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EFFECT OF DIFFERENT HYDROLYSIS TIME AND ENZYMES ON CHEMICAL PROPERTIES, ANTIOXIDANT AND ANTIHYPERGLYCEMIC ACTIVITIES OF EDIBLE BIRD NEST HYDROLYSATE

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ABSTRACT

Edible bird nest (EBN) is a dried glutinous secretion from the salivary glands of swiftlet species commonly found in the Southeast Asian region, including Malaysia. It is consumed traditionally by the Chinese as food delicacy and also considered as an important ingredient in traditional Chinese medicine for its beneficial health effects. The aim of this study was to evaluate the effect of enzymatic hydrolysis using alcalase, papain and papaya juice hydrolyze at 0.5 to 3 hr on the degree of hydrolysis (DH), protein solubility, concentration of reducing sugar, antioxidant and anti-hyperglycemic activity. In general, an increase in hydrolysis time increases the DH and soluble protein digestion rate. However, the concentration of reducing sugar was not affected by hydrolysis time. The solubility of proteins was highest for alcalase and papain at 3 h, while papaya juice at 2 h. Papain showed the highest antioxidant activity in 1 and 2 h of hydrolysis time whereas at 3 h, results for both papain and alcalase were higher compared to papaya juice. Result of anti-hyperglycemic activity showed that only EBN hydrolyzed using papaya juice has positive activity. Based on this study, enzymatic hydrolysis had improved the functional properties of EBN and results showed the potential of EBN to be developed as natural antioxidants and anti-hyperglycemic agents.

Key words: Antioxidant, anti-hyperglycemic, degree of hydrolysis, edible bird nest, hydrolysate

INTRODUCTION

Edible bird's nest (EBN) or also known as '*Yan Wo*' among Chinese population is produced from the saliva of the swiftlet species of the White-nest swiftlet *(Aerodramus fuciphagus)* and the Black-nest swiftlet *(Aerodramus maximus)* (Kang *et al*., 1991). EBN has long been consumed in the form of 'bird nest soup' for its medicinal properties since the dynasty of Tang (618–907 A.D.) and Sung (960–1279 A.D.) and has been considered as a symbol of wealth, power, and prestige (Lim *et al*., 2002). Most studies on EBN were done on the extracts while only a few were done on the hydrolysate or peptides. The health-enhancing properties of EBN extracts includes anti-aging, anti-influenza viral properties, growth promoting, enhancing complexion, neuroprotective effect, strengthening bone, immune system and overall general health (Lim *et al*., 2002; Marcone *et al*., 2005; Guo *et al*., 2006; Matsukawa *et al*., 2011; Yew *et al*., 2014; Hu *et al*., 2016). A study by Yida *et al*. (2015) showed the ability of EBN extracts on the prevention of insulin resistance in high-fat diet induced rats. However, at present, there is no scientific report on the anti-hyperglycemic properties of EBN hydrolysates.

Reactive oxygen species (ROS) attacks biological and chemical systems in human body, leading to oxidative stress-related diseases including cancers, arthritis, cardiovascular disorders, neuro-degenerative diseases, insulin resistance and diabetes (Verdile *et al*., 2015; Mateen *et al*., 2016; Hecht *et al*., 2016; Jiang *et al*., 2016; Asmat *et al*., 2016). Synthetic antioxidant has been used to scavenge free radicals and prevent oxidation on biomolecules, caused by overproduction of ROS (Öztaşkın *et al*., 2015; Yehye *et al*., 2015). However

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this synthetic antioxidant cause negative side effects including carcinogenicity (Zheng & Wang, 2001). Treatment of diabetes mellitus also involves the use of oral antidiabetic agents such as sulphonylureas, biguanides, thiazolidinediones and α-glucosidase inhibitor (Li *et al*., 2005). Despite their effectiveness in reducing hyperglycemia, the use of these drugs is also associated with side effects such as hypoglycemia, hepatotoxicity, gastrointestinal adverse reaction and weight gain (Kimmel & Inzucchi 2005; Andrade *et al*., 1998).

Bioactive peptides or hydrolysates from natural sources have been increasingly popular due to their therapeutic properties particularly for the treatment and prevention of diet-related diseases (Zambrowicz *et al*., 2015). Due to these reasons, the search for various natural peptides derived from protein hydrolysates of plant or animal origin as an alternative to the synthetic antioxidants and antidiabetic agent with health benefits and little or no side effects have been the interest of many researchers today. The high protein content of EBN could yield various peptides that are able to scavenge free radicals and inhibit α-glucosidase activity thus controlling free radical damage and hyperglycemia. To the best of our knowledge, EBN hydrolysate has not been appraised for its α-glucosidase inhibitory activity. In the present study, EBN was hydrolyzed by alcalase, papain and papaya juice at different hydrolysis time, and the resulting hydrolysates' protein solubility, antioxidant and α-glucosidase inhibitory activity was evaluated.

MATERIALS AND METHODS

Preparation of sample and control

Raw cleaned edible bird nest from Terengganu were provided by Mobile Harvesters Malaysia Sdn. Bhd. Raw EBN was ground to powder using laboratory grinder (Waring Blender 7011S, America) and then soaked in distilled water at a ratio of 1:100. for 16 hours at 4°C. The soaked EBN was then boiled using the double boiling method for 30 minutes at 100°C. The resulting boiled EBN was then cooled to room temperature before adjusting with the suitable pH for further enzymatic hydrolysis and experiments. Papaya fruit was washed, peeled and seeds were removed before cutting into small pieces and then blended with the laboratory grinder (Waring Blender 7011S, America) to get the juice. The juice was then filtered using a muslin cloth and the filtrate juice was then used for further experiments. Boiled EBN without any enzyme treatment was freeze-dried and used as a control in all assays conducted in this study.

Enzymatic hydrolysis of EBN

EBN hydrolysates were prepared according to the method reported by Adler-Nissen (1986). Alcalase (2.4 U/g) and papain (3.37 U/mg) were used in this experiment. The previously prepared EBN was added with 1% alcalase (6 µL/100 mL), 1% papain (6 mg/100 mL) and 30% of papaya juice (30 g/100 mL). The hydrolysis process was then carried out at 60°C for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. The enzyme reaction was stopped by heating in boiling water for 5 minutes. EBN hydrolysate was then filtered with Whatman #4 filter paper, frozen at -20°C for 48 hours and then freeze-dried for further analysis.

Degree of hydrolysis

Degree of hydrolysis was determined using the autotitrator (Metrohm 799 GPT Titrino, USA) according to Guerard *et al*. (2001). The hydrolysis process was carried out at different times of 0.5 to 3.0 hours. The volume of sodium hydroxide (0.1 N) titrated was used to calculate the DH and controlled the pH according to the enzyme used (alcalase pH 8, 60°C), (papain pH 7, 60°C) and (papaya juice pH 6, 60°C). The percentage DH was calculated according to the following formula:

$$DH\ (\%) = (B \times N_b \times 1/\alpha \times 1/M_p \times 1/H_{tot}) \times 100$$

Where,
B = mL of 0.1 N NaOH consumed during the reaction
N_b = normality of the titrant
α = pK for amino groups at given temperature
M_p = crude protein mass (g) in sample (N X 6.25)
H_{tot} = total number of peptide bonds in protein substrate

Protein solubility

The soluble protein content of EBN hydrolysate was determined using the Bradford assay (Bradford, 1976). The colorant reagents were prepared by diluting 1 part saturated dye reagent (Bio-Rad, Richmon, USA) with 4 parts of distilled water and filtered using Whatman #4. Then, 5 µL sample was added to 250 µL dye reagent the microplate and left for 5 minutes at room temperature. The absorbance was measured at 595 nm (BioTek® Instruments, USA) using a spectrophotometer. Bovine serum albumin (BSA) with a concentration of 1 mg/mL to 5 mg/mL was used to make a standard curve.

Total reducing sugar

Total reducing sugar in EBN hydrolysate was determined using the DNS method (3,5-dinitrosalyzed acid) (Breuil & Saddler, 1985). Sample (250 µL) was mixed with 1000 µL of the DNS reagent and left for 5 minutes in boiling water.

The formation of 3-amino-5-nitrosalicylic acid resulted in a change in light absorption at a wavelength of 540 nm (BioTek® Instruments, USA). Glucose with a concentration of 200 mg/mL to 1000 mg/mL was used to make a standard curve.

Free radical scavenging test (DPPH)

Determination of antioxidant activity was done based on the method by Brand-Williams *et al*. (1995) with some modifications. Ascorbic acid and BHA were used as a positive control. Hydrolyzed EBN (500 µL) was added to 2.5 mL DPPH reagent (0.01 mM). The mixture was left for 30 minutes at room temperature in a dark place. The absorbance was detected using spectrophotometer at 517 nm (BioTek® Instruments, USA).

Inhibition of the α-glucosidase activity

The inhibition of α-glucosidase activity was performed based on the method by Deautschlander *et al*. (2009) with slight modification. Substrate solution (10 mM p-nitrophenyl-α-D-glucopyranoside) was prepared in 50 mM of phosphate buffer of pH 6.5. After that, 130 µL of 30 mM buffer phosphate solution, 10 µL of the sample, and 10 µL α-glucosidase solution (3 U/mL) were mixed and pre-incubated in 96 well plates for 5 minutes at 25°C. The reaction mixtures were then incubated for another 15 min at 25°C. A total of 50 µL of 2 M glycine (pH 10) was added to stop the reaction and the absorption was measured at 405 nm using EpochTM Microplate Spectrophotometer, Biotech Instrument (USA).

Statistical analysis

All the experimental data were expressed as mean ± standard deviation. Data were analyzed for one-way ANOVA using Minitab 17.0. Tukey test was used to assess the difference between means. A significant difference was considered at the level of $p<0.05$.

RESULTS AND DISCUSSION

Figure 1 shows that DH (%) of EBN hydrolyzed with alcalase, papain and papaya juice at different hydrolysis time (0.5 to 3.0 hours). These different hydrolysis time were selected due to previous studies that reported optimum DH, antioxidant and antihypertensive activities of EBN hydrolysates was between 1.5 to 2.0 hour (Babji *et al*., 2018; Nurfatin *et al*., 2016). From the figure, a trend can be seen whereby as the hydrolysis time increases, the DH also increases. Increase in hydrolysis time allows the enzyme to act more extensively on the protein, thus resulting in an increment in the DH. DH for alcalase ranges from 11.6 to 16.2%, papain from 5.8 to 14.3% whereas papaya juice from 2.9 to 16.3%. For alcalase, DH at 3.0 hours showed the highest percentage, however, there is no significant difference between the different hydrolysis time. DH

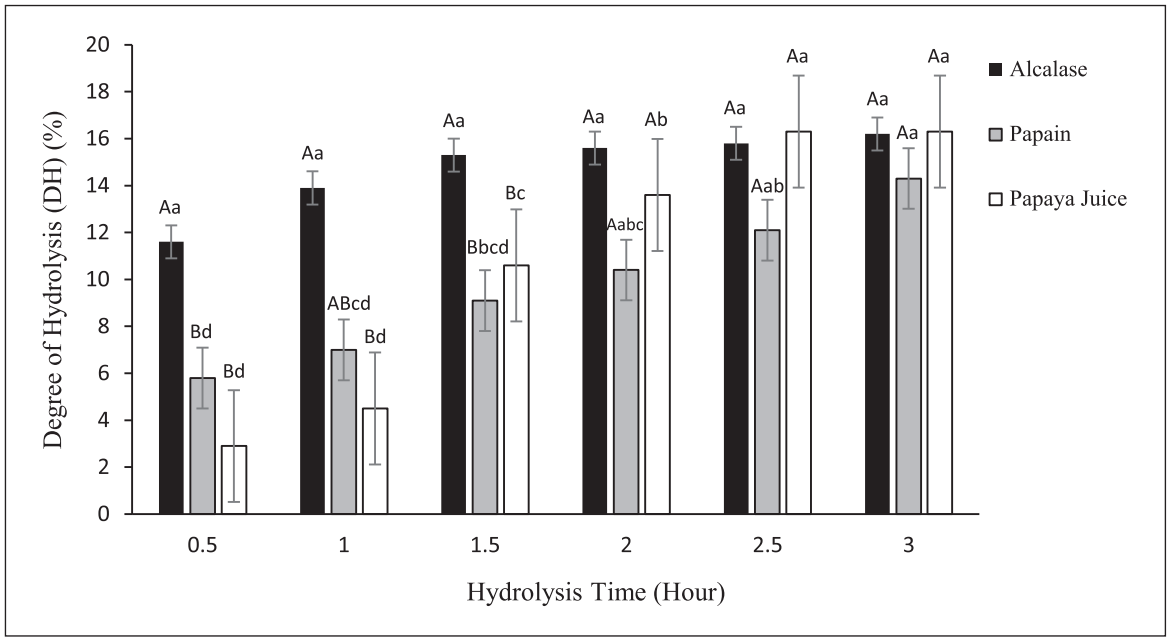

Fig. 1. Degree of hydrolysis for different EBN hydrolysate at 0.5 to 3.0 hours.
The values showed that mean±standard deviation. Mean (n=3) with different alphabets showing a significant difference ($p<0.05$) was analyzed by Tukey test.
[A-B] shows significant difference between different enzymes at $p<0.05$.
[a-d] shows significant difference between different hydrolysis time at $p<0.05$.

for papain was highest in 3.0 hour compared to 0.5, 1.0 and 1.5 hour ($p<0.05$). Meanwhile, for papaya juice, DH was significantly higher at 2.5 and 3.0 hour compared to others ($p<0.05$). Alcalase showed significantly higher ($p<0.05$) DH at 0.5 and 1.5 hour compared to papain and papaya juice. This indicates that alcalase is the most suitable enzyme to be used for hydrolysis of EBN compared to others as it requires shorter time to hydrolyze 11.6% glycoprotein compared to papain (5.8%) and papaya juice (2.9%). According to Rebeca *et al*. (1991), alkaline proteases such as alcalase was able to give higher activities compared to those of neutral of acidic proteases such as papain. Besides, alcalase is also a non-specific endopeptidase enzyme with broad specificity, having capabilities in breaking down the peptide bonds at random whereas papain is more specific and able to break down the peptides bonds near leucine and glycine (Clemente *et al*., 2000). Based on the previous study (unpublished data), papaya juice showed the capability to hydrolyze EBN when compared to other commercial enzymes. Papaya juice was also chosen in this study to add colour, taste and other beneficial nutrients to EBN hydrolysate which will be further developed into functional food ingredients. The DH values obtained in this study are comparable to those reported by Noor Hidayati Syamimi *et al*. (2018) on EBN hydrolysate and Hanafi *et al*. (2018) on green soybean hydrolysate. The trend of DH is similar to the previous reports for the hydrolysates from mung bean protein (Xie *et al*., 2019) and EBN (Nurul Nadia *et al*., 2017). Enzymatic hydrolysis will help in releasing the bioactive peptides from the inactive parent protein sequence exposing the active sites, which enables them to exhibit biological properties in therapeutic purposes (Sila & Bougatef, 2016).

Table 1 summarizes the result obtained on the soluble protein content (mg/g) of EBN hydrolysate at different hydrolysis time and enzymes used measured using the Bradford method. Results obtained showed that increase in hydrolysis time increases the protein solubility of EBN hydrolysate except for papaya juice that showed an increase from 1.0 to 2.0 hour then decrease from 2.0 to 3.0 hour. This result was expected as the DH and protein solubility generally will have a trend whereby it will increase rapidly at the beginning of hydrolysis and then decrease before entering stationary phase. For alcalase, the increase in protein solubility was not significant ($p>0.05$) from 1 to 3 hours (315.70 to 450.80 mg/g). However, a study by Nurfatin *et al*. (2016) reported the solubility of protein profiles in EBN hydrolysates showed a significant increase from 0.5 to 1.5 hour. The difference in the results obtained might due to the different method used which is the Folin-Lowry method to analyze the solubility of proteins in EBN hydrolysate when compared to Bradford method used in this study. For papain and papaya juice, increase in hydrolysis time significantly increases the protein solubility. The results are in agreement with findings reported by Etty Sharmila *et al*. (2014) on EBN hydrolysate using pancreatin enzyme. Meanwhile, for papaya juice, the solubility of protein from 1 hour to 2 hours increased from 217.70 to 630.90 mg/g and then decreased to 381.20 mg/g. This may be due to the presence of strong peptide bond as a limiting factor, therefore any further increase in hydrolysis time was not able to increase the digestion rate or concentration of soluble protein. The boiled EBN protein solubility was significantly lower in comparison with hydrolyzed EBN. This is because boiled EBN does not undergo any enzymatic hydrolysis and the boiling temperature was not able to break the strong peptide bonds. These enzymatic hydrolyses will break down peptides in proteins, thereby increasing the protein solubility of EBN hydrolysates.

The concentration of reducing sugar in EBN hydrolysate was measured using the DNS method. The information on reducing sugar is important as the protein structure in EBN composed of glycoprotein in which it has both protein and carbohydrate properties. Houdret *et al*. (1975) indicated that 40% of the hydroxyl amino acids in EBN are in the position of carbohydrate-peptide linkages. Hydrolysis of the polysaccharides in EBN

Table 1. Protein solubility of EBN hydrolyzed at 1.0 to 3.0 hours compared to boiled EBN

Hydrolysis Time (Hour)	Protein Solubility (mg/g)		
	Alcalase	Papain	Papaya Juice
1.0	315.70 ± 120.10^{Aa}	313.50 ± 28.80^{Ac}	217.70 ± 40.30^{Ac}
2.0	395.10 ± 106.60^{Ba}	473.40 ± 52.20^{ABb}	630.90 ± 70.70^{Aa}
3.0	450.80 ± 111.20^{ABa}	607.50 ± 69.30^{Aa}	381.20 ± 89.30^{Bb}
Boiled EBN	4.00 ± 1.03^{Ab}	4.18 ± 1.00^{Ad}	3.75 ± 0.88^{Ad}

The values showed that mean±standard deviation. Mean (n=3) with different alphabets showing a significant difference ($p<0.05$) was analyzed by Tukey test.
$^{A-B}$ shows significant difference between columns at $p<0.05$.
$^{a-d}$ shows significant difference between rows at $p<0.05$.

will release the reducing sugar. Hydrolyzed EBN using papain alcalase and papaya juice showed higher ($p<0.05$) reducing sugar concentration compared to boiled or unhydrolyzed EBN (Table 2). The reducing sugar of EBN hydrolysate was between 25.42 to 190.80 mg/g. Results from this study showed that the concentration of reducing sugar did not increase significantly as a function of hydrolysis time. Apart from that, EBN hydrolyzed with papaya juice showed significantly ($p<0.05$) higher concentration of reducing sugar compared to alcalase and papain. The higher concentration of reducing sugar content in EBN hydrolyzed with papaya juice is due to the hydrolysis of polysaccharides including pectins, cellulose and starch that were naturally present in the fruit (Bal *et al*., 2014). In addition, papaya contains higher carbohydrate content at 70.7% (Maisarah *et al*., 2014) compared to EBN at 25.8% (Nurfatin *et al*., 2014).

In this study, the antioxidant properties of EBN hydrolysates prepared at different hydrolysis times were evaluated based on their radical scavenging capacity against DPPH (Table 3). Ascorbic acid and BHA at concentrations of 0.01 mg/mL was used as the positive control. All of the samples tested exhibited moderate scavenging ability against DPPH (10.4–29.9%). Increases in hydrolysis time significantly ($p<0.05$) increases the ability of EBN hydrolyzed by alcalase in scavenging free radicals from 10.4% at 1 hour to 28.4% at 3 hours. However, for papain and papaya juice, increase in hydrolysis time did not affect the antioxidant activity ($p>0.05$) although DH increases over time. This indicates that 1 hour hydrolysis of EBN using papain and papaya juice was enough to produce hydrolysate with maximum DPPH activity compared to alcalase at 3 hours. Papain showed antioxidant activity at 25.4 to 29.9% whereas papaya juice at 15.5 to 17.2%. Besides, at 1 and 2 hours of hydrolysis, papain showed higher ($p<0.05$) free radical scavenging activity compared to alcalase and papaya juice. At this point, EBN hydrolyzed with papain may have more amino acids or peptides with active sites, which were able to form more stable compounds when reacting to the free radicals of DPPH. Even though the DH of papain was lower than alcalase at 1 hour, it showed higher antioxidant activity. However, at 3 hours, the antioxidant activity was not different between alcalase and papain ($p > 0.05$). The activity of EBN hydrolysates is comparable to the hydrolysates reported from Bluefin leatherjacket

Table 2. The concentration of reducing sugar of EBN hydrolyzed at 1.0 to 3.0 hours compared to boiled EBN

Hydrolysis Time (Hour)	Concentration of Reducing Sugar (mg/g)		
	Alcalase	Papain	Papaya Juice
1.0	25.42 ± 6.04^{Ba}	26.81 ± 8.23^{Ba}	190.80 ± 32.90^{Aa}
2.0	32.31 ± 4.29^{Ba}	26.13 ± 1.09^{Ba}	146.20 ± 26.10^{Aa}
3.0	25.78 ± 2.56^{Ba}	33.65 ± 1.33^{Ba}	145.20 ± 23.30^{Aa}
Boiled EBN	15.31 ± 4.12^{Ab}	15.76 ± 4.00^{Ab}	15.89 ± 4.56^{Ab}

The values showed that mean±standard deviation. Mean (n=3) with different alphabets showing a significant difference ($p<0.05$) was analyzed by Tukey test.
$^{A-B}$ shows significant difference between columns at $p<0.05$.
$^{a-b}$ shows significant difference between rows at $p<0.05$.

Table 3. DPPH inhibitory activity of EBN hydrolyzed at 1.0 to 3.0 hours compared to boiled EBN

Hydrolysis Time (Hour)	Inhibition (%)		
	Alcalase	Papain	Papaya Juice
1.0	10.4 ± 2.1^{Bd}	27.9 ± 2.6^{Ac}	15.5 ± 4.3^{Bc}
2.0	13.7 ± 2.3^{Bd}	29.9 ± 4.9^{Ac}	17.2 ± 1.0^{Bc}
3.0	28.4 ± 3.0^{Ac}	25.4 ± 1.7^{Ac}	16.9 ± 3.2^{Bc}
Boiled EBN	ND	ND	ND
Ascorbic Acid	80.1 ± 5.0^{Aa}	76.5 ± 5.3^{Aa}	71.0 ± 5.7^{Aa}
BHA	35.2 ± 6.7^{Ab}	39.7 ± 6.9^{Ab}	41.1 ± 5.9^{Ab}

The values showed that mean±standard deviation. Mean (n=3) with different alphabets showing a significant difference ($p<0.05$) was analyzed by Tukey test.
$^{A-B}$ shows significant difference between columns at $p<0.05$.
$^{a-d}$ shows significant difference between rows at $p<0.05$.

Table 4. Inhibition of the á-glucosidase activity of EBN hydrolyzed at 1.0 to 3.0 hours compared to boiled EBN

Hydrolysis Time (Hour)	Inhibition (%)		
	Alcalase	Papain	Papaya Juice
1.0	ND	ND	18.6 ± 2.9^{b}
2.0	ND	ND	12.3 ± 2.6^{c}
3.0	ND	ND	16.6 ± 0.9^{cb}
Boiled EBN	ND	ND	ND
Quercetin	–	–	43.5 ± 2.4^{a}

The values showed that mean±standard deviation. Mean (n=3) with different alphabets showing a significant difference ($p<0.05$) was analyzed by Tukey test.
$^{a-c}$ shows significant difference between rows at $p<0.05$.

(*Acipenser sinensis*) and Cod (*Gadus morhua*) which were 25.7% and 19-32%, respectively (Chi *et al*., 2015; Girgih *et al*., 2015). EBN hydrolysate is reported to contain tyrosine and phenylalanine (Babji *et al*., 2018) and these amino acids play important role in radical scavenging activity because the indole and benzene ring of these amino acids can donate protons to electron deficient radicals, making reactive oxygen species become stable (Hernández-Ledesma *et al*., 2005). From the result obtained, it is suggested that EBN hydrolysates contained electron donors substances that are able to react with free radicals, converting them to stable products and terminate the radical chain reaction by producing non-radical products.

Anti-hyperglycemic activity of EBN hydrolysate was analyzed by their ability to inhibit α-glucosidase activity (Table 4). No inhibitory effects against α-glucosidase enzyme were observed for boiled (unhydrolyzed) EBN and EBN hydrolyzed with alcalase and papain. However, EBN hydrolyzed with papaya juice showed moderate inhibitory activity ranging from 12.3 to 18.6%. The highest activity was seen on 1 hour compared to 2 and 3 hour of hydrolysis time, which indicates that hydrolysis time did not affect anti-hyperglycemic activity. The anti-hyperglycemic potential of natural products usually measured using α-glucosidase, α-amylase and a recent approach using glucose-lowering agent Dipeptidyl-peptidase IV (DPP-IV) inhibitory assays. These different assays show different mechanisms of action in their anti-hyperglycemic activity and according to Jao *et al*. (2015) peptides derived from animal and plant sources have been well recognized for its ability to inhibit DPP-IV activity. However, for both α-glucosidase and α-amylase, the studies on the inhibitory activity of peptides or hydrolysates using these assays are limited. This shows that (DPP-IV) inhibitory assay is more suitable to access the anti-hyperglycemic potential of peptides or hydrolysates. This has been proven by Vilcacundoet *et al*. (2017) which showed that all of the peptides from quinoa (*Chenopodium quinoa* Willd.) showed positive effects as DPP-IV inhibitor while only a few peptides were effective against the α-glucosidase enzyme. The results in this study also indicated the need to evaluate the anti-hyperglycemic potential of EBN hydrolysate using other methods including α-amylase and DPP-IV inhibitory assay as EBN hydrolysate might rely on different mechanisms of action.

CONCLUSION

EBN hydrolyzed with alcalase, papain and papaya juice showed moderate antioxidant activity wheres only EBN hydrolyzed with papaya juice showed inhibitory activity against α-glucosidase. In general, DH and protein solubility increases with increasing hydrolysis time for both alcalase and papain. However, the concentration of reducing sugar was not affected by hydrolysis time and papaya juice hydrolysate showed the highest reducing sugar content. The results of this work proved that, with the right enzyme and hydrolysis time employed, a standardized EBN hydrolysate could be produced which can serve as a functional ingredient with protective effects against diabetes and free-radical damage.

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