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Angiotensin-I converting enzyme (ACE) inhibitory peptides from chicken skin gelatin hydrolysate and its antihypertensive effect in spontaneously hypertensive rats

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Abstract

Chicken skin gelatin hydrolysates and peptides with angiotensin converting enzyme inhibitory (ACEI) activity were produced enzymatically using alcalase, pronase E, and collagenase before fractionation into <2, <5, and <10 kDa fractions. The fraction with the highest ACEI activity was further purified sequentially using gel filtration chromatography RP-HPLC. The ACEI activity of <2, <5 and <10 kDa hydrolysate fractions were 69.64, 76.80 and 87.69%, respectively. Gel filtration chromatography of <2 kDa fraction produced peptides with ACEI activity (92%), and showed a similarity to the synthetic drug captopril (93%). The relative molecular mass range of the <2 kDa peptide fraction was 300-900 Da, comprising mainly Glu, H.Pro, Gly, Ala, Pro and Lys. Further separation using HPLC revealed a fraction with the sequence of one peptide: Gly-Pro-Ile-Gly-Pro-Pro-Ser-Gly-Gly-Phe-Asp (IC_{50}, 0.04 mg/mL). The antihypertensive effect of the purified peptide was supported by spontaneously hypertensive rats (SHR). Oral administration of a 15 mg/kg dose of the purified peptide decreased systolic blood pressure significantly for 24 h. These results suggest that the purified peptide derived from chicken skin gelatin hydrolysate has potential antihypertensive properties which can be used as antihypertensive agent in the food and pharmaceutical industries.

Introduction

Angiotensin-I converting enzyme (EC3.4.15.1; ACE) plays an important physiological role in regulation of blood pressure by converting angiotensin I to angiotensin II, a potent vasoconstrictor. Therefore, the inhibition of ACE activity is a major target in the prevention of hypertension (Wijesekara and Kim, 2010). Currently, synthetic inhibitors of ACE used as pharmaceuticals to treat hypertension include captopril, lisinopril, enalapril and fosinopril. However, the search for natural ACE Inhibitors leads to several undesirable side effects such as loss of taste, chronic dry cough, renal impairment and angioneurotic oedema. In contrast, the ACE inhibitory peptides derived from food proteins have not demonstrated these side effects.

The exploration of bioactive peptides from animal and plant food protein sources has been extensively performed. Studies have shown that a variety of ACE inhibitory peptides exist in different food protein sources. Peptides from the digestion of gelatin possess potent inhibitory activity against ACE, especially fish gelatin hydrolysate of different species. Numerous *in vivo* studies of marine-derived antihypertensive peptides in spontaneously hypertensive rats (SHR) have shown potent ACE inhibitory activity (Lee *et al*., 2010). Several studies have also suggested that among SHR there is suppression of hypertension with a diet rich in ACE inhibitory peptides (Hong *et al*., 2008). Many ACE inhibitory peptides have been identified from the enzymatic hydrolysis of various natural sources (Lee *et al*., 2010).

Enzymatic hydrolysis is widely used to improve and upgrade the functional and nutritional properties of food proteins (Kamara, 2011). Previously, preparation of ACE inhibitory peptide via hydrolysing fish gelatin with alcalase, pronase E, and collagenase

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was successfully conducted (Kim *et al*., 2001). We have recently successfully extracted gelatin from waste chicken skin and reported its physicochemical properties with a view to using it as an alternative to mammalian gelatin and to improve food supplies (Sarbon *et al*., 2013). However, there has been no study reporting bioactive peptide production from chicken skin gelatin, a waste by-product of chicken fillet production.

The aim of the present work was therefore to upgrade chicken skin to value-added products with potential health benefits, including ACEI peptides. The peptide produced were subjected to SHR for further investigation on its properties. This finding will add value to a poultry industry by-product that is usually discarded.

Materials and methods

Materials

Chicken skin was obtained from a local supplier, Surrey, UK. It was then chilled in ice while transported to the laboratory. The visible fat was mechanically removed, and the skin was thoroughly washed. The cleaned skins were weighed (wet weight) and stored at -80°C for further use.

ACE (Angiotensin Converting Enzyme, 6 units/mg) from rabbit lung, substrate peptide (hippuryl –L-histidyl- L- leucine, HHL), pronase E (from *Streptomyces griseus*, Type XIV, 4.8 units/mg) and collagenase from *Clostridium histolycum* (276 units/mg) were purchased from Sigma–Aldrich Company Ltd. Alcalase™ CLEA (9.87 U/g) was purchased from Fluka (Sigma–Aldrich Company Ltd). All other chemicals and reagents used were of analytical grade.

Methods

Chicken skin preparation

Thawed chicken skin was prepared by thoroughly rinsing in excess water in order to remove any remaining impurities. The skin was then cut into 2-3 cm pieces before freeze-dried. The dried skin was further ground and defatted using the Soxhlet method (Sarbon *et al*., 2013).

Gelatin extraction

Chicken skin gelatin was extracted following Sarbon *et al*. (2013) with slight modification. Defatted dried skin was mixed with sodium hydroxide (0.15%, w/v) at a ratio of 1:14 for 40 min before centrifuging at 3,500 g for 10 min. The resulting pellets were then treated with sulphuric acid (0.15%, v/v), followed by citric acid (0.7%, w/v), then centrifuged at 3,500 *g* for 10 min after rinsing with distilled water in between different solutions. In order to remove non-collagenous proteins and pigments, the treated alkaline and acid steps were repeated three times, and the solution was changed every 40 min. To remove any residual salts, the pellets were washed with distilled water and centrifuged at 3,500 *g* for 15 min. Final extraction of gelatin was conducted in distilled water at 45°C overnight. The resultant mixture was then filtered before further deionised to 50 µ Siemens/cm, using an Amberlite mixed bed resin (M B-6113) with pH adjusted to 6.0. The solution was finally evaporated at 45°C before being freeze-dried.

Enzymatic hydrolysis of chicken gelatin

Gelatin from chicken skin was hydrolysed with three different enzymes, namely alcalase™, pronase E and collagenase (Kim *et al*., 2001). A 1% (w/v) gelatin solution was prepared in deionised water and digested with alcalase (enzyme to substrate ratio, 1:50) at 50°C, for 6 h with constant shaking. The pH was maintained at 8.0 with 0.1 M NaOH. The hydrolysate was fractionated through a membrane ultrafilter (Vivaspin, Epsom, UK) with a 10 kDa molecular weight cut off (MWCO). The resultant fraction (F1) was then hydrolysed with pronase E (enzyme to substrate ratio, 1:33) for 6 h at 50°C at a constant pH of 8.0 and then fractionated using a 5 kDa MWCO membrane. Subsequently, the resultant fraction (<5 KDa) was hydrolysed with collagenase (enzyme to substrate ratio, 1:100) at 37°C for 6 h at pH 7.5. The resultant fraction from <5 KDa was ultra-filtered using a 2 kDa MWCO membrane to obtain three fractions (<2 KDa). Fractions <10 KDa, <5 KDa, and <2 KDa were then lyophilised and stored at -20°C until required for ACE inhibitory activity testing.

ACE inhibitory activity assay of peptides

The reaction mixture was made up of 50 µL 2.17 mM HHL, 10 µL 2 mU of ACE and 10 µL peptide fractions (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH, 8.3) to a total volume of 70 µL. The peptide fraction and HHL were combined and incubated at 37°C for 10 min in 2 mL polyethylene microcentrifuge tubes. ACE was also treated in the same way and incubated at 37°C for 10 min before the two solutions (HHL and peptide) were mixed together and incubated further at 37°C for 30 min with continuous agitation. After 30 min, 85 µL 1 M HCl was added to terminate the reaction, and vortexed. A positive control (HHL and enzyme) and blank (HHL and buffer) were also prepared in the same manner. ACE inhibitory activity was assayed

using HPLC (Waters: Alliance, Waters, UK) with an integrated detector (Dual l absorbance-Waters 2487) and separation module (Waters 2695).

Samples (10 µL) were injected on a C_{18} column (3.0 × 150 mm, 5 µm, Phenomenex). The elution peak of HA and HHL were detected at 228 nm. The column was eluted (1 mL/min) with a two-solvent system: (A) 0.05% (v/v) TFA in water, and (B) 0.05% (v/v) TFA in acetonitrile, with a 5-60% acetonitrile gradient for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 min. This was followed by isocratic elution for 4 min at a constant flowrate of 1 mL/min. ACE inhibitory activity was calculated using Eq. 1:

$$\text{ACE inhibitory activity (\%)} = 100-[(S-B)/(C-B) \times 100] \quad \text{(Eq. 1)}$$

where C = peak area of the control (buffer added instead of test sample); B = peak area of the reaction blank (without ACE and sample); and S = peak area of the sample. Data collection and analysis were undertaken using integration software (ChromoQuest 2004). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity under the assayed conditions and determined by regression analysis of ACE inhibition (%) versus inhibitor concentration (µM or mg/mL).

Gel filtration

The lyophilised low molecular weight peptide fraction (<2 KDa) with high ACE inhibitory activity was dissolved (200 mg) in 5 mL 50 mM sodium phosphate buffer (pH, 7.0). The solution was applied onto a SP-Sephadex G-25 (Sigma Chemical Co., St. Louis, MO) gel filtration column (2.0 × 90 cm), which was previously equilibrated with 50 mM sodium phosphate buffer (pH, 7.0). The column was then eluted with the same buffer at 1 mL/min at room temperature (22°C) for 10 h using an FPLC system (FPLC AKTA, Dionex Co., Camberley, Surrey, UK). Fractions (5 mL) were collected at a flow rate of 1 mL/min for all eluted peaks. The absorbance of the collected fractions was measured at 215 nm, and the ACE inhibitory activities of all fractions were quantified. The fractions showing the highest ACE inhibitory activity were collected from several GF chromatograms, pooled and lyophilised.

Molecular mass and sequencing of chicken gelatin peptide

The molecular mass of the purified peptide with high ACE inhibitory activity was determined by LC-MS spectrometry coupled with an electrospray ionisation (ESI) source. Each sample was made up to 5 mg/mL in buffer A (2%, (v/v) acetonitrile) and 0.1%, (v/v) formic acid and then filtered through 0.2 µm Millex-GV PVDF 4 mm filter (Millipore). One microlitre (1 µL) sample was mixed with 11 µL water, and 8 µL was injected into the LC (Ultimate 3000, Dionex UK) which is equivalent to 0.75 µL sample (3.75 µg). The analytical column used was a 25 cm, 75 µm ID Acclaim PepMap 100 C_{18} (Dionex, UK). The LC gradient was 2-50% buffer B, (90% (v/v) acetonitrile and 0.1% (v/v) formic acid), over 30 min followed by 90% (v/v) buffer B for 5 min. The mass spectrometry-IDA (independent data acquisition) experiment used a survey scan of 250-1200 m/z amu and 55 min data acquisition. Each cycle included two product ion scans with a mass range of 65-1200 m/z amu. The peptide sequence was analysed using MASCOT (v2.2.04, Matrix Science, London, UK) software.

Determination of amino acid composition of ACE inhibitory peptides

The amino acid content of chicken gelatin hydrolysate and peptide was determined following Sarbon *et al*. (2013). Approximately 2.0 g chicken gelatin hydrolysate and peptides were hydrolysed with 15 mL 6 N HCl in a screw cap tubes to yield free amino acids. The tubes were closed under nitrogen and heated in an oven at 110°C for 24 h. The derivatisation of hydrolysed samples and amino acid standards (20 µL) were conducted with phenylisothiocyanate (PITC) and were analysed in duplicate, by reverse phase HPLC.

Animals and measurement of blood pressure

Spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) (3 months old, male, 280-350 g body weight) were supplied by Animal Research and Service Centre, Health Campus, Universiti Sains Malaysia. The rats were housed in group of three in standard rat cages and allowed to acclimatise for 1 w under standard environmental conditions (24°C with 60 to 70% humidity) on a 12 h light-dark cycle. The animals were given standard rat Goldcoin (Chipsi Classic Heimtierbett, Germany) and tap water *ad libitum*. Single oral administration of peptide sample was done in 8 w SHR. Dosages of 15 mg/kg or 20 mg/kg dissolved in 1 mL saline solution were orally gavaged. Another group of rats was treated with losartan potassium at a dose of 20 mg/kg as a positive control. Three animals were used in each group, as this is a preliminary study. Losartan is a commercial anti-hypertensive drug which belongs to

the selective angiotensin II subtype 1 (AT1) receptor blocker group. Control rats were administrated with the same volume of saline solution. Systolic blood pressure (SBP) were measured at 0, 2, 6, and 24 h after administration using the tail-cuff method with a plethysmography BP system (IITC Life Science, California) (Ichimura *et al*., 2006). The study was approved by the Animal Ethics Committee, USM (USM/Animal Ethics Approval/ 2015/ (95) (635)).

Statistical analysis

All measurements were conducted in triplicate. One-way analysis of variance (ANOVA) was conducted, and data were expressed as mean ± standard deviation. The SPSS package version 16 was used for statistical tests determination for all analyses, and Least Significant Difference (LSD) test was used to evaluate significant difference ($p < 0.05$) between means.

Results and discussion

Enzymatic hydrolysis of chicken skin gelatin

The yield of fractionated chicken gelatin hydrolysates obtained after enzymatically hydrolysed with alcalase, pronase E and collagenase were 18 ± 0.05, 32 ± 0.11, 24 ± 0.23 and 26 ± 0.58% for crude hydrolysate, Fraction 1 (<10 KDa), Fraction 2 (<5 KDa) and Fraction 3 (<2 KDa), respectively. Fraction 1, Fraction 2 and Fraction 3 were the first, second and third permeates obtained for hydrolysates filtered through the 10, 5 and 2 kDa ultrafiltration membranes respectively, thus resulting in progressively smaller molecular size fractions. The findings show that the enzymatic hydrolysis using several enzymes resulted in higher yields due to greater peptide bond breakdown. The increased breakdown of peptide bond resulted in higher levels of hydrolysed protein in smaller fractions.

More recently, Gehring *et al*. (2009) reported that fish protein hydrolysates have been shown to inhibit ACE. Many studies have shown that lower molecular weight fractions have superior bioactive properties such as ACE inhibitory activity and antioxidant and anticancer activity as compared to larger molecular weight peptides. Common molecular weights of protein hydrolysate with better ACE inhibitory properties have been reported at <1 kda. The most common enzymes used in producing ACE inhibitory peptide includes alcalase, neutrase, pepsin, papain, α-chymotrypsin and trypsin. In addition, there were significant effects on ACE inhibitory activity peptides for single and combined enzymes as well as yield. The combination of alcalase and pronase E, as studied by Byun and Kim (2001), resulted in a lower and more effective ACE inhibitory activity with IC_{50} value of 0.555 mg/mL. In addition, collagenase (endopeptidase) produced peptides with a C-terminal Pro-X or N-terminal Gly-Pro sequence from fish skin peptide. Clostridial collagenase can also break up the native, triple-helical types I, II, and III collagen structure in several places into a mixture of small peptides. The present work showed that the combination of three enzymes alcalase, pronase E and collagenase yielded effective digestion of gelatin, resulting in high ACE inhibitory activity and low IC_{50} values.

In vitro ACE inhibitory activity of chicken gelatin peptides

The results of ACE inhibitory activities based on the formation of hippuric acid (HA) from the substrate HHL for the fractionated chicken skin gelatin hydrolysate (CGH), and a positive control captopril, a widely used antihypertensive drug, and their IC_{50} values are shown in Table 1. The ACE inhibitory activity of fractions 1, 2 and 3 was 69.64, 76.80 and 87.69%, respectively. Thus, digestion with alcalase and pronase E, followed by collagenase and sequential separation by ultrafiltration to smaller molecular weight peptides, increased ACE inhibitory activity with the <2 kDa fraction. This compares well with the positive control captopril (93.44%). Many studies of peptides from different proteins have shown that low molecular weight fractions possessed higher ACE inhibitory activity than high molecular weight fractions. In addition, the IC_{50} values, i.e., concentrations (mg/mL), at which enzyme activity is 50% inhibited were calculated using inhibition curves (regression lines). The fractions 1, 2 and 3 and captopril showed IC_{50} values of 5.36, 2.85, 1.31 and 0.02 µM, respectively. IC_{50} values decreased with the increase of ACE inhibitory activity. Based on these results, the most effective fraction (<2 kDa) was selected for further purification.

Table 1. ACE inhibitory activity of chicken skin gelatin hydrolysate fractionated using 2, 5 and 10 molecular weight cut off (MWCO) ultrafiltration membranes and GF28 fraction after gel filtration.

Hydrolysate	ACE inhibitory activity (%)	IC_{50} (µM)
F1	69.64 ± 0.95	4.83
F2	76.80 ± 0.44	2.57
F3	87.69 ± 0.71	1.18
(GF 28)	92.47 ± 0.34	0.04
Captopril	93.44 ± 0.11	0.02

IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity. F1 = fraction 1, F2 = fraction 2, F3 = fraction 3.

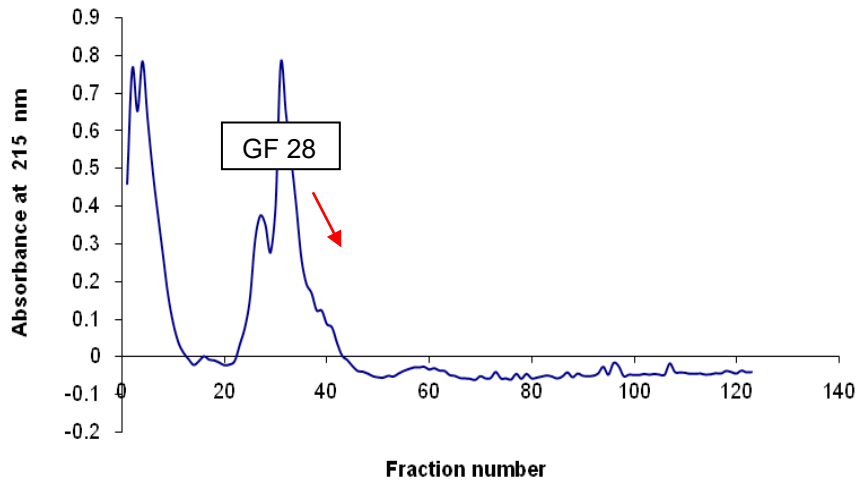

Figure 1a. FPLC chromatogram of F3 separated on a Sephadex G-25 column and detected at 215 nm. Separation was performed at a flow rate of 1 mL/min and collected at a fraction volume of 5 mL.

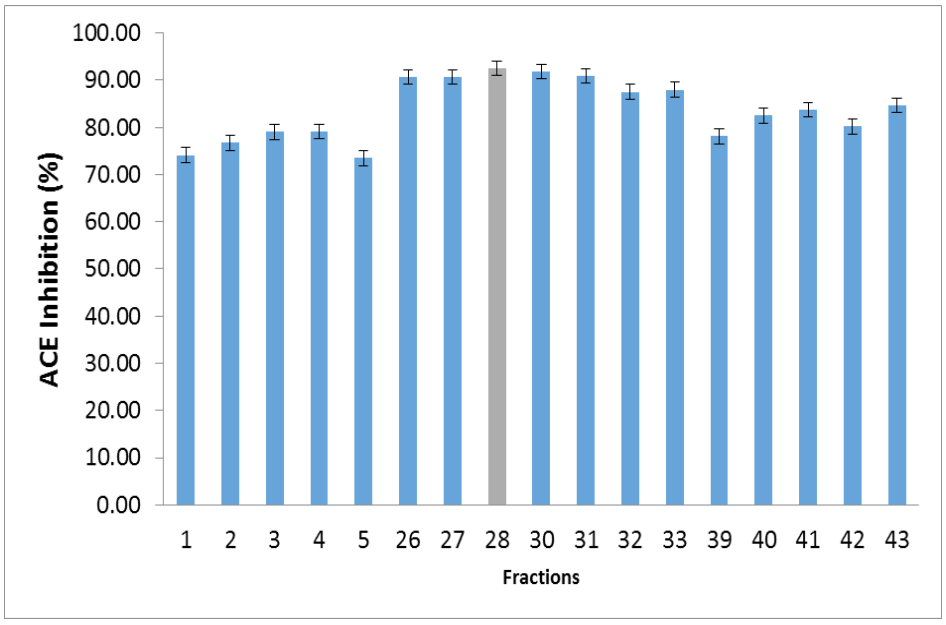

Figure 1b. ACE inhibition activity of F3 separated on a gel filtration Sephadex G 25 column. Tube number 28 showed the highest ACE inhibition activity of peptide with the IC50 value 0.04 µM.

Gel filtration

To separate the active peptides from chicken skin gelatin hydrolysates, partial purification of fraction 3 (<2 kDa) was undertaken by gel filtration (GF) using a size exclusion Sephadex G-25 column. Each separated peak was collected, lyophilised, and evaluated for ACE inhibitory activity, resulting in 17 selected tubes which numbered 1-5, 26, 27, 28, 30-33, 39, 40-43 (Figure 1a). Among the selected fractions collected, tube number 28 (GF 28) showed the highest ACE inhibitory activity with IC_{50} value of 0.04 µM (Figure 1b). The ACE inhibitory activity of peptides in fraction 3 increased from 87.69 to 92.47% with further purification (Table 1).

Molecular mass distribution of chicken gelatin peptides

In addition to the specific amino acid composition, the molecular weight of hydrolysate is a critical factor in producing peptides possessing ACE inhibitory activity (Byun and Kim, 2001). The molecular mass distribution of chicken gelatin peptides with higher ACE inhibitory activity (GF 28) is shown in Figure 2. The molecular mass of GF 28 is estimated to be in the range of 300-900 Da. which corresponds to 3-8 amino acids in the peptide fraction. The low molecular mass of the useful fraction produced by a combination of alcalase, pronase E and collagenase enzymes and ultrafiltration contributed to the high ACE inhibitory

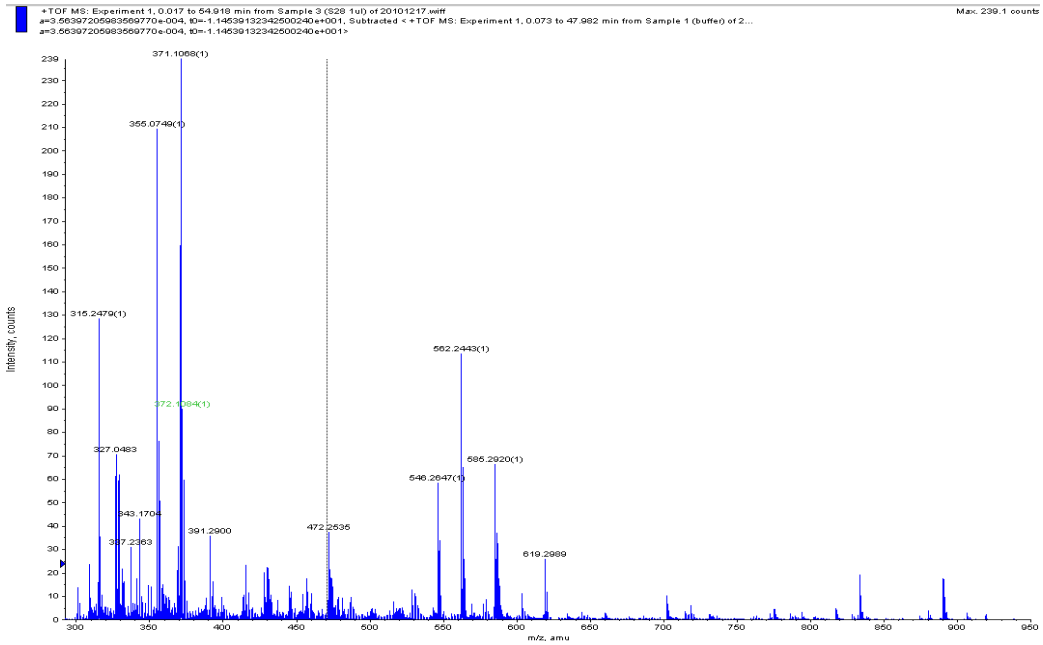

Figure 2. Mass spectrum of ACE inhibitory peptides of F3 obtained by LC/MS spectrometry.

activity. Most ACE inhibitory peptides have been reported to be short sequences containing 2–12 amino acids (Darewicz *et al*., 2014). Previously, many studies have reported that most peptides in the alcalase hydrolysate were small, with a molecular weight below 6.6 KDa and the highest activity was associated with small peptides (Matsui *et al*., 1993). This is because smaller peptides more easily bind to the ACE active site, resulting in decreased ACE inhibitory activity. This might partly explain the low inhibitory activity of the Fraction 1 (<10 KDa) conducted in the present work.

Identification of angiotensin-I converting enzyme (ACE) inhibitory peptides

Fraction GF 28 was collected over several runs, pooled, and subjected to LC-ESI-MS/MS to characterise the ACE inhibitory peptides. Even after HPLC, there were several peaks observed in the LC chromatogram (Figure 3a), which contained several peptides. Of these, one peptide was examined and found to be comprised of 11 amino acid residues with the sequence of Gly-Pro-Ile-Gly-Pro-Pro-Ser-Gly-Gly-Phe-Asp. Some researchers have reported that ACE inhibitory peptides have at least

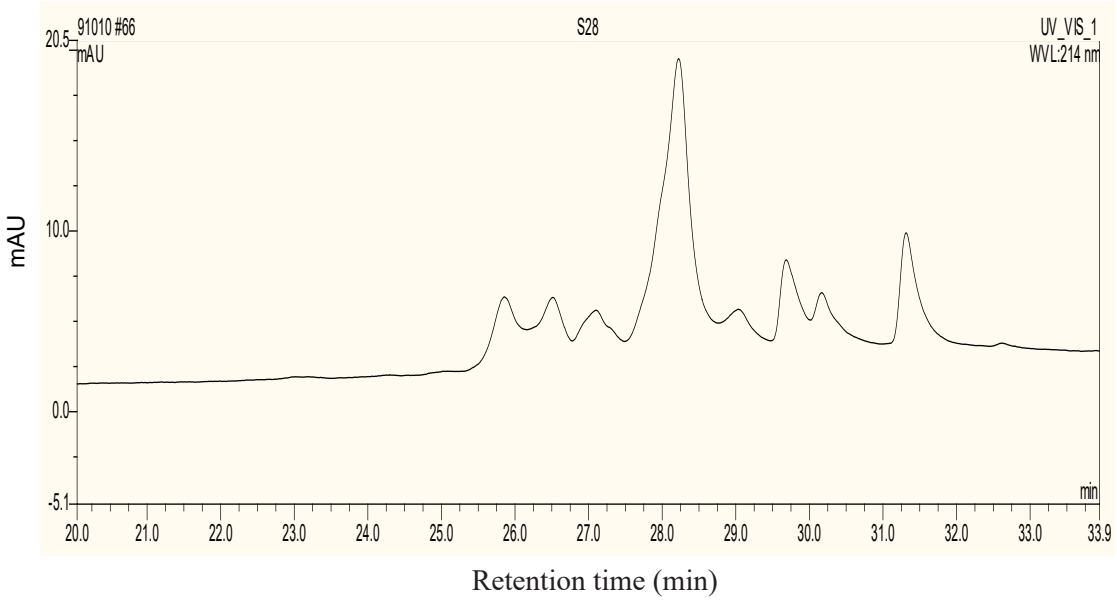

Figure 3a. HPLC chromatogram of peptides from fraction GF 28 separated on a 25 cm, 75 µm ID Acclaim PepMap 100 C18 column using a gradient 2-50% buffer B (90% ACN, 0.1% formic acid) for 30 min.

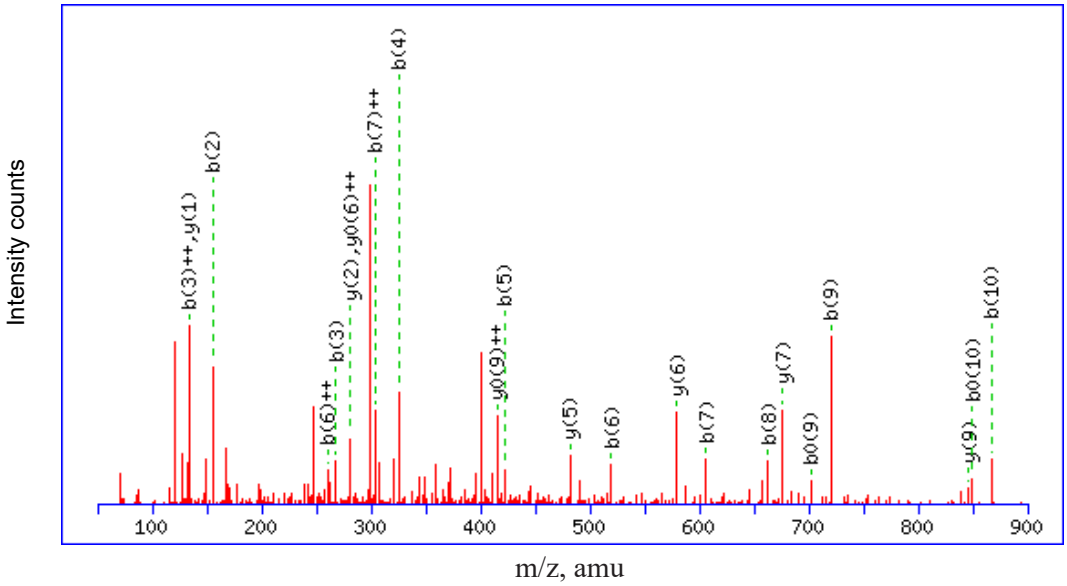

Figure 3b. ESI/MS/MS spectrum of the GF 28. Measurements were performed in positive ion mode using electrospray ionisation (ESI) and ESI/MS/MS, respectively.

one proline residue (Cheng *et al*., 2008). Similar results were observed in the present work where the isolated ACE inhibitory peptides comprised Gly-Pro at the N-terminal. Figure 3b shows the ESI/MS/MS mass spectrum of the $(M+H)^+$ ion and displays notation of fragment ion of the peptide according to the spectrum. The analysis of chromatograms generated for the mass-to-charge ratio (m/z) of many fragment ions derived from a selected precursor ion allowed the experimental retention time for peptides to be determined. Di- and tripeptides, for which large differences were found between predicted and experimental retention time, were regarded as identified if the experimental retention times in the tested samples were comparable (±1 min) to the predicted values.

Amino acid composition of chicken gelatin hydrolysate and peptide

The amino acid composition and sequence of chicken skin hydrolysates determine the efficacy of the peptide for ACE inhibitory activity. In the present work, the GF purified chicken gelatin peptide fraction with high ACE inhibitory activity was rich in Gly (33.64%), Pro (16.75%), H.Pro (13.06%), Ala (7.8%), Glu (5.48%), and Lys (6.88%) (Table 2). Chicken gelatin hydrolysate (<2 kDa fraction) contained predominantly Gly (33.04%), Pro (12.58%), H.Pro (10.11%), Tyr (8.32%), Ala (9.59%) and Arg (5.97%). The results show that enzymatic hydrolysis of chicken skin gelatin increased the amount of the hydrophobic amino acids. Further, purification of the hydrolysate to the GF peptide fraction increased Pro from 13.42% to 16.75% ($p < 0.05$); Trp from 0.09 to 0.43%; and Met from 0.38 to 2.4%. In addition, lysine (6.88%) and Asp (2.88%) were also significantly ($p < 0.05$) higher in the GF28 peptide fraction as compared to the gelatin hydrolysate (Lys 2.70%; Asp 1.29%). His, Tyr and Cys were detected in the hydrolysate but not in the GF28 peptide fraction. Thus, the higher

Table 2. Amino acid composition of chicken gelatin F3 fraction and GF 28 fraction as compared to the parent gelatin.

Amino acids	Gelatin (%)	Chicken gelatin F3 (%)	Chicken gelatin GF28 peptides (%)
Asp	2.11 ± 0.02	1.29 ± 0.00	2.88 ± 0.04
Glu	5.84 ± 0.01	3.81 ± 0.01	5.48 ± 0.10
H.Pro	12.13 ± 0.02	10.11 ± 0.03	13.06 ± 0.47
Ser	2.20 ± 0.00	2.53 ± 0.00	2.20 ± 0.04
Gly	33.70 ± 0.02	33.04 ± 0.04	33.64 ± 1.07
His	0.30 ± 0.01	0.41 ± 0.00	-
Arg	5.57 ± 0.00	5.97 ± 0.01	3.35 ± 0.10
Thr	1.01 ± 0.00	1.30 ± 0.00	0.80 ± 0.04
Ala	10.08 ± 0.02	9.59 ± 0.02	7.80 ± 0.19
Pro	13.42 ± 0.01	12.58 ± 0.00	16.75 ± 0.44
Tyr	1.22 ± 0.01	8.32 ± 0.05	-
Val	1.94 ± 0.02	2.25 ± 0.01	1.05 ± 0.03
Met	0.07 ± 0.00	0.38 ± 0.00	2.64 ± 0.06
Cys	0.16 ± 0.00	0.24 ± 0.00	-
Ileu	1.15 ± 0.00	1.30 ± 0.01	0.55 ± 0.02
Leu	2.63 ± 0.00	2.32 ± 0.00	2.09 ± 0.09
Phe	1.77 ± 0.00	1.78 ± 0.00	1.04 ± 0.03
Trp	0.04 ± 0.00	0.09 ± 0.00	0.43 ± 0.00
Lys	4.66 ± 0.00	2.70 ± 0.00	6.88 ± 0.23

Values are mean ± SD of three determinations ($n = 3$).

levels of Pro, H.Pro, Glu, Trp, Meth, Lys and Asp in chicken gelatin GF peptides as compared to the parent gelatin or ultrafiltered hydrolysate (<2 kDa fraction) may have contributed to the increased ACE inhibitory activity of the GF peptides. Several studies have reported that the hydrophilic group usually makes peptides inaccessible to the active site which results in a weaker inactive peptide, whereas the high potency of the peptide with ACE inhibitory activity is related to hydrophobic amino acids at their C-terminal and branched aliphatic amino acid at the N-terminal (Kasase, 2009). However, the results obtained in the present work also indicate high levels of lysine and aspartic acids in the GF28 fraction with the highest ACE inhibitory values that compared well with captopril. The present work demonstrates the feasibility of using chicken skin, a by-product of poultry processing, to produce value-added novel peptides with ACEI activity, which has the potential to improve health.

Antihypertensive effect of chicken skin gelatin peptide on SHR

The antihypertensive effect of the purified peptide was evaluated by measuring the changes of systolic blood pressure (SBP) in spontaneous hypertensive rats (SHR) at 0, 2, 6, and 24 h after oral administration of peptide. This was a preliminary study which aimed to assess whether the administration of single dose has an effect in reducing blood pressure within 24 h period (acute period). In the present work, Losartan was used as a positive control at a dose of 20 mg/kg of body weight. There was no change in SBP in the control group over the investigation period. As shown in Figure 4, SBP was significantly reduced in SHR rat of peptide at 15 mg/kg and 20 mg/kg as compared to SHR on vehicle after 2 h of oral administration for 162.67 ± 2.52 mmHg and 152.00 ± 1.41 mmHg, respectively. Furthermore, after 6 h of administration, peptide levels were significantly reduced in SBP as compared to WKY rat (15 mg/kg; $p < 0.001$), (20 mg/kg; $p < 0.0001$). The SBP of SHR rats remained significantly reduced after 24 h of peptide administration; as compared to WKY and Losartan ($p < 0.0001$). In contrast, Losartan reduced the SBP of SHR rat 6 h after administration, but this was not significant as compared to normal WKY rats. Losartan did not sustain lower blood pressure after 24 h as compared to peptides at both dosages; the lowering of blood pressure remained up to 24 h which means Losartan must be taken twice daily in order to maintain blood pressure low within 24 h, as SBP of SHR back to high (baseline) (182.00 ± 2.65 mmHg). The result suggested that the studied peptide produced

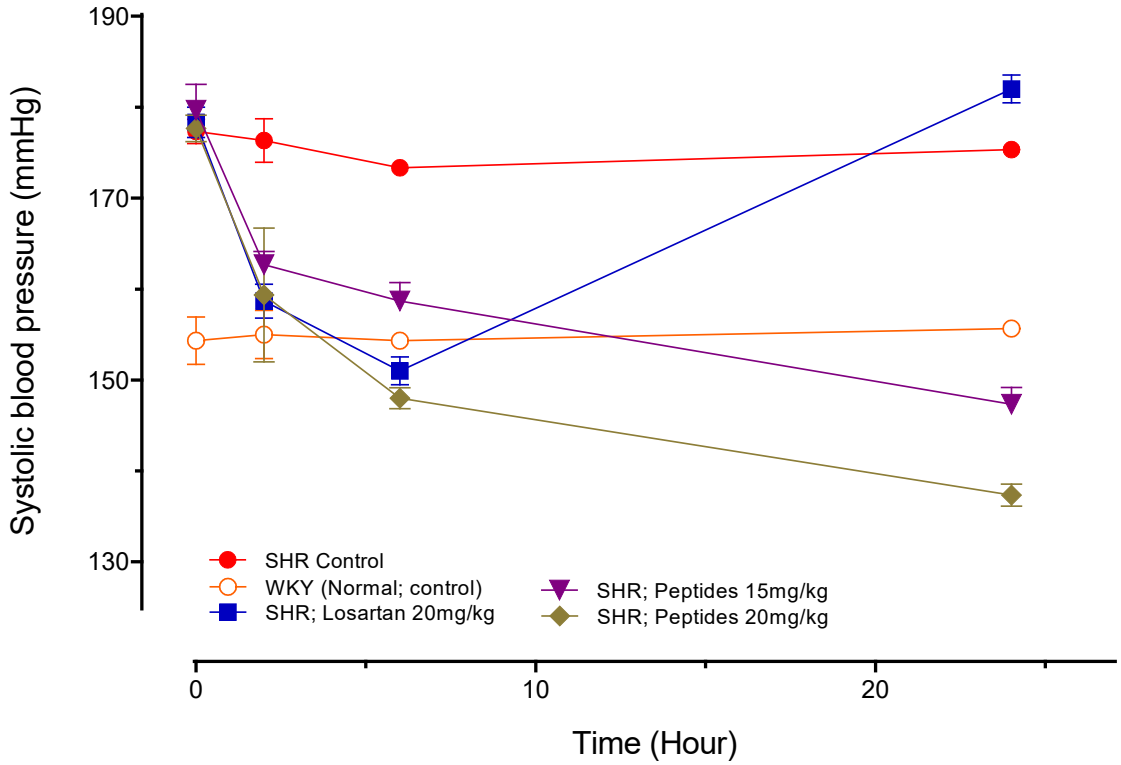

Figure 4. SBP trend after administration of Losartan (20 mg/kg), peptide (15 mg/kg and 20 mg/kg), and control (SHR and WKY without treatment), respectively. SBP was measured before the administration (0 h) and at interval of 2 h, 6 h and 24 h after administration.

a clear antihypertensive effect in oral administration of peptide at 15 mg/kg and 20 mg/kg to reduce SBP of SHR, and the reduction is sustained even after 24 h of administration. Furthermore, no allergic reactions were observed during the experiment. This suggests that ACE inhibitory peptide derived from hydrolysed chicken skin gelatin could have strong potential to be utilised to develop nutraceuticals and pharmaceuticals. However, the present work will be continued with repeated dose administration for a period of six weeks (sub-acute study) with more animals.

Conclusions

In conclusion, the present work has demonstrated that hydrolysate and peptide purified from chicken skin gelatin potentially allowed angiotensin converting enzyme inhibition (ACEI) activity, which is a new alternative source for hypertension treatment. The ACEI activity of chicken skin gelatin peptide was similar to the synthetic drug captopril used for hypertension treatment. The findings of the present work also demonstrated that chicken skin gelatin peptide had blood pressure lowering effect on SHR after 2, 6 and 24 h of post-administration and the effect was sustainable up to 24 h. These novel hydrolysates and peptides are not only useful for ACEI activity but will also help to improve poultry waste management in poultry industry.

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References

Byun, H. G. and Kim, S. K. 2001. Purification and characterization of angiotensin I converting enzyme inhibitory peptides from Alaskan pollack (*Theragra chalcogramma*) skin. Process Biochemistry 36(12): 1155–1162.

Cheng, F. Y., Liu, Y. T., Wan, T. C., Lin, L. C. and Sakata, R. 2008. The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. Animal Science Journal 79(1): 122–128.

Darewicz, M., Borawska, J., Vegarud, G. E., Minkiewicz, P. and Iwaniak, A. 2014. Angiotensin I-converting enzyme (ACE) inhibitory activity and ACE inhibitory peptides of salmon (*Salmo salar*) protein hydrolysates obtained by human and porcine gastrointestinal enzymes. International Journal of Molecular Sciences 15(8): 14077–14101.

Gehring, C. K., Davenport, M. P. and Jaczynski, J. 2009. Functional and nutritional quality of protein and lipid recovered from fish processing by-products and underutilized aquatic species using isoelectric solubilization/precipitation. Current Nutrition and Food Science 5(1): 17–39.

Hong, F., Ming, H., Yi, S., Zhanxia, L., Yongquan, W. and Chi, L. 2008. The antihypertensive effect of peptides: A novel alternative to drugs. Peptides 29(6): 1062–1071.

Ichimura, T., Yamanaka, A., Ichiba, T., Toyokawa, T., Kamada, Y., Tamamura, T. and Maruyama, S. 2006. Antihypertensive effect of an extract of Passiflora edulis rind in spontaneously hypertensive rats. Bioscience, Biotechnology and Biochemistry 70(3): 718–721.

Kamara, M. T., Amadou, I., Kexue, Z., Kelfala Foh, M. B. and Huiming, Z. 2011. The influence of debittering and desalting on defatted foxtail millet (*Setaria italica* L.) protein hydrolysate. American Journal of Biochemistry and Molecular Biology 1(1): 39–53.

Kasase, C. 2009. Antihypertensive and antioxidant activity of peptides derived from fish. United Kingdom: University of Surrey, PhD Thesis.

Kim, S. K., Kim, Y. T., Byun, H. G., Park, P. J. and Ito, H. 2001. Purification and characterization of antioxidative peptides from bovine skin. Journal of Biochemistry and Molecular Biology 34(3): 219–224.

Lee, S. H., Qian, Z. J. and Kim, S. K. 2010. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chemistry 118(1): 96–102.

Matsui, T., Matsufuji, H., Seki, E., Osajima, K., Nakashima, M. and Osajima, Y. 1993. Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolyzates derived from sardine muscle. Bioscience, Biotechnology, and Biochemistry 57(6): 922–925.

Sarbon, N. M., Badii, F. and Howell, N. K. 2013. Preparation and characterisation of chicken skin gelatin as an alternative to mammalian gelatin. Food Hydrocolloids 30(1): 143–151.

Wijesekara, I. and Kim, S. K. 2010. Angiotensin-I-Converting Enzyme (ACE) inhibitors from marine resources: Prospects in the pharmaceutical Industry. Marine Drugs 8(4): 1080–1093.