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Effect of enzymatic hydrolysis on Angiotensin converting enzyme (ACE) inhibitory activity in swiftlet saliva

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Abstract

Angiotensin I-converting enzyme (ACE) plays an important role in reducing blood pressure and gives an anti-hypertensive effect. Inhibition of ACE mainly results in an overall antihypertensive effect. The objectives of this study were to determine the ACE inhibition activity in edible bird nest hydrolysates and the effect of different hydrolysis time of 30, 60, 90, 120, 180 and 240 minutes. Edible bird nest protein was hydrolysed by enzymatic hydrolysis with alcalase and papain to obtain ACE inhibitory peptides. The results suggested that 60 minutes of hydrolysis time using alcalase contributed to the best ACE inhibitory activity (IC_{50} = 0.02mg protein/ml) which shows edible bird nest protein hydrolysate to be a potent ACE inhibitor that may be used to decrease blood pressure.

Introduction

Angiotensin I-converting enzyme (ACE) is an important component in regulation of blood pressure as well as cardiovascular function. It is an enzyme that cause hypertension or increase blood pressure. ACE is a dipeptidylcarboxypeptidase that converts the inactive decapeptide angiotensin I into a potent and active vasoconstrictor. It also deactivates bradykinin, which functions as a vasodilating agent (Li *et al*., 2004). Thus, the inhibition of ACE may give an antihypertensive effect to individuals. The ACE inhibitor can be obtained in food containing protein, through the presence of bioactive peptide.

Bioactive peptide is specific and dependent on the arrangement of the peptide chain. The presence of peptide of interest can inhibit the ACE activity. Although the peptides can be found in meat, the peptide presence is hidden in the parent protein network and can only be released in the presence of proteolysis enzymes, such as microbial and proteinase digestion (Norris and Fitzgerald, 2013). ACE inhibitory peptides derived from food proteins are suitable candidates for such products. Many ACE inhibitory peptides have recently been discovered from enzymatic hydrolysates of different food proteins (Guan *et al*., 2007).

Edible bird nest (EBN) is the most expensive animal products made up by salivation of Aerodramus swiftlet species ranging from 90% to 95% of the edible nests and 5% to 10% wool and dirt (Lim and Cranbrook, 2002; Ibrahim *et al*., 2009). Traditionally, EBN was prepared by soaking it in water for several hours or overnight to remove excess feather from the nest. EBN is usually prepared by boiling them in a solution of rock sugar, and is known as the 'bird's nest soup' (Lim and Cranbrook, 2002).

The major ingredients of edible bird nest are glycoproteins, unique compound build up from carbohydrate and protein components (Kathan and Weeks, 1969) and also contain essential trace elements such as calcium, sodium, magnesium, zinc, manganese and iron (Marcone, 2005). Traditionally used as a food delicacy, EBN is also an important ingredient in traditional Chinese medicine for health-enhancing effects ranging from enhancing complexion, alleviating asthma, and strengthening the immune system (Lim and Cranbrook, 2002).

For medicine purposes, EBN extract has been shown to stimulate mitosis hormones and the growth factor for epidermal growth (Ng *et al*., 1986; Kong *et al*., 1987) and is known to have exerted strong inhibitory effect on influenza viruses in a host range-independent manner (Guo *et al*., 2006). At the same time, carbohydrate in EBN was found to contain among others, sialic acid and glucosamine

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(Tung *et al.*, 2008) which has been associated with enhanced brain functions in infants (Colombo *et al*., 2003). High in demand and highly priced in the global market, EBN cultivation has increased dramatically in EBN-producing countries including Malaysia (Hobbs, 2004). Despite many health claims associated with EBN consumption, the mechanism of action is unknown and there is no clinical evidence to support those claims.

This study was conducted to determine the ACE inhibition activity in edible bird nest hydrolysate and the effect of different time of hydrolysis on the ACE inhibition activity from the hydrolysate obtained.

Materials and Methods

Sample preparation

Raw cleaned Edible Bird Nest (EBN) from bird house at Rompin, Pahang was purchased from Nest Excel Resources Sdn. Bhd. located in Kuala Lumpur. Edible bird nest was transferred into air tight containers and kept at ambient temperature (25°C to 30°C) until further analysis.

Edible bird nest (EBN) peptides were prepared according to the method by Choa *et al.* (2004) with some modifications. Raw EBN was soaked in distilled water (1:100) and kept in chiller (4°C) for 16 hours. Then, the soaked materials was double boiled in 100°C water for 30 minutes. The sample was then be cooled down to room temperature and ready for enzymatic hydrolysis and further experiments.

Enzymatic hydrolysis

The EBN suspension was incubated with two different enzymes; papain using the ratio of 1:100 (w/v); pH 7; 60°C and alcalase (1:100 w/v, pH 8, 60°C) for 30, 60, 90, 120 180 & 240 minutes. The ratio of each enzyme to the EBN protein fractions was 1:100 (w/w) (Jang and Lee, 2005). The enzymatic hydrolysis was stopped by boiling for 5 min. The hydrolysate was filtered with Whatman 4 filter paper and the filtrate was freeze dried.

Degree of hydrolysis

The degree of hydrolysis (DH) was estimated as described by Hoyle and Merrit (1994). At the end of each hydrolysis time, an aliquot of 10 ml of the aqueous suspension of enzyme-treated fractions was removed and mixed with 10 ml of 20% trichloroacetic acid (TCA) to obtain 10% TCA-soluble nitrogen and 10% TCA-insoluble nitrogen and then centrifuged at 15770 x g for 15 minutes at 4°C. The supernatant was decanted and analyzed for soluble nitrogen using Kjeldahl method (Kjeltec TM 2100, Foss, Denmark). The percentage DH is expressed as follows:

Degree of Hydrolysis (%) = (10% TCA-soluble N in sample/ total N in sample) x 100

Peptide content

The peptide content of hydrolysates were measured by the method of Church *et al.* (1983) 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 µl of hydrolysate, containing 5-100 g 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 a standard to quantify the peptide content.

Protein solubility

The Folin–Lowry method was used to determine the soluble protein content (Lowry, 1951). 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 a standard.

Assay of ACE inhibitory activity

ACE inhibitory activity was tested using the method of Cushman and Cheung (1971). Briefly, a sample solution (50 µl) with 50 µl of ACE solution (25 mU/ml) was incubated at 37°C for 10 min, and the mixture was pre-incubated with 150 µl of substrate (8.3 mM HHL in a 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37°C for 30 min. Finally, the reaction was stopped by the addition of 1 M HCl (250 µl) and the resulting hippuric acid was extracted by the addition of 500 µl ethyl acetate. After centrifugation (3000 rpm, 10 min), 200 µl of the upper layer was transferred into a glass tube and dried at 90°C for 15 min. The hippuric acid was re-dissolved in 1 ml of distilled water and the absorbance was measured at 228 nm using a fluorescence micro-plate reader (Tecan Austria

GmbH, Grodig/Salzburg, Austria). The IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

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 SAS.

Results and Discussions

Edible bird nest contain mainly glycoprotein component (Marcone, 2005) and very low fat content (Huda *et al.*, 2008). The low fat content in the sample is desirable in hydrolysis process to reduce the formation of protein-lipid complex, which affects the formation of a stable protein hydrolysate, and subsequently affects the angiotensin converting enzyme (ACE) inhibitory activity (Goh *et al*., 1999). Edible bird nest refers to the nest produced by several different swiftlet species which is composed almost entirely of a glutinous materials found in saliva secreted from the swiftlet's two sublingual salivary glands (Goh *et al.*, 1999) as shown in Figure 1 (A). This salivary nest cement is the most important ingredient in the edible bird nest and is undoubtedly one of the most expensive food ingredients in the world (Marcone, 2005). Figure 1 (B) show the hydrolysed EBN after being treated by specific enzyme (papain/ alcalase) through enzymatic hydrolysis process. Enzymatic hydrolysis released bioactive peptides which commonly contain 3-20 amino acid units that are inactive in the parent protein sequence. (Jun *et al*., 2004; Mamelona *et al*., 2010 and Lee *et al.*, 2012). These peptides can exhibit various biological activities and nutritional properties (Jun *et al*., 2004; Mamelona *et al*., 2010 and Lee *et al.*, 2012). The biological activities of the proteins can be increased using enzymatic hydrolysis, with some peptides or fractions having stronger activity than others.

Table 1 showed the degree of hydrolysis (DH) of edible bird nest hydrolysate at time of hydrolysis of 30, 60, 90, 120, 180 and 240 minutes. Degree of hydrolysis is a measurement of the extent of hydrolytic degradation of protein. It is considered the most practical and convenient means for measuring the hydrolysis process. Based on Table 1, the highest degree of hydrolysis (DH) is at the 90 minutes for alcalase with a value of 82.7 ± 1.86 %, meanwhile for the papain is at 60 minutes of hydrolysis time with a value of 32.6 ± 3.26 %. The high value at

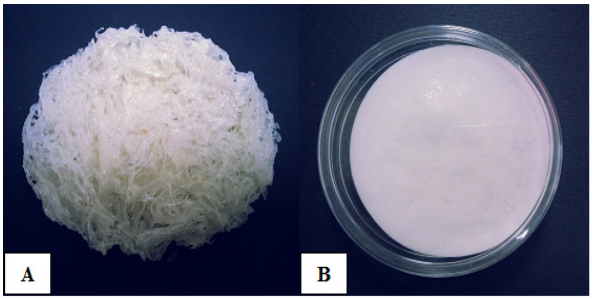

Figure 1. Unhydrolysed EBN and hydrolysed EBN AUnhydrolysed EBN (Raw edible bird nest) B Hydrolysed EBN with enzyme

Table 1. Degree of hydrolysis (DH) for edible bird nest hydrolysed with alcalase and papain at different time

Hydrolysis time (minutes)	Degree of Hydrolysis (% DH)	
	Alcalase	Papain
0 (Unhydrolysed EBN)	7.6 ± 5.00^{c}	7.6 ± 5.00^{c}
30	71.2 ± 1.84^{b}	30.1 ± 1.86^{ab}
60	72.0 ± 3.72^{b}	32. 6 ± 3.26^{a}
90	82.7 ± 1.86^{a}	28.5 ± 1.90^{ab}
120	73.5 ± 1.90^{b}	27.4 ± 5.02^{ab}
180	73.3 ± 3.19^{b}	25.8 ± 3.22^{b}
240	72.6 ± 3.16^{b}	25.2 ± 1.90^{b}

$^{a\text{-}c}$Same letter at different column indicates no significant difference ($p> 0.05$).
*Mean ± SD for 3 sample/treatment

90 minutes (alcalase) and 60 minutes (papain) of hydrolysis indicated that more peptide bonds are broken down by the enzyme in that time duration. The degree of hydrolysis depended on the type of enzyme, the cutting site of enzymes and the reaction time. At a given hydrolysis time, the DH values of the alcalase digestion were significantly higher than that of the papain treatment ($p<0.05$). This indicated that alcalase enzyme was more efficient for preparing EBN protein hydrolysates. These data indicated that the susceptibility to hydrolyze the EBN protein depends on the type of enzyme used.

In this study, a decrease in rate of hydrolysis of EBN with alcalase was observed when incubated more than 90 minutes ($p<0.05$) and for papain was observed more than 60 minutes ($p<0.05$). Meaning that after an initial rapid phase of hydrolysis, the rate of hydrolysis tends to decrease, entering a stationary phase. Despite of having more cleavage site, the extent of hydrolysis of EBN depends on cleavage specificity of enzyme and accessibility of peptide bonds to each enzyme (Mackei, 1982).

Peptide content test was done to quantitate the peptide in samples, which may affect ACE inhibitory activity (Hussain *et al*., 2014). The absorbance value measured by the spectrophotometer was used in determination of the concentration of a sample

Table 2. Peptide content (%) for edible bird best hydrolysed with alcalase and papain at different time

Hydrolysis time (minutes)	Peptide content (%)	
	Alcalase	Papain
0 (Unhydrolysed EBN)	9.70 ± 3.04[e]	9.70 ± 3.04[c]
30	62.02 ± 0.72[d]	65.26 ± 2.66[a]
60	74.29 ± 1.41[b]	66.29 ± 4.51[a]
90	86.68 ± 0.38[a]	64.15 ± 2.38[a]
120	69.33 ± 0.14[c]	63.68 ± 3.44[a]
180	75.96 ± 2.00[b]	55.04 ± 2.09[b]
240	70.1 ± 1.00[c]	52.54 ± 2.95[b]

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

Table 3. Protein solubility (%) for edible bird nest hydrolysed with alcalase and papain at different time.

Hydrolysis time (minutes)	Protein solubility (%)	
	Alcalase	Papain
0 (Unhydrolysed EBN)	14.75 ± 1.13[g]	14.75 ± 1.13[f]
30	81.9 ± 1.79[d]	77.8 ± 0.23[b]
60	85.7 ± 0.27[c]	84.06 ± 0.08[a]
90	104.1 ± 0.13[a]	74.15 ± 0.08[d]
120	81.2 ± 0.93[d]	75.6 ± 0.08[c]
180	87.8 ± 0.78[b]	59.9 ± 0.13[e]
240	85.7 ± 0.68[c]	60.3 ± 0.93[e]

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

because it indicates the amount of photon absorbing molecules in the sample.

The peptide content of the sample are summarized in Table 2 where the peptide content of EBN hydrolysate treated with alcalase increased with time of hydrolysis for the first 90 minutes; 9.70 mg/g to 86.68 mg/g but decrease after that. The same trend showed by EBN hydrolyzed by papain; increased from 9.70 mg/g to 66.29 mg/g. The highest peptide content was recorded by alcalase with 86.68 mg/g. The peptide content for hydrolysate treated by both enzymes is slightly low. The low peptide content was related to the type of enzyme used and the specific cutting site of that enzyme in separation of peptide bonds in the sample. Peptide content was influenced by the specific action of the enzyme used in the cleavage of peptide bond (Arihara *et al.*, 2006).

The soluble protein content in sample with different hydrolysis time measured using Folin-Lowry method summarized in Table 3. Solubility profile of a protein provides some insight into the extent of denaturation on irreversible aggregation and precipitation that may have occurred. Solubility is primarily dependent on the distribution of hydrophobic and hydrophilic amino acids on the surface of a protein and on the thermodynamics of the protein-water interaction. Solubility profile of EBN hydrolysates were significantly different ($p<0.05$). By increasing the time of hydrolysis, the soluble protein content of EBN hydrolyzed by alcalase and papain increased from 14.75 mg/g to 104.1 mg/g and 14.75 mg/g to 84.06 mg/g, respectively (Table 3). The protein solubility of EBN after 90 minutes incubation with alcalase and 60 minutes incubation with papain was decreased. Enzymatic hydrolysis is often used to improve the solubility of protein. However, 100% solubility will never be achieved due to peptide-peptide interactions and partly to the presence of glycoprotein in EBN protein that cannot be hydrolyzed (Arihara *et al.*, 2006). Glycoproteins are proteins that contained covalently attached sugar residues. The hydrophilic and polar characteristics of sugars may dramatically change the chemical characteristics of the protein to which they are attached. Glycoproteins are frequently present at the surface of cells where they function as membrane proteins or as part of the extracellular matrix. These cell surface glycoproteins play a critical role in cell–cell interactions and the mechanisms of infection by bacteria and virus.

Table 4 summarizes the result obtained for the percentage of ACE inhibitory activity in the hydrolysates with different hydrolysis time, tested with a concentration of 2.0 mg/ml. Among the hydrolysates tested, alcalase hydrolysate showed the highest ACE inhibitory activity, 86.24% (60 minutes of hydrolysis) even though the highest DH was at 90 minutes. Study by Zhang *et al*. (2008) reported that DH value does not affect ACE inhibitory activity, but the ACE inhibitory activity is affected by 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.* (2006) who reported that ACE inhibitory activity is affected by the enzyme specific activity in producing specific peptide chain. Several studies have reported that alcalase hydrolysates derived from food proteins showed potent bioactivities, such as antioxidant and ACE inhibitory activities. These bioactivities are attributed to the ability of alcalase to produce various bioactive peptides because of its endo-peptidase properties. Alcalase produces shorter peptide sequences as well as terminal amino acid sequences responsible for various health beneficial bioactivities, including ACE inhibition (Hyun and Shin, 2000). Moreover, alcalase affords the highest ACE inhibitory activity in the production of antihypertensive peptides when compared with other specific (pepsin and trypsin) and non-specific (pronase E) proteases (Qian *et al*., 2007). Previous studies have reported that alcalase

Table 4. Percentage of angiotensin converting enzyme (ACE) inhibitory activity and IC_{50} value for different hydrolysis time of sample fraction of edible bird nest hydrolysate.

Hydrolysis Time (minutes)	Papain		Alcalase	
	ACE-I (%)	IC_{50} value(mg/ml)	ACE-I (%)	IC_{50} value (mg/ml)
0 (Unhydrolysed EBN)	6.88 ± 4.85^{d}	-	6.88 ± 4.85^{c}	-
30	39.68±1.6^{b}	1.29	83.07±2.4^{a}	0.07
60	46.56±2.4^{a}	0.78	86.24±3.2^{a}	0.02
90	28.04±2.4^{c}	1.68	71.43±4.2^{b}	0.15
120	41.80±3.7^{ab}	1.01	81.48±4.0^{a}	0.09
180	41.80±6.0^{ab}	0.97	73.54^{b}±5.7^{b}	0.18
240	31.75±1.6^{c}	1.07	71.43^{b}±4.0^{b}	0.19

$^{a-d}$ Same letter at different samples indicates no significant difference ($p > 0.05$).
*Mean ± SD for 3 sample/treatment.

is capable of producing bioactive peptides when it is used to hydrolyze food proteins (Li *et al.*, 2006).

The IC_{50} value of hydrolysate fractions, the concentration of peptide required to reduce 50% of the ACE activity. From Table 4, Alcalase hydrolysate with 60 minutes hydrolysis time exhibited the highest ACE inhibitory activity compared to papain hydrolysate. The alcalase hydrolysate showed potent ACE inhibitory activity after 60 minutes incubation with the IC_{50} value being 0.02 mg protein/ml while papain hydrolysate showed potent ACE inhibitory activity after 60 minutes incubation with the IC_{50} value being 0.78 mg protein/ml. This result suggested that peptides released from EBN by enzymatic hydrolysis can be a potent ACE inhibitor. Hydrolysis is necessary in order to release ACE inhibitory peptides from an inactive form within the sequence of edible bird nest protein.

Conclusion

This study suggested that bioactive peptides produced by enzymatic hydrolysis from edible bird nest could be useful as a potential functional ingredient in food, nutraceutical and pharmaceutical industries against hypertension and related diseases. Hydrolysis time affect the release of ACE inhibitory peptides from an inactive form within the sequence of edible bird nest protein where 60 minutes of hydrolysis time gave the best ACE inhibitory activity. Edible bird nest protein could be used as a health enhancing ingredient in the formulation of functional foods in order to prevent hypertension. However, further study should be carried out to demonstrate the antihypertensive activity of EBN hydrolysate in vivo.

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