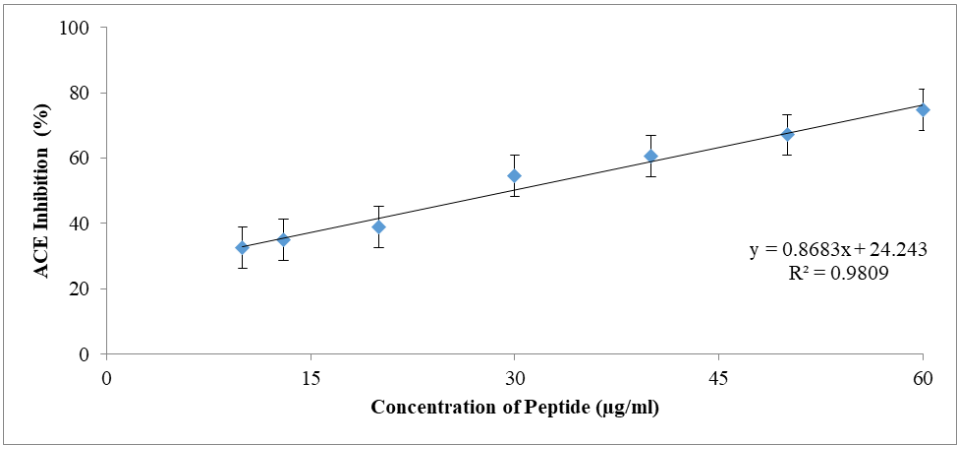

Figure 1. IC_{50} value determination of chymotrypsin hydrolysate

3.2. Fractionation of RP-HPLC and SCX

The lyophilized chymotrypsin hydrolysate that had been passed through <3 kDa MWCO was fractionated using the C18 column of RP-HPLC for 1 hour. The sample was dissolved in 5% ACN + 0.2% TFA and collected every 5 minutes due to the existence of high peaks. There were 12 fractions that appeared in the RP-HPLC chromatogram and that were used to measure the ACE inhibitory activities. The ACE inhibition of 12 fractions varied from 12.43

to 92.12% as seen in Figure 2 (a). On the other hand, chymotrypsin hydrolysate was separated using an offline strong cation exchange (SCX) column containing SP Sephadex. The sample was dissolved in 5% ACN + 0.2% FA in deionized water (v/v). The elution was divided into 7 fractions. The ACE inhibition of 7 fractions varied from 40.54 to 83.20%, as seen in Figure 2 (b).

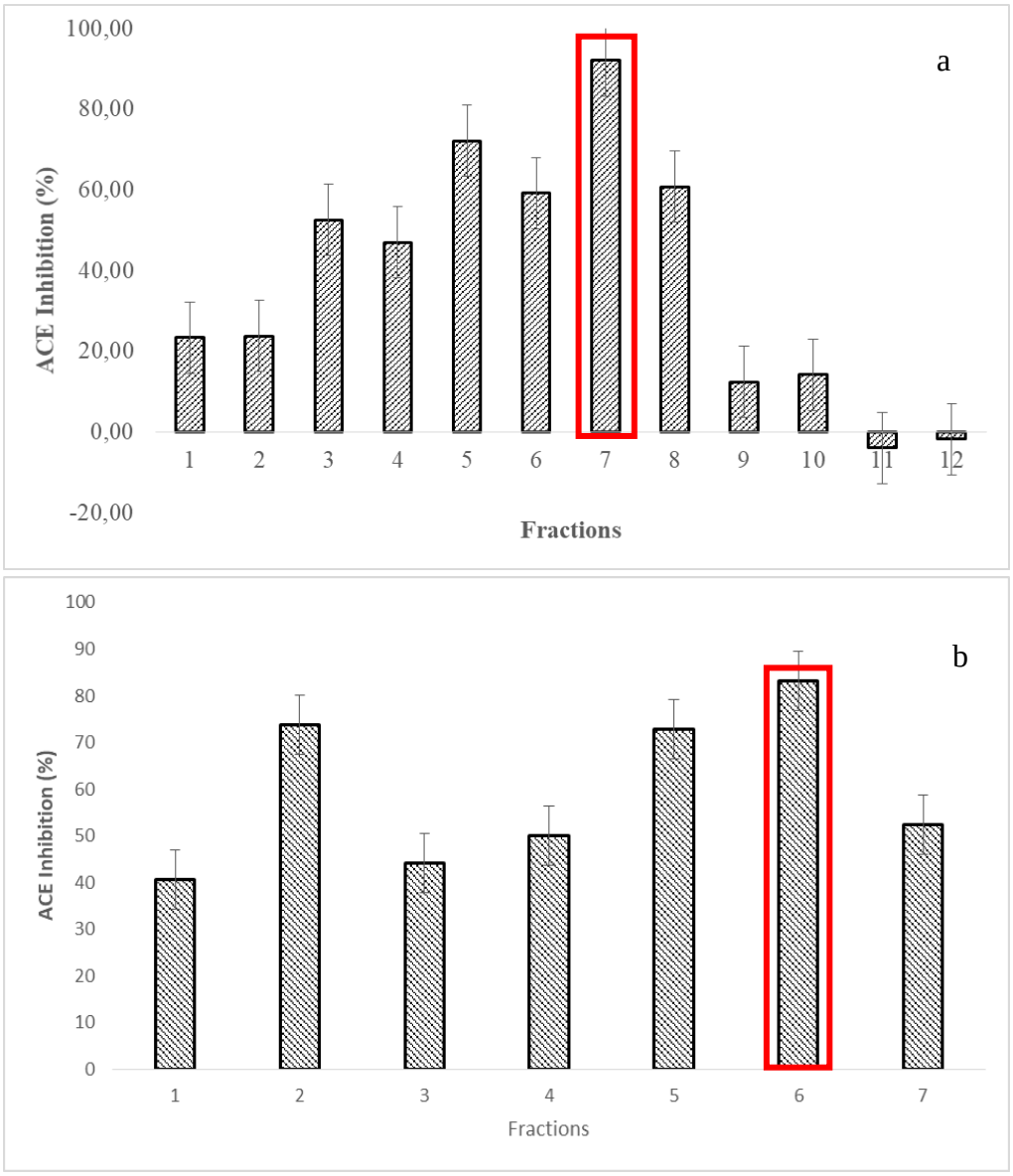

Figure 2. (a) the ACE inhibitory activities of the 12 fractions collected from RP-HPLC; (b) the ACE inhibitory activities of the 7 fractions collected from offline SCX.

3.3. Peptide Identification

Fraction 7 from RP-HPLC and fraction 6 from SCX showed the highest ACE inhibition activities, the identification of the peptide that may exist in the fractions was performed using LC-MS/MS. Two peptides that have been overlapping between fraction 7 and fraction 6 were identified as seen in Table 1 from RP-HPLC and Table 2 from SCX. Thus, the peptides were also characterized with their double charge at m/z 611.32 and 632.15 as seen in Figures 3 (A) and (B), respectively. Based on the Mascot

Distiller database search, the two peptides were identified as KIATEVVTGRF (KF-11) and KIRSPQLEEY (KY-10).

Table 1. Peptide identified from fraction 7 RP-HPLC; database-assisted

Identified Protein	Identified Peptide	Position Start-End	Observed m/z	Peptide Mass (Calc)	Score of Peptide
Vitellogenin-2 (gi: 558149531)	TLVGSEKY	18-11	299.19	895.46	14
	KIRSPQLEEY	70-79	632.13	1261.66	40
	AVQEDKRNNGIF	166-177	696.22	1389.70	26
	NEPGGVAVTDARQEL	247-261	778.56	1554.76	74
	KDPRKVQDIL	528-537	606.61	1210.70	31
	ISNVIVNY	664-671	461.65	920.49	39
	QLRADVSPSIY	841-851	625.61	1247.65	26
	HRLIGEHEAQL	1019-1029	652.65	1301.68	56
	GDSTSPTL	1282-1289	389.46	776.35	7
	KIATEVVTGRF	1361-1371	611.04	1219.69	73
	VAKLPERTIF	1437-1446	392.51	1172.69	22

Table 2. Peptides identified from fraction 6 of the offline SCX; database-assisted

Identified Protein	Identified Peptide	Position Start-End	Observed m/z	Peptide Mass (Calc)	Mascot Score of Peptide
Vitellogenin-2 (gi: 558149531)	KIRSPQLEEY	70 - 79	631.99	1261.66	20
	NGIWPRDPF	80 - 88	552.04	1100.54	28
	VEVKNEHVSPPEIHL	264 - 278	576.08	1725.90	42
	KIHSEDL	384 - 390	421.67	840.43	33
	KIHSEDLSY	384 - 392	546.58	1090.52	47
	ANVDKDLIQQL	742 - 752	629.02	1255.67	37
	ATGPADRHTW	756 - 765	556.37	1110.52	48
	ATGPADRHTW	756 - 765	371.68	1110.52	13
	DAKIDLKEKSF	884 - 894	432.32	1292.69	28
	KPASADAAIDKIQL	1032 - 1045	721.08	1439.79	56
	KIATEVVTGRF	1361 - 1371	408.05	1219.69	33
	ADHPAIQVKL	1372 - 1381	546.46	1090.61	13
	EWPKVPSRF	1382 - 1390	573.80	1144.60	22

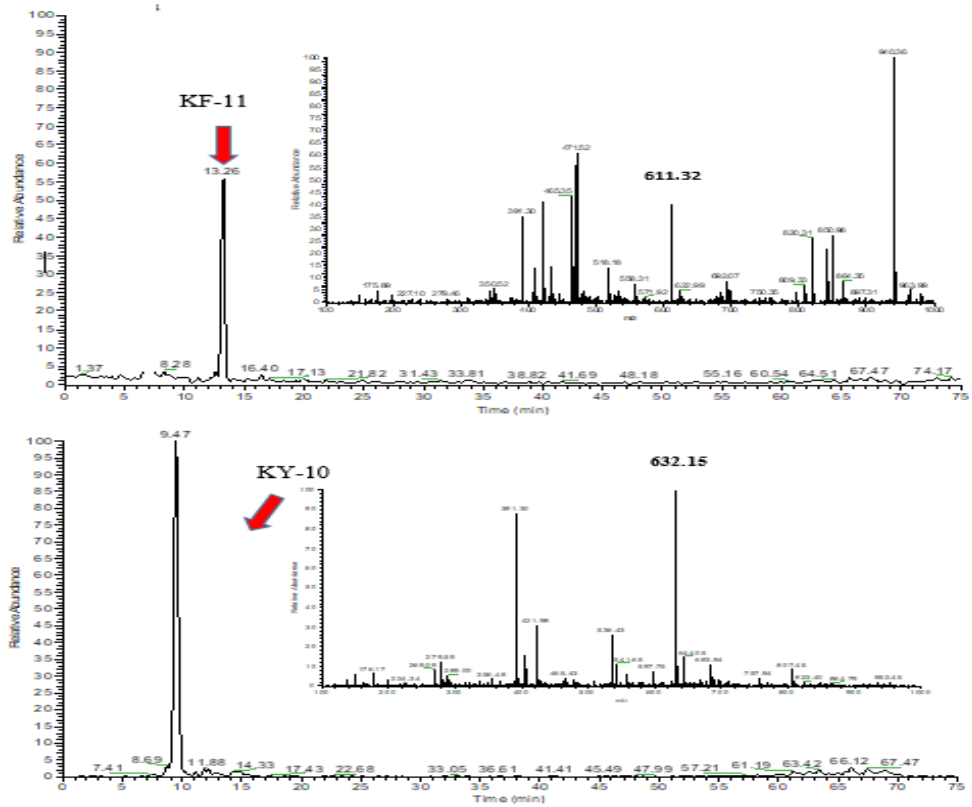

Figure 3. (a). LC-MS chromatogram and mass spectrum of KIATEVVTGRF with m/z 611.32; (b). LC-MS chromatogram and mass spectrum of KIRSPQLEEY with m/z 632.15

One of the basic theories of nutrition in traditional Chinese medicine is food as a tonic. Food as a tonic refers to using food to make a person stronger and feeling better in general. Chinese soft-shelled turtles have always been viewed as a tonic food and they have been eaten for thousands of years in China. They are believed to nourish yin (body fluid) and enhance liver function, which makes a person feel calmer. The eggs of the turtle are also thought to nourish yin and supplement deficiency. The medicinal effects of turtle eggs have been depicted in Materia Medica [9].

In the previous study, the egg whites of the soft shelled turtle showed the potential of containing an ACE inhibitory peptide. Considering that yolks are a rich source of proteins, it is worth obtaining bioactive peptides in order to increase the value of the yolks, even though turtle yolks are already abundant in Taiwan [10]. Egg whites are now understood to contain substances with biological functions beyond basic nutrition, and extensive research has been undertaken to identify and characterize these biologically active components. The IC_{50} from turtle yolk hydrolysate with a molecular weight of less than 3 kDa was compared with other hydrolysate peptides. The Chinese turtle hydrolysate (CTH) showed there to be a limited inhibition effect on ACE with an IC_{50} value at 280±8 μg/ml, while thegraded fraction of molecular weight was less than 5000 Da (CTH5K) obtained through membrane ultrafiltration exhibited better inhibitory activity (IC_{50}=190±5 μg/ml) [11]. The data thus suggests that a lower molecular weight of less than 3 kDa from the turtle yolk digested by chymotrypsin indicates its potential as ACE inhibitory peptides.

It is well known that the biological role of chymotrypsin in the intestine is to catalyze the cleavage of peptide bonds in which the carbonyl group is supplied primarily by aromatic or bulky hydrophobic amino acids (tyrosine, tryptophan, phenylalanine, leucine, isoleucine, and methionine). Chymotrypsin hydrolyze proteins with peptide bonds on the carboxyl side of the aromatic amino acids and, therefore might generate potent ACE inhibitory peptides in hydrolysates. It has been previously reported that peptides containing proline or aromatic residue (Trp, Tyr, or Phe) at their C-terminus and a branched

aliphatic (Val, Ile, or Leu) amino acid residue at the N-terminus involve or are involved in potent ACE inhibitory activities [12].

The fractionation of hydrolysate peptide using RP-HPLC and offline SCX chromatography are that methods use different working principals to separate the peptide fractions. However, in this study, the suggestion to use the different methods of fractionation was because some of the peptides may overlap in each fractions. Chymotrypsin hydrolysate showed that there were 2 peptides that have been overlapping during fractionation using RP-HPLC and SCX chromatography which were identified as KF-11 and KY-10. The result indicate that the overlapped peptides in fractions RP-HPLC and SCX chromatography can be potential ACE inhibitory peptides.

4. Conclusion

The low molecular weight <3 MWCO of chymotrypsin hydrolysate from soft shelled turtle yolk exhibits ACE inhibitions in vitro due to the presence of peptides that have the potential to be antihypertensive. The application of orthogonal bioassay guided fractionation using RP-HPLC and SCX methods can be considered an excellent separation technique.

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