Effect of Molecular Weight Reduction of Polypeptides on Angiotensin Converting Enzyme (ACE) Inhibitory Activity in Chicken Skin Hydrolysate (Collagen)

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Abstract: Inhibition of Angiotensin Converting Enzyme (ACE) reduces blood pressure and gives an anti-hypertensive effect. Chicken skin is an undesirable by-product of the poultry industry, disliked by consumer because of the high fat content. The aim of this research is to determine the effect of molecular weight reduction on ACE inhibition activity in chicken skin hydrolysate. Chicken skin is prepared by manually defatting, soaked in acetone and in 0.1M phosphate buffer. Sample hydrolysis is carried out using alcalase enzyme for a duration of 4 hours at 60˚C and pH 9.5. The best degree of hydrolysis (DH), at 4th hour, with value of 49.54 ± 0.79 %, is ultrafiltrated and used in ACE inhibition activity detection. The sample weight ≥ 10 kDa , 3 – 10 kDa and ≤ 3 kDa contains 5.63 ± 0.01 g/L, 2.84 ± 0.06 g/L and 1.07 ± 0.18 g/L peptide content respectively whereas soluble protein content is 0.51 mg/mL for sample weight ≥ 10 kDa, 0.27 mg/mL for sample weight 3 – 10 kDa and 0.23 mg/mL for sample weight ≤ 3 kDa. The ACE inhibition activity in sample weight ≤ 3 kDa is highest with value of 80.38 ± 2.69% followed by sample weight 3 – 10 kDa with a value of 49.40 ± 2.63% and sample weight ≥ 10 kDa with value of 42.73 ± 5.08%. Significant differences (P ≤ 0.05) exist between sample weight ≤3 kDa and > 3 kDa. This research shows that molecular weight reduction increases ACE inhibition activity.

Keyword: Angiotensin Converting Enzyme (ACE), chicken skin, inhibitory, weight.

INTRODUCTION

Angiotensin Converting Enzyme (ACE) is an enzyme that effects hypertension or increases blood pressure. ACE is a dipeptidylcarboxypeptidase that converts the inactive decapeptide angiotensin I into the potent and active vasoconstrictor. It also deactivates the bradykinin, which functions as a vasodilating agent [1]. The inhibition on the ACE activity gives anti-hypertensive affect to individuals. The activity of ACE inhibitor can be observed in food containing protein, through the presence of bioactive peptides. Bioactive peptides are specific and dependent on the arrangement of the peptide chain. Certain peptide presence can inhibit the ACE activity. Although these peptides can be found in meat, the peptide presence is hidden in the core protein network and is only released in the presence of proteolysis enzymes, such as microbial and proteinase digestion [2].

Chicken skin is used as a raw material in this study because it is an unwanted by-product of the poultry industry, disliked by consumer and causes waste disposal problems [3]. Research by Onuh *et al*. [4] has reported that defatted chicken skin has high protein content which directly increases amino acid content and bioactive peptide availability which will increase the nutritional and functional value of chicken skin. Apart from that, research by Abedin and Riemschneider [5] reported that chicken skin contains 75% type I collagen and 15 % type III collagen, which can release the amino acid proline and hydroxyproline, that plays a role in the ACE inhibitory peptide [6, 7]. This is also supported by Kim [8] that states chicken skin is a source of collagen and can be used as alternative to collagen from bovine and porcine.

The objectives of this study is to determine the ACE inhibition activity in chicken skin hydrolysate and to determine the effect of molecular weight reduction of polypeptides on the ACE inhibition activity in chicken skin hydrolysate.

MATERIALS AND METHODS

Sample Preparation

The chicken skin, obtained from Bandar Baru Bangi market was manually defatted to produce 250 g of raw material. The chicken skin was then defatted according to Onuh *et al*. [4] with modification. The manually defatted sample was then soaked in acetone with a ratio of 1 g to 5 ml for 2 hours until fat layer is effectively removed. Acetone was used because it is an organic solvent that can solubilise the fat portion while the protein portion is precipitated. It is also a convenient fat removal technique because it evaporates easily and can be removed from the sample rapidly without affecting the protein fraction of the sample compared to the traditional method of using

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detergent where there is a difficulty in the separation of the solvent from the sample [9]. Sample is dried overnight in the fume hood to evaporate excess acetone. Sample in petri dish is freezed at -20 ˚C for 24 hours and subsequently freeze dried. The freeze dried sample is blended using a Warring blender to produce dried powder before soaking in 0.1M phosphate buffer, pH 6.6 at a ratio of 1g to 40 mL for 2 hours at 37 ˚C. The sample is then centrifuged at 10000 x g for 40 minutes to produced sample palets, that are air dried in fume hood and used in the consequent test.

Hydrolysis

The pallet was suspended in distilled water with ratio of 1:100 (w/v) and incubated with 1 % alcalase (pH 9.5; 60˚C) for duration of 4 hours. The enzymatic hydrolysis was stopped by boiling for 5 minutes. The hydrolysate was centrifuged at 3000 g for 20 minutes and the soluble aqueous fraction was decanted and lyophilized.

Degree of Hydrolysis

The degree of hydrolysis was determined using method modified o-phthaldialdehyde (OPA) spectrophotometric assay [10, 11]. Fifty milliliters of fresh OPA reagent was prepared by mixing 25 ml of 100 mM sodium tetra hydroborate, 2.5 ml of 20% (w/w) sodium dodecyl sulphate, 40 mg of OPA solution (dissolved in 1 ml of methanol) and 100 ml of b-mercaptoethanol and then adjusted the volume to 50 ml with deionized water. 50 microlitres of hydrolysate, containing 5–100 lµg protein, was mixed with 2 ml of OPA reagent and incubated for 2 min at room temperature. The absorbance at 340 nm was measured with a spectrophotometer (Model UV-160A). The degree of hydrolysis was calculated as follow:

$$\%\ \text{Degree of Hydrolysis (DH)} = \frac{h}{h_{total}} \times 100$$

Where h_{total} is total peptide bind per protein equivalent and h is number of peptide hydrolysate.

Ultrafiltration

The hydrolysate solution was filtered by 0.2 µm membrane and separated into large and low molecular weight fractions by ultrafiltration at 4˚C using 10 kDa Molecular weight cut-off (MWCO) membrane (Vivaflow 200) and 3 kDa MWCO (Vivaflow 50) membrane. Both membranes were activated by spinning 100 ml of deionized water prior to use. This permeate was defined as small peptides with molecular weight ≤ 3kDa, 3 - 10 kDa and ≥10 kDa.

Peptide Content

The peptide contents of hydrolysates were measured by the method of Church *et al.* [12] with some modifications using o-phthaldialdehyde (OPA) spectrophotometric assay. Fifty milliliters of fresh OPA reagent was prepared by mixing 25 ml of 100 mM sodium tetra hydroborate, 2.5 ml of 20% (w/w) sodium dodecyl sulphate, 40 mg of OPA solution (dissolved in 1 ml of methanol) and 100 ml of b-mercaptoethanol and then adjusted the volume to 50 ml with deionized water. 50 microlitres of hydrolysate, containing 5-100 µg protein, was mixed with 2 ml of OPA reagent and incubated for 2 min at room temperature. The absorbance at 340 nm was measured with a spectrophotometer (Model UV-160A) using Casein tryptone in phosphate buffer (pH: 7.4) as the standard to quantify the peptide content.

Protein Solubility

The Folin–Lowry method was used to determine the soluble protein content [13]. An aliquot of 0.5 ml of the sample was mixed with 0.7 ml of an alkaline-copper reagent and incubated for 20 min at room temperature. The mixture was added to 0.1 ml of the Folin–Ciocalteu's phenol reagent at 2-fold dilution with deionized water and allowed to stand for 30 min or longer at room temperature. The absorbance at 750 nm was measured with a spectrophotometer (Model UV-160A). The soluble protein content was quantified using bovine serum albumin as the standard.

Determination of Ace Inhibitory Activity

The ACE inhibitory activity was assayed with RP-HPLC modified from the spectrophotometric method described by Wu *et al*. [14]. Commercial ACE (1 unit) was diluted in 50 mM Tris–HCl (pH: 7.5) containing 300 mM NaCl to obtain concentration of 100 mU/ml. Aliquots of 100µl were then stored at -20˚C. A volume of 25 µl containing 1.0 mg/mL concentration of different weight hydrolysate was added to 100 µl solution containing 5 mm Hippuryl-L-Histidyl-LLeucine (HHL). Sample hydrolysate and HHL were prepared in 100 mM Na–borate buffer, pH 8.3, containing 300 mM NaCl. After incubation at 37˚C for 10 min, 10 µl of ACE (100 mU/ml) was added and samples were incubated

for 30 min at 37˚C. The enzyme reaction was stopped by the addition of 100 µl of 1 M HCl. The solution was filtered through a 0.45 µm nylon syringe filter and injected directly onto a symmetry shield C18 column to separate the product and hippuric acid (HA) from HHL. The column was eluted with 50% methanol in water (v/v) containing 0.1% TFA at a flow rate of 1 ml/min and the absorbance was measured at 228 nm. The instrument consisted of a Jasco PU-980 intelligent pump equipped with Waters 2487 dual absorbance detector. The evaluation of ACE inhibition was based on the comparison between the concentration of HA in the presence or not (control sample) of an inhibitor. After injection in HPLC of the control and the assay with inhibitor, the HA peak areas obtained in the two cases were measured.

$$\text{Percentage of ACE inhibition activity} = \frac{B - A}{B} \times 100$$

where A is the relative area of HA peak generated in the presence of ACE inhibitor component and B the relative area of HA peak generated without ACE inhibitors.

Statistical Analysis

Results were compared using mean ± standard deviation between samples containing enzyme and samples not containing enzyme. The significance value ($p<0.05$) between samples was determined using Duncan Multiple Test and the program used is SPSS.

RESULTS AND DISCUSSION

2.0 g of chicken skin was manually defatted before soaking in acetone to remove the fat layer from the skin. The sample is then freeze dried and soaked in 0.1 M phosphate buffer, at pH 6.6 and temperature 37˚C to produce 51.17 g of dried raw chicken skin sample. The dried chicken skin contains 24.55% protein and 3.25% fat. The low fat content in sample indicates an efficient fat removal. The low fat content in the sample is also desirable in hydrolysis process to reduce the formation of protein-lipid complex, which affects the formation of a stabil protein hydrolysate, and subsequently affects the angiotensin converting enzyme (ACE) inhibitory activity [15, 16].

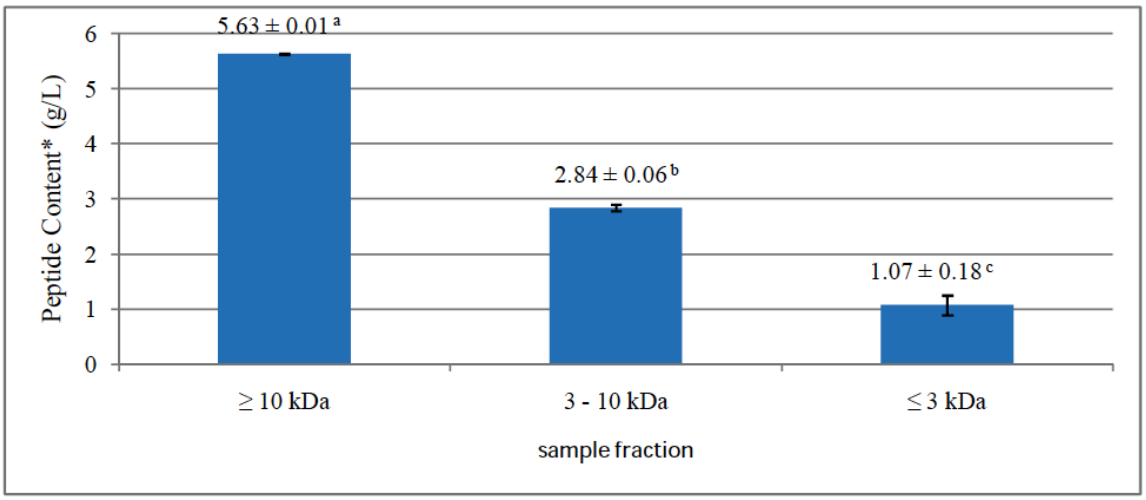
Based on Table **1**, the highest degree of hydrolysis is at the fourth hour with a value of 49.54 ± 0.78%. The high value at fourth hour indicates who more peptide bonds are broken down by the enzyme in that time duration. A similar result was reported by Zhang *et al*. [17], where increase in time indicates a higher degree of hydrolysis (DH) value. Although the DH value does not achieve the desired 80% to increase the detection of functional properties of bioactive peptides as indicated by Kristinsson & Rasco [18], study by Zhang *et al*. [17] reported that DH value does not affect ACE inhibitory activity, but the ACE inhibitory activity is affected the the ability of the enzyme used in producing specific protein chain containing hydrophobic amino acid at the C- terminal of tripeptides. This is supported by Arihara *et al*. [19] that reported that ACE inhibitory activity is affected by the enzyme specific activity in producing specific peptide chain.

Table 1: Degree of Hydrolysis (DH) for Chicken Skin Hydrolysed with Alcalase At Different Time

Time	Degree of Hydrolysis (%)*
1 hour	21.65 ± 2.37[a]
2 hour	28.11 ± 2.00[b]
3 hour	48.99 ± 4.26[c]
4 hour	49.54 ± 0.79[c]

[a-c]Same letter at different column indicates no significant difference (P > 0.05).
*Mean ± SD for 3 sample/treatment.

Peptide content are then analysed using o-phthaldialdehyde (OPA) spectrophotometric assay to ensure presence of peptide in samples, which may affect ACE inhibitory activity. Spectrophotometer operates on the basic principle of passing a beam of light, which consist of a stream of photons, through a sample and measuring the intensity of light reaching the detector. The fraction of light from the original beam that passes through the sample and reaches the detector is known as transmitter, whereaas absorbance is the fraction of light that do not reach the detector and is absorbed by the sample. The absorbance value measured by the spectrophotometer is used in the determination of the concentration of a sample because it indicates the amount of photon absorbing molecules in the sample. The peptide content of the sample are summarized in Graph **1**, where the highest peptide content is in sample with molecular weight ≥ 10 kDa with value of 5.63 ± 0.01g/L and sample with molecular weight ≤ 3 kDa has peptide content of 1.07 ± 0.18g/L. The low peptide content present in sample with smaller molecule weight may be attributed to the ultrafiltration process which may have separated the larger weight peptides from the sample causing a low peptide content value. This theory is also supprted by Wu & Ding and Tsai *et al*. [14, 20] which states that

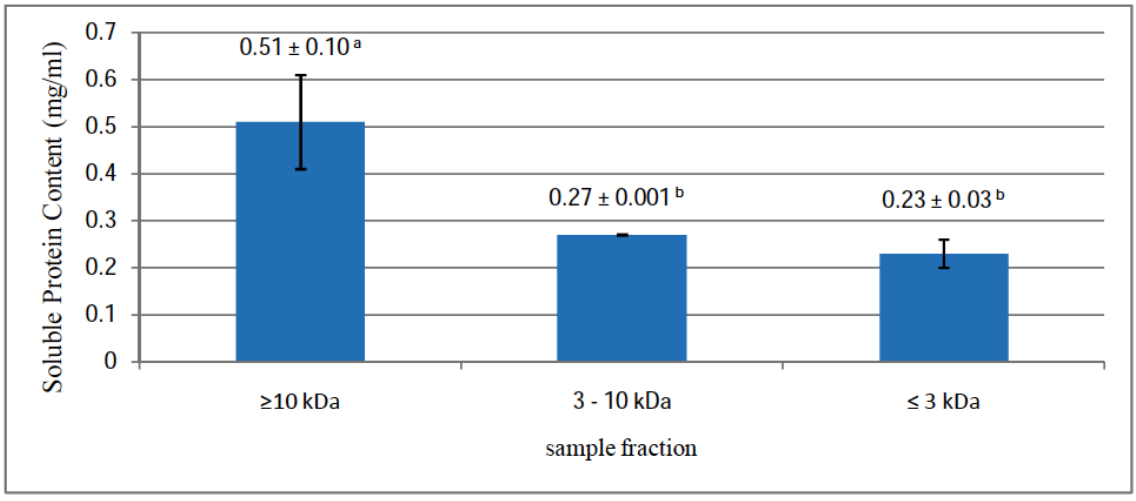


Graph 1: Peptide Content in ultrafiltrated sample fraction of chicken skin hydrolysate*.
[a-c]Same letter at different samples indicates no significant difference (P > 0.05).
*Mean ± SD for 3 sample/treatment.

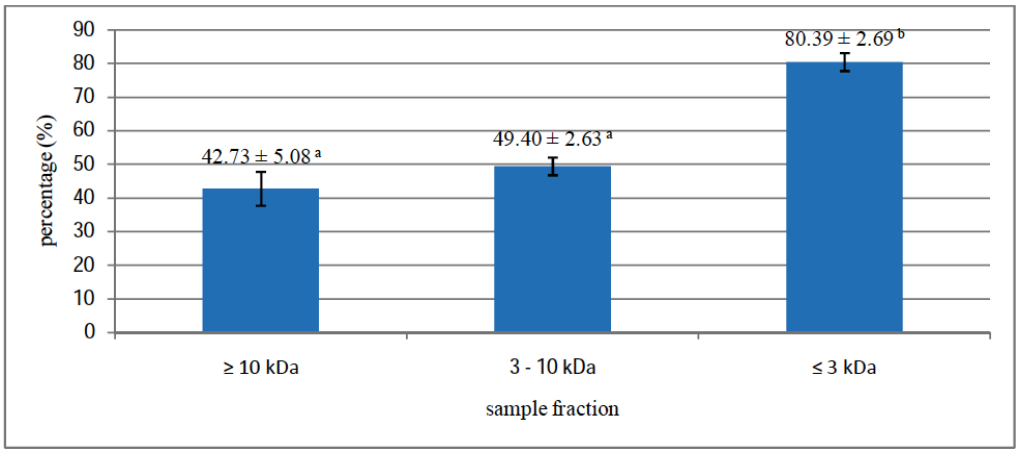
ultrafiltration process causes a lower peptide content and amino acid content in sample and sample with smaller molecular size, will have a lower peptide content.

The soluble protein content in sample with different molecular weight are then measured using Folin- Lowry method and summarized in Graph **2**. The sample with molecular weight ≥ 10 kDa has the highest soluble protein content that is 0.51 ± 0.10 mg/mL whereas sample with molecular weight ≤ 3 kDa has the lowest soluble protein content that is 0.23 ± 0.03mg/mL. The low soluble protein content obtained from sample with lower molecular weight may be caused by the separation of large molecular weight peptides from sample during the ultrafiltration process. Soluble protein content is analysed in the research to evaluate the content of the tyrosin, cysteine, cystine, histidine dan tryptophan in the samples. These amino acids are able to convert the molybdate in folin-phenol reagent into the blue molybdium [21, 22]. According to [23-26], ACE inhibitory activity is affected by the presence of the amino acid proline, phenylalanine or tyrosin at the terminal-C of peptide chain. Therefore the increase of the soluble protein content, may increase presence of these amino acids which can affect the ACE inhibitory activity.in the ultrafiltrated sample fraction hydrolysates.

Graph **3** summarizes the result obtained for the percentage of ACE inhibitory activity in the ultrafiltrated sample fraction hydrolysates at concentration of 1.0 mg/mL. The ACE inhibitory activity is calculated by



Graph 2: Soluble Protein Content in ultrafiltrated sample fraction of chicken skin hydrolysate*.
[a-c]Same letter at different samples indicates no significant difference (P > 0.05).
*Mean ± SD for 3 sample/treatment.



Graph 3: Percentage of angiotensin converting enzyme (ACE) inhibitory activity in ultrafiltrated sample fraction of chicken skin hydrolysate*.

[a-c]Same letter at different samples indicates no significant difference (P > 0.05).

*Mean ± SD for 2 sample/treatment.

comparing the area of the hippuric acid peak and the control. Sample with low molecular weight (≤ 3 kDa) has the highest ACE inhibitory activity percentage with value of 80.39 ± 2.69% followed by sample with molecular weight between 3 – 10 kDa with value of 49.40 ± 2.63% and sample with molecular weight ≥ 10 kDa with value of 42.73 ± 5.08%. Sample with molecular weight of 3 – 10kDa and sample with molecular weight ≥ 10 kDa does not have any significant difference (P ≤ 0.05) and this may indicate that peptides with larger molecular weight are present in the 3 – 10 kDa sample fraction. Similar findings are reported by Ghassem *et al*. [24] whereby sample with molecular weight less than 3 kDa has the highest ACE inhibitory activity and [27] where sample with molecular weight less than 1 kDa has the highest ACE inhibitory activity. Campos *et al*. [27] reported that the ACE inhibitory activity is affected by the peptide chain containing less than 6 hydrophobic amino acids and this is supported by Zhang *et al*. [17] that reported the ACE inhibitory activity is affected by the presence of hydrophobic amino asid at the C- terminal of a tripeptide.

Based on Graph **3** and Graph **1**, the result obtained for the ACE inhibitory activity and the peptide content in the sample fraction are inversely proportional. The low molecular weight sample (≤ 3 kDa) has the lowest peptide content but the highest ACE inhibitory activity. Kapel *et al*. and Tsai *et al*. [28, 29] also obtained a similar result whereby the high peptide content in a sample does not affect ACE inhibitory activity but sample with molecular weight less than 400 Da gave high ACE inhibitory activity result. Byun and Kim [30] reported that the ultrafiltation process using a 3 kDa membrane does not only separates the molecular weight of peptides in samples, but also concentrates the lower molecular weight sample which helps in the ACE inhibitory activity detection. The result obtained from this research indicates that ACE inhibitory activity is affected by the molecular weight of the polypeptides in the sample and not the peptide content in the sample.

CONCLUSION

Chicken skin contains peptides that are able to inhibit angiotensin converting enzyme (ACE). ACE inhibitory activity is affected by the molecular weight of polypeptide in the sample. The percentage of ACE inhibitor is highest in sample fraction with weight ≤ 3kDa followed by sample fraction weight 3 – 10 kDa and sample fraction weight ≥ 10 kDa. The peptide content and soluble protein content does not affect the ACE inhibitory activity in this analysis. This is summarized from the result of the peptide content and soluble protein content which is lowest for the sample with molecular weight ≤ 3kDa and highest for sample with molecular weight ≥ 10 kDa. The low value for peptide content and soluble protein content for sample with molecular weight ≤ 3kDa may be caused by the loss of peptides with a larger molecule size during ultrafiltration. Based on the result of peptide content and ACE inhibitory activity in the samples, it can be concluded that ACE inhibitory activity is not affected by peptide with large molecule size in the sample.

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