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Effect of enzymatic hydrolysis on antioxidant capacity of cave edible bird's nests hydrolysate

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Abstract : Considering its high source of glycoproteins, the hydrolysates of Edible Birds Nest (EBN) is anticipated to potentially possess good commercialized functional properties. This study was conducted to determine the antioxidant capacity in EBN protein hydrolysates produced via enzymatic hydrolysis using alcalase and papain. EBN protein hydrolysate that had been hydrolysed for 2 hours showed 77.9% - 84.5% increments in degree of hydrolysis value compared to raw EBN. Meanwhile, for electrophoresis analysis, it showed that raw EBN samples contained molecular weight between 51.3 kDa to 123.0 kDa. Use of protease enzymes in this study successfully produced molecular weight of EBN protein that was lower than 51.3 kDa. Regarding to antioxidant analysis, alcalase hydrolysed EBN showed stronger radical scavenging activity as indicated by the IC_{50} (0.2643 mg/g ± 0.05 and 1.619 mg/g ± 0.06) for DPPH and ABTS respectively, compared to papain (1.3972 mg/g ± 0.10 and 2.9073 mg/g ± 0.20). EBN hydrolysates contained more antioxidative amino acid residues such as amino acid aromatic and hydrophobic compared to raw EBN. As a conclusion, enzymatic hydrolysis had improved the functional properties of EBN while producing antioxidative peptides.
Key words: Edible bird's nest, alcalase, papain, peptides, antioxidant activities.

Introduction

Enzymatic hydrolysis of proteins produces peptides with smaller molecular sizes and less secondary structure than the original proteins[1]. These peptides exhibit different biological activities and physicochemical properties depending on their amino acid sequence and molecular weight such as antimicrobial, antioxidant, antithrombotic, antihypertensive, hypocholesterolemic, hypoglycemic, immunomodulatory, opioid, and anti-proliferative activities[2].

Edible birds nest (EBN), the salivary mucin-like substance produced from male swiftlets were reported to consists of high value glycoprotein rich in amino acids, carbohydrate, calcium, sodium and potassium[3]. The proximate protein composition of EBN was 59.8%-65.8%[4]. The most abundant amino acids of the nest are serine, threonine, aspartic acid, glutamic acid, proline, and valine[5]. The white nest is rich in two aromatic amino acids, i.e., phenylalanine and tyrosine[6]. Each kind of edible bird's nest has its own unique protein fingerprint on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) because they are composed of different protein species[7]. EBN is categorized into two types based on their harvesting location, either from house nest or cave nest. Generally cave EBN had higher nitrite and nitrate content and thus, exert more yellowish colour to the EBN compared to the house nest type[4].

To date, research on EBN suggested that EBN contains epidermal growth factor-like substances which could help to trigger cell division that results in skin rejuvenation[8]. Apart from this, EBN extract was found to exhibit versatile medical potentials such as enhancing bone strength and dermal thickness[9], treating erectile dysfunction as well as serving as an alternative chondroprotective agent in curing osteoarthritis[10]. Meanwhile, a few researches had been done on antioxidant activity such as in Yida[11] which extracted the EBN using enzymatic digestion and reported that digested EBN enhanced antioxidant capacity compared to undigested EBN. Hu[12] suggested that EBN can promote SOD and CAT activity and decreased MDA level when observed the flies treated with different doses of EBN. Therefore, this study was conducted in purpose to identify the potential antioxidant capacity in EBN peptides since EBN consists of 60% glycoprotein and research on bioactivity in peptides EBN is still limited. Enzymatic hydrolysis was carried out in this study since it is the best way to produce antioxidative peptides from food proteins.

Materials and Methods

Materials and Chemicals:

Cleaned raw edible birds nest (EBN) was obtained from cave nest in Sarawak. The EBN was grounded and homogenized before hydrolysis. Alcalase® 2.4 L (declared activity of 2.4 U/g, density of 1.18 g/ml), an endoproteinase from *Bacillus licheniformis* and papain 100 g (declared activity 3.37 U/mg), derived from Carica papaya were purchased from Sigma Aldrich. All reagents used were of analytical grade.

Preparation of EBN protein hydrolysate:

Raw EBN was soaked in a water using 1/100 ratio before incubated at 4°C for 16 hours. After that, the sample was boiled at 100°C for 30 minutes and had been cooled to room temperature before adjusting with the suitable pH for enzymatic hydrolysis.

Enzymatic hydrolysis of EBN protein hydrolysate:

Alcalase (pH 8, 60°C) and papain (pH 7, 60°C) were used in this experiment. The enzymes were added to substrate ratios (E/S ratios) at 1% and the hydrolysis was carried out for 2 hours. The resulting hydrolysates were heated in boiling water for 5 min to inactivate the enzymes, and then centrifuged at 4°C and 4,000 rpm for 10 min. The supernatant was filtered using Whatman No.1 and the filtrate was freeze-dried before stored for further analysis.

The hydrolysed EBN samples were labelled as:

a) A-EBNPH: Alcalase EBN protein hydrolysate

b) P-EBNPH: Papain EBN protein hydrolysate

Peptides Content and Degree of Hydrolysis (DH):

The peptides content and %DH of hydrolysates were determined using the o-phthaldialdehyde (OPA) method described by Spellman[13]. Gluthathione (100-1000 μg/ml) was used as a standard. The degree of hydrolysis (DH) was expressed in terms of percent hydrolysis. The DH was calculated as the following equation:

DH (%) = (peptides in hydrolysate) / (total peptides in EBN) x 100

Total peptides content in EBN was carried out by strong acid for complete hydrolysis and measuring by OPA method as described by Chen[14].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis:

The molecular weight distribution was assessed using Sodium Dodecyl sSlfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Schägger[15] and the molecular weight protein was separated by a 16.5% Tricine-SDS-PAGE. The polypeptide of molecular weight standards from 26.6 to 1.42 kDa (Bio-Rad No.161-0326, USA) was used as the marker protein. A graph of log MW against relative migration distance

(R_f) was plotted, based on the values obtained for the bands in the MW standards and the MV of the unknown protein band was then calculated by interpolation using the graph. R_f value was calculated using following equation:

R_f = migration distance of protein / migration distance of the dye front

Determination of the antioxidative activities

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity:

DPPH radical scavenging activity of hydrolysates was determined as described by Zhang[16]. The value was described as IC_{50} which defines as the median inhibition concentration that causes a decrease in the initial radical concentration by 50% is a parameter widely used to measure the antiradical efficiency. The lower the IC_{50} is, the higher the free radical-scavenging ability is.

ABTS radical-scavenging activity:

ABTS radical-scavenging activity of EBN protein hydrolysates was determined as described by Najafian[17].

Reducing power assays:

The ability of the EBN protein hydrolysates to reduce iron (III) was measured according to the method of Oyaizu[18].

Determination of Amino Acid Composition

Amino acid compositions in raw EBN and EBN hydrolysates were determined using the Alaiz[19] method with slight modifications. EBN samples were hydrolysed with 5 ml 6M hydrochloric acid at 110 ± 1 °C for 24 h. Next, α-aminobutyric acid as an internal standard was added to the hydrolysed samples and filtered through a 0.2 µm cellulose acetate membrane (Whatman No. 1). Derivatisation of the amino acids was done for 10 min at a temperature of 55 °C, which was maintained using a heating block. On a C18 AccQ-Tag amino acid analysis column (150 × 3.9 mm, Waters, USA), the amino acids were run with the temperature set at 37 °C and a flow rate of 1 ml/min. The absorbance was measured at 248 nm, and the associated fluorescence detector was operated at excitation and emission wavelengths of 250 nm and 395 nm (for amino acid quantification), respectively. Alkaline hydrolysis was also performed to quantify the level of tryptophan[20].

Statistical Analysis:

Data obtained from the study were analysed statistically with analysis of variance and Duncan test by using SPSS Version 20 (SPSS 2011) to identify the significance difference among the samples ($p<0.05$). Data were reported as mean ± standard deviations from triplicate determination.

Results and Discussion

Effect of enzymatic hydrolysis on degree of hydrolysis:

Figure 1 shows the % DH of EBN protein during the two hour hydrolysis using alcalase and papain. Use of protease enzymes in this study successfully increased the DH values as shown in the graph. At 0-min, the DH value in EBN hydrolysate was 14% and it had been increased to 64% - 91% after been hydrolysed for 2 hours. It was observed that at 30-min, the DH of both EBN samples was significantly ($p<0.05$) increased with the highest increment of A-EBNPH sample (61.57%) compared to the P-EBNPH (15.1 %). The higher DH observed in alcalase hydrolysed EBN indicated that higher proteolytic activity of the enzyme. Generally alkaline proteases, including Alcalase, exhibit higher activities than the acid or neutral proteases such as papain[21]. Alcalase is a group of endopeptidase, which has a wide spectrum of specificity, capable to break down the bonds near serine, glycine and aromatic amino acid. Meanwhile Papain is able to break down the peptides bonds near leucine and glycine[22].

The DH value that obtained from A-EBNPH and P-EBNPH was regarded as higher compared to DH value obtained from patin hydrolysates (89.17%) reported by Najafian[17] and salmon skin protein hydrolysates (76.43%) reported by See[23] which both studies used alcalase enzyme to break down the peptide bonds. The higher DH of EBN protein hydrolysates probably due to the boiling treatment during the preparation of protein hydrolysates which help to break down the glycopeptide bond of EBN.

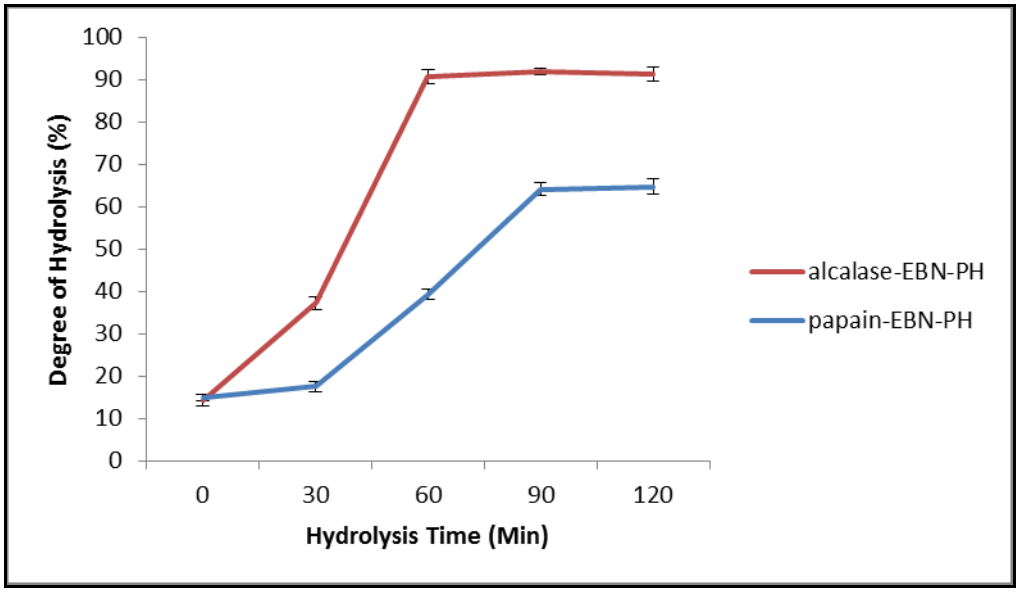


Figure 1. Effect of time on the degree of hydrolysis of EBN protein hydrolysates

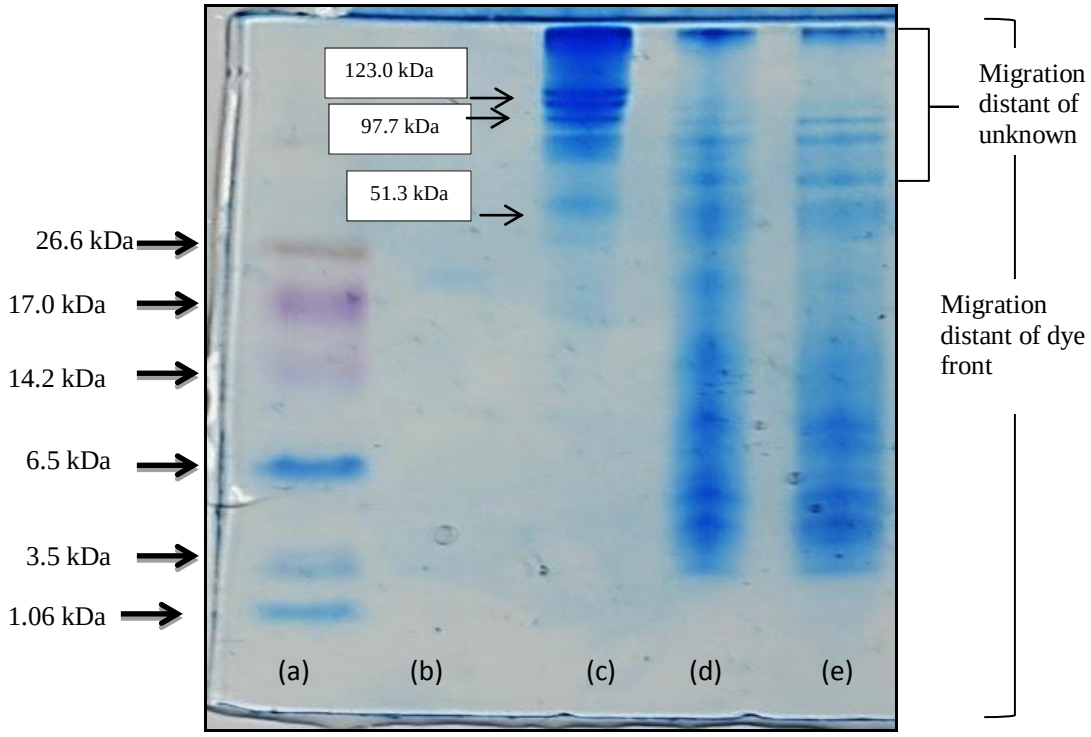


Figure 2. Molecular weight distribution of EBN samples which (a) MW standard; (b) no pretreatment raw EBN sample; (c) pretreatment raw EBN sample; (d) A-EBNPH sample; (e) P-EBNPH sample.

Electrophoresis Analysis of EBN Protein Hydrolysates

SDS-PAGE analysis was conducted to establish the molecular weight distribution of various EBN protein samples as shown in Figure 2. In the lane (b) and (c), both samples were raw EBN samples but sample in lane (b) was not undergo pre-treatment analysis meanwhile sample in lane (c) was been soaked and heated before hydrolysed with the enzymes. There was no band shown in lane (b) indicated that without pre-treatment analysis, the raw EBN was still in the rigid glycoprotein structure and did not dissolved properly and thus, it will affect the antioxidant analysis. Therefore, pre-treatment analysis had been done to raw EBN samples and regarded as control sample in this study. A graph of MW standard against R_f value was plotted and from the graph, the unknown band protein band was been calculated. For the raw EBN sample (lane c), the high intensity protein bands was observed at MW 51.3 kDa, 70.8 kDa, 79.4 kDa, 114.8 kDa and 123.0 kDa. Marcone[6] reported that the white nest and red blood nest contains 5 types of glycoprotein with their molecular weight from 20 kDa to 90 kDa. Meanwhile, Utomo[24] stated that MW of white nest from Callocalia sp. was 21.2 kDa, 64.8 kDa and 140.8 kDa which were quite similar with the MW obtained in this study.

Enzymatic hydrolysis using alcalase and papain successfully broke down the EBN protein into fragments as shown in lane (d) and (e). There was several high intensity bands observed in EBN hydrolysates starting from 2.51 kDa to the highest MW which was 97.7 kDa meanwhile there were no clear protein band observed in MW below than 51.3 kDa in raw EBN samples.

Antioxidant activities of unhydrolysed and hydrolysed EBN:

The antioxidant activities of the hydrolysates were measured using DPPH and ABTS radical-scavenging activity and also reducing power assays. Table 1 showed the IC_{50} values from the DPPH and ABTS radical scavenging activity respectively. IC_{50} is defined as the concentration of antioxidants required for 50% scavenging of DPPH/ABTS radicals in the specific time period. The higher the antioxidant activity, the lower is the value of IC_{50}[25]. From the table, it demonstrated that EBN protein hydrolysate hydrolysed from both enzymes (alcalase and papain) did contributed to the higher antioxidant activity as shown by decreasing values of IC_{50}. There were significantly differences ($p<0.05$) between hydrolysed and unhydrolysed EBN which showed that antioxidative peptides in EBN was released upon hydrolysis as more active amino acid R groups will be exposed that leads to increased antioxidant activity[26].

Table 1. IC_{50} values of DPPH and ABTS radical scavenging activity on EBN protein hydrolysates

Samples	DPPH Radicals Scavenging Activity (mg/g)	ABTS Radical Scavenging Activity (mg/g)
Unhydrolysed EBN	$4.0240^{a}\pm 0.20$	$4.4273^{a}\pm 0.11$
Alcalase-EBNPH	$1.6185^{c}\pm 0.06$	$0.2643^{c}\pm 0.05$
Papain-EBNPH	$2.9073^{b}\pm 0.20$	$1.3972^{b}\pm 0.10$
Ascorbic Acid (Positive Control)	$0.0469^{d}\pm 0.05$	$0.0289^{d}\pm 0.02$

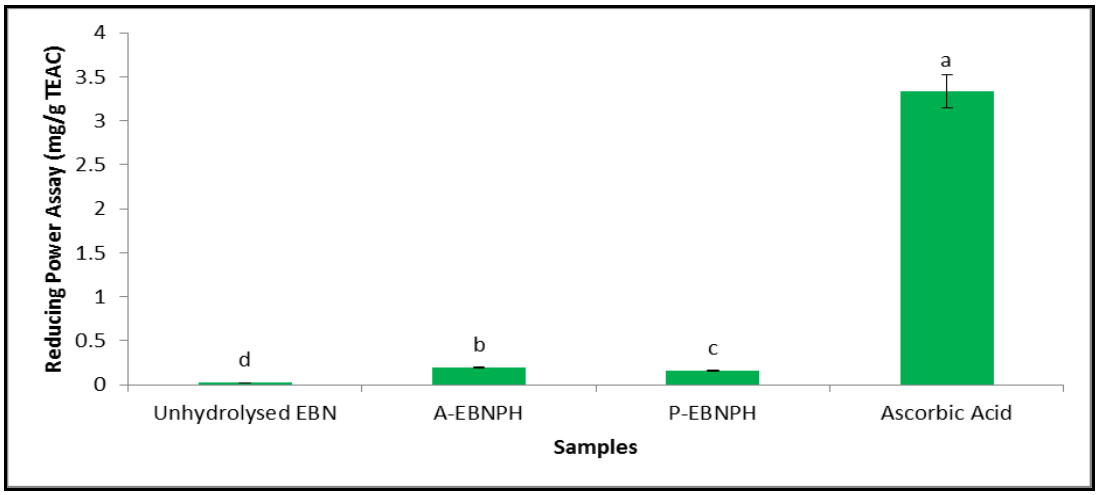
[a-d] Different letters within the same column indicate significant differences ($P < 0.05$).

Meanwhile, in comparison between the two enzymes used in this study, alcalase-EBN demonstrated lower IC_{50} compared to papain-EBN. IC_{50} for alcalase was 0.2643 mg/g ± 0.05 and 1.619 mg/g ± 0.06 whereas IC_{50} for papain was 1.3972 mg/g ± 0.10 and 2.9073 mg/g ± 0.20 for ABTS and DPPH radical scavenging activity respectively. This result was quite similar with the study reported by Najafian[17] which also using alcalase and papain to hydrolyse fish protein. It showed that the IC_{50} valuesfor alcalase was lower than papain in DPPH and ABTS radical scavenging activity. However, if comparison was done between the IC_{50} value for both study, EBN protein hydrolysates produced lower IC_{50} compared to fish protein hydrolysates which suggested that the EBN protein hydrolysate showed greater scavenging activity.

Besides that, it showed that alcalase had better scavenging activity both in fish hydrolysates and EBN protein hydrolysates. Hydrolysates prepared using different enzymes and processing conditions may display a similar degree of hydrolysis but different peptide compositions[27] which explained the differences of antioxidant activity in EBN hydrolysed by alcalase and papain. Alcalase as an endopeptidase is capable of hydrolysing peptide bonds at random into large peptides[22]. With the wide spectrum of alcalase specificity, it can destroy peptides bonds near serine, glycine and aromatic acids which probably exposing the antioxidative peptides in EBN more than papain. Compared with the papain preparations, EBN protein hydrolysates prepared with

Alcalase may have had more active amino acids or peptides which could form more stable products when reacted with free radicals.

For reducing power assay, the presence of antioxidants in the EBN protein hydrolysates results in reduction of the ferric cyanide complex to the ferrous form. Figure 3 shows that after hydrolysis, the degree of electron donation capacity increased up to 86.25% to 89.0% which suggested that enzymatic hydrolysis did improve the degree of electron donation capacity of EBN. However, the results were much lower compared to the positive control (ascorbic acid). Study by Chen[14] reported that the reducing power in duck egg white hydrolysates was in a range of 0.106 – 0.170 mg/ml TEAC which was much lower than EBN protein hydrolysates.



[a-d] Different letters within the same parameter indicate significant differences (P < 0.05).

Figure 3. Reducing power assay of EBN protein hydrolysates at conc. 1 mg/ml

Table 2 Amino acid profiles of raw and hydrolysed EBN (alkalase and papain).

Amino acid Residues	Raw EBN (mg/100 g)	A-EBNPH (mg/100g)	P-EBNPH (mg/100g)
Aspartic + Asparagines	11.4364[a] ± 0.127	8.4756[b]± 0.343	8.6183[b] ± 0.019
Serine	10.2105[a]± 0.107	8.5073[b]± 0.390	8.7457[b]± 0.202
Glutamic + Glutamine	7.5065[a] ± 0.025	6.8787[b]± 0.244	6.0307[c]± 0.008
Glycine	4.2124[a] ± 0.129	3.3235[b] ± 0.464	3. 5432[ab]± 0.002
Histidine	3.1692[b] ± 0.006	3.4311[a]± 0.098	3.1885[b]± 0.057
Arginine	7.4958[a] ± 0.096	6.5769[b]± 0.0944	6.2918[b]± 0.126
Threonine	5.7032[b] ± 0.136	6.4712[a]± 0.132	6.3606[a]± 0.145
Alanine	3.1879[a] ± 0.025	2.5222[a]± 0.120	2.7876[a]± 0.368
Proline	7.9341[b] ± 0.024	8.0822[b]± 0.008	8.6880[a]± 0.140
Tyrosine	6.7398[b]± 0.071	7.7074[a]± 0.036	7.0997[ab] ± 0.429
Valine	6.8360[a] ± 0.039	6.9800[a]± 0.157	6.7188[a]± 0.408
Methionine	0.7043[b] ± 0.018	4.9650[a]± 0.017	5.2335[a]± 0.329
Lysine	4.7715[c]± 0.027	5.5175[b]± 0.042	5.3776[a]± 0.084
Isoleucine	2.5648[c] ± 0.002	2.9244[a]± 0.058	2.7085[b]± 0.005
Leucine	7.0560[a] ± 0.095	6.7372[b]± 0.142	6.3985[c]± 0.023
Phenylalanine	5.8113[b] ± 0.108	6.2960[a]± 0.121	5.9892[ab]± 0.047
Sistine	3.5756[a] ± 0.044	2.4714[b]± 0.343	3.0104[ab]± 0.156
Tryptophan	1.0847[a] ± 0.164	2.4923[ab]± 0.103	3.2097[a]± 0.776
Total AAH[1]	40.8343[c]	46.2147[a]	45.6239[b]
Total AAR[2]	16.8051[b]	19.9269[a]	19.4871[a]

[ab]Different letters within the different column indicate significant differences (P < 0.05).
[1]AAH= Hydrophobic amino acid (Alanine, Valine, Methionine, Isoleucine, Leucine, Phenylalanine, Proline and Tyrosine)
[2]AAR = Aromatic amino acid (Phenylalanine, Histidine, Tryptophan, Tyrosine

Amino acid Composition

The amino acid composition of raw EBN and EBN hydrolysates were summarised in Table 2. Aspartic acid and glutamic acid was significantly reduced ($p<0.05$) in EBN hydrolysates compared to raw EBN due to the proteolysis activity. Pre-treatment of raw EBN (soaking and heating) before undergo enzymatic hydrolysis was believed to break down the glycoprotein bonds in EBN and thus released more amino acid residues such as proline, tyrosine, methionine, lysine, isoleucine and tryptophan.

Antioxidant activity showed in EBN protein hydrolysates was highly related to the presence of amino acid residues such as amino acid hydrophobic (AAH) and amino acid aromatic (AAR) which contributing to the antioxidant properties. As shown in Table 2, total AAR and AAH showed significantly higher in EBN hydrolysates compared to raw EBN. AAR which consists of phenylalanine, histidine, tryptophan, and tyrosine were able to donate the proton to the radical-electron-deficient thus increased the radical scavenging activity. Meanwhile for AAH which consists of Alanine, Valine, Methionine, Isoleucine, Leucine, Phenylalanine, Proline and Tyrosine, higher AAH value in EBN hydrolysate was expected since hydrolysis process exposed more protein bonds hence increased the hydrophobicity.

Conclusion

In this study, the results demonstrated that enzymatic hydrolysis increased the functional properties of Edible birds nest (EBN). EBN hydrolysed with alcalase showed greater DH and higher antioxidant activities as measured by DPPH, ABTS and reducing power assays compared to papain. These findings of EBN hydrolysates as potentially strong natural antioxidants increase the novelty of edible birds nest as very few scientific data in currently available on the antioxidative activities of EBN hydrolysates.

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