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ANTIOXIDANT ACTIVITY OF BIOACTIVE PEPTIDE DERIVED FROM PEKIN DUCK FEET GELATIN HYDROLYSATE

FATIN ARINA MOHD ZAIN[1], NORSHAZILA SHAHIDAN[1*], NURUL HUDA[2], ZARINAH ZAKARIA[1] and ABDI WIRA SEPTAMA[3]

[1]*Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Terengganu, Malaysia*
[2]*Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Sabah, Malaysia*
[3] *Research Centre for Chemistry, Indonesian Institute of Science, Jakarta, Indonesia*
[*]*E-mail: norshazila@unisza.edu.my*

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ABSTRACT

Malaysia is one of the main producers of duck meat globally with increasing demands. Increasing in duck production will also increase the number of duck by-products such as skin, feet and bones. Pekin duck (*Anas platyrhyncos domestica*) is one of the famous duck breeds. Gelatin that was extracted from Pekin duck feet has shown a potential raw material for the production of bioactive peptide that can involve in various functions of the organism physiologically for example antioxidant effects. Pekin Duck feet gelatin was hydrolyzed by using five commercial enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) to identify radical scavenging potencies of derived bioactive peptides. All the five enzymes were studied under three different enzyme-substrate ratio (1:10, 1:15, 1:20) with every enzyme optimum pH and temperature. Scavenging activities studied included DPPH radical scavenging activity and ABTS radical scavenging activity. In DPPH radical scavenging activity, all the five enzymes showed the highest percentage of radical scavenging activity at (1:20) enzyme-substrate ratio condition. Among the five enzymes studied, gelatin hydrolyzed with Protamex showed the highest activity (54.83%), followed by Alcalase (53.12%), Esperase (49.81%), Flavourzyme (49.32%) and lastly Neutrase (47.49%) at sample concentration 4.5 mg/ml. The half-maximal inhibitory concentration (IC_{50}) value of the bioactive peptide for ABTS radical scavenging activity was measured. Alcalase has produced the duck feet gelatin hydrolysate that has the lowest IC_{50} value against ABTS radical scavenging activity with value (0.45%) followed by Esperase (0.54%), Neutrase (0.57%), Protamex (0.60%) and lastly Flavourzyme (0.74%).

Key words: Gelatin hydrolysate, duck feet, enzymatic hydrolysis, antioxidant, bioactive peptide scavenging activity

INTRODUCTION

Peptides have the same ability to produce antioxidant activity like other antioxidants with the replicate mechanism (Jemil *et al*., 2016). Antioxidant peptides can be used to manage lipid and protein oxidation that causes oxidative stress in the human body by being endorsed as food ingredients, enhance in the functional food and nutraceuticals (Liu *et al*., 2016). Hence it can help to enhance public health as well as reducing economic losses in food production (Lorenzo *et al*., 2018).

The type of proteases play a vital role for the composition, amount, amino acid sequence, size, and also influence the antioxidant activity of hydrolysate (Liu *et al*., 2016). Alcalase, Flavourzyme and Protamex are industrial food-grade proteinases that have been used in the generation of antioxidant peptides in duck breast meat (Wang *et al*., 2015). The use of Esperase is usually rare, although Mosquera *et al*. (2015) reported the use of this enzyme on dried giant squid tunic gelatin hydrolysate to produce bioactive peptide. Lastly, the usage of Neutrase enzyme is due to the study of blood clam muscle hydrolysate (*Tegillarca granosa*) that has high antioxidant activity (Chi *et al*., 2015). Food protein that was hydrolyzed with different peptidases will produce various peptides pattern (Toldra *et al*., 2017).

Kim *et al*. (2016) reported that the reason for choosing duck feet as a raw material for collagen and gelatin production is due to its complex part of bones and tendons. There are several studies about

* To whom correspondence should be addressed.

extraction of gelatin from Pekin duck feet by using the acidic, alkaline and enzymatic treatment. All the extraction process has produced duck feet gelatin with a distinguished amount of yield for example acetic acid (4%), sodium hydroxide (3%) and Pepsin (5%) (Abedinia *et al*., 2017; Nik Muhammad *et al*., 2017). The previous study from Kuan *et al*. (2017) focuses on physicochemical properties of duck feet gelatin and less on peptide bioactive compound. Nevertheless, there is little information about antioxidant peptides from duck feet gelatin. Therefore, the main objectives of this study were to investigate the scavenging activities (DPPH and ABTS) of duck feet gelatin hydrolysate prepared with five different types of enzyme (Alcalase, Esperase, Flavourzyme, Neutrase and Promex) with three different enzyme-substrate ratio treatment (1:20, 1:15 and 1:10).

MATERIALS AND METHODS

Materials

The Pekin duck feet was purchased from Perak Duck Food Industries Sdn Bhd, Khaki Campbell duck feet was purchased from 4R Agro Enterprise, Pasir Mas, Kelantan and Muscovy duck feet was purchased from duck farm at Kota Bharu, Kelantan. The raw materials were transported under the refrigerated condition to Muscle Lab, Faculty Bioresources and Food Industry, University Sultan Zainal Abidin and stored at -18°C before use. Commercial proteases (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex), DPPH, ABTS and Trolox were purchased from Sigma-Aldrich (St. Louis, United State).

Preparation of duck feet gelatin hydrolysates

Gelatin from Pekin duck feet was extracted by using the method described by Kuan *et al*. (2017). Duck feet gelatin was hydrolyzed by using five different commercial enzymes which are Alcalase, Esperase, Flavourzyme, Protamex and Neutrase. The sample was hydrolyzed for 6 hr under every enzyme optimal temperature and pH conditions with an enzyme-substrate ratio of 1:10, 1:15 and 1:20 (w:w) according to the method of Jin *et al*. (2016). The optimum condition for Alcalase and Flavourzyme is at pH 7 at temperature 50°C and Neutrase at pH 6 and temperature 50°C. Meanwhile, optimum condition for Protamex is at pH 7 and temperature 60°C and for Esperase the optimum condition is at pH 8 and temperature 60°C (Hwang *et al*., 2010; Lee *et al*., 2012). The pH of the reaction was kept constant by adding a 1 N NaOH and 1N HCl solution to the reaction. The enzymes were inactivated by heating at 100°C for 10 min, and the samples were centrifuge (Sigma Sartorius, Germany) at 3000 g for 20 min. The supernatants which comprise the hydrolysates was lyophilized (Freeze dryer Christ alpha 1–4 LD plus) and stored at –80°C for further assays.

DPPH scavenging activity

The DPPH radical scavenging activity of CSGH was determined using the method described by Razali *et al*. (2015) with some modifications. About 500 µL (4.5 mg/mL) of sample solution was mixed with 500 µL of ethanol and 125 µL 0.02% (w/v) of DPPH in 99.5% ethanol. Then, the mixture was shaken vigorously and incubated in a dark place. After 60 min, the absorbance was measured at 517 nm using a spectrophotometer (ThermoSpectronic Genesys 20). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) acts as a positive control. All determinations were based on the means of three measurements. The calculation of the DPPH radical scavenging activity was calculated as follows:

Radical Scavenging activity (%) =
$$[\,(A_{blank} - A_{sample}) \,/\, A_{blank}\,] \times 100$$

where A_{blank} = absorbance of the control (blank) and A_{sample} = absorbance of the sample.

ABTS scavenging activity

ABTS radical [2,22 -azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging assay was carried out according to the method described by Chi *et al*. (2015). The stock solution of ABTS radical consisted of 7 mM ABTS in potassium persulfate 2.45 mM, kept in the dark at room temperature for 16 hr. An aliquot of stock solution was diluted with methanol to give the working solution of ABTS radical an absorbance at 734 nm of 0.70±0.02. A 20 µL aliquot sample was mixed with 200 µL of ABTS reagent and then left to stand in the dark at 30°C for 10 min. A spectrophotometric assay was carried out in a 96-well plate and absorbance values were read at 734 nm using a microplate reader (Multiskan Go Thermo Scientific). The ABTS scavenging activities of the samples were calculated using the following equation:

ABTS scavenging activity (%) =
$$[\,(A_{control} - A_{sample}) \,/\, A_{control}\,] \times 100$$

where $A_{control}$ = absorbance without sample, A_{sample} = absorbance with sample.

RESULT AND DISCUSSION

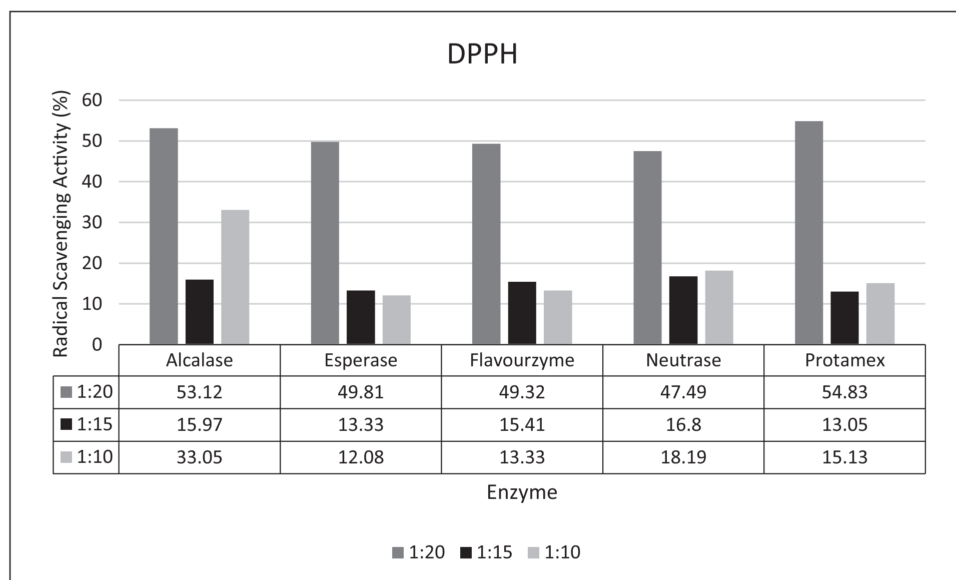
DPPH scavenging activity

DPPH method is the convenient method to estimate the capability of antioxidants to scavenge free radicals because it is a stable free radical that exhibit maximum absorbance at 517 nm (Christodouleas *et al*., 2014; Wang *et al*., 2015). Free radicals will be scavenged by antioxidants through hydrogen atom donation and the absorbance will be reduced (Wang *et al*., 2015; Nenadis & Tsimidou, 2018). Figure 1 below presents the DPPH scavenging activity for five enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) under three different enzyme-substrate ratios (1:20, 1:15, 1:10) at concentration sample 4.5 mg/mL respectively.

The highest DPPH scavenging activity for Alcalase is at enzyme-substrate ratio 1:20 (53.12%), followed by enzyme-substrate ratio 1:10 (33.05%) and enzyme-substrate ratio 1:15 (15.97%) accordingly. The scavenging ability obtained in this study was higher than the study reported by Hasyera and Omar (2016) using chicken skin gelatin hydrolysate which had scavenging activity of 47.33% under treatment enzyme-substrate ratio 1:20. Duck feet gelatin hydrolysate has a higher scavenging activity compare to chicken skin gelatin hydrolysate because duck feet consist of complex parts of tendons and bones hence it can produce more bioactive peptide.

DPPH scavenging activity of duck feet gelatin hydrolysates examined possessed antioxidant activity which was independent on the hydrolysis treatment employed. The hydrolysate yield from Protamex enzyme drop from 1:20 (54.83%) to 1:10 (15.13%) and finally 1:15 (13.05%). Wang *et al*. (2015) reported that the duck meat hydrolysate that was hydrolyzed using Protamex enzyme has 82.09% DPPH radical scavenging activity at a lower concentration sample (1 mg/mL) which are higher than DPPH radical scavenging activity of duck feet gelatin hydrolysate.

All the five enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) that were used to produce duck feet gelatin hydrolysate showed the highest DPPH scavenging activity at enzyme-substrate ratio (1:20). The radical scavenging activities for Alcalase, Esperase, Flavourzyme, Neutrase and Protamex are 53.12%, 49.81%, 49.32%, 47.49% and 54.83% accordingly. The most suitable enzymes that can be used to produce duck feet gelatin hydrolysate with optimum DPPH scavenging activity are Protamex and Alcalase. Alcalase is an endopeptidase which has broad specificity and preference for uncharged residues for example Ser, Thr, Asp and Glu (Luna-vital *et al*., 2014). Meanwhile, Protamex is a mixture of endopeptidase and exopeptidase that work faster in peptide chain compared to Alcalase (Piotrowicz & Mellado, 2015). This study was supported by Latorres *et al*. (2017) finding where white shrimp (*Litopenaeus vannamei*) protein hydrolysate hydrolyzed with Protamex has a higher radical scavenging activity than protein hydrolysate hydrolyzed with Alcalase. The difference in the radical scavenging activity is caused by the amino acid composition, hydrophobicity, length and sequence of peptides within protein hydrolysates (Salem *et al*., 2017).



	Alcalase	Esperase	Flavourzyme	Neutrase	Protamex
1:20	53.12	49.81	49.32	47.49	54.83
1:15	15.97	13.33	15.41	16.8	13.05
1:10	33.05	12.08	13.33	18.19	15.13

Fig. 1. DPPH scavenging activity for five enzyme (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) with three different enzyme-substrate ratio (1:20, 1:15, 1:10) at concentration sample 4.5 mg/mL.

Table 1. ABTS scavenging activity for five enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) with three different enzyme-substrate ratios (1:20, 1: 15, 1:10) at concentration sample 4.5 mg/mL

Enzyme – Substrate Ratio	1:20 (%)	1:15 (%)	1:10 (%)
Alcalase	0.45 ± 0.02^{a}	0.58 ± 0.01^{b}	0.47 ± 0.01^{a}
Esperase	0.54 ± 0.02^{a}	0.60 ± 0.01^{b}	0.61 ± 0.02^{b}
Flavourzyme	0.76 ± 0.01^{a}	0.74 ± 0.01^{a}	0.81 ± 0.03^{b}
Neutrase	0.70 ± 0.02^{c}	0.57 ± 0.01^{a}	0.61 ± 0.01^{b}
Protamex	0.75 ± 0.01^{c}	0.60 ± 0.02^{a}	0.64 ± 0.01^{b}

ABTS scavenging activity

Antioxidant activity of hydrophilic and lipophilic compounds was measure by ABTS radical capture method (Centenaro *et al.*, 2014). This method involving electron transfer method based on the decrease of ABTS radical solution absorbance within the range 710-760 nm (Christodouleas *et al.*, 2014). Table 1 below presents the ABTS scavenging activity for five enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) under three different enzyme-substrate ratios (1:20, 1:15, 1:10) at concentration sample 4.5 mg/mL respectively.

Duck feet gelatin hydrolysate that was obtained from Alcalase enzyme showed the IC_{50} value for ABTS scavenging activity at concentration sample 4.5 mg/mL. IC_{50} result for the enzyme-substrate ratio (1:20), (1:15) and (1:10) are 0.45%, 0.58% and 0.47% respectively. Thus, the enzyme-substrate ratio (1:20) has the highest scavenging activity compare to other hydrolysates produce with Alcalase enzyme. Mosquera *et al.* (2015) reported that dried giant squid tunic hydrolysate that was hydrolyzed using Alcalase enzyme has 43.79% of ABTS radical scavenging activity at concentration sample 45 mg/g. Duck feet gelatin hydrolysate has a higher ABTS radical scavenging activity than dried giant squid tunic that was hydrolyzed by using the same enzyme.

Duck feet gelatin hydrolysate that was obtained from Esperase enzyme showed the IC_{50} value for ABTS scavenging activity at concentration sample 4.5 mg/mL. The enzyme-substrate ratio (1:20) has the lowest IC_{50} value which is 0.54%. Meanwhile, both the enzyme-substrate ratio (1:15) and (1:10) had the comparable IC_{50} value which was 0.60% and 0.61% respectively. Mosquera *et al.* (2015) reported that dried giant squid tunic hydrolysate that was hydrolyzed using Esperase enzyme has 43.36% of ABTS radical scavenging activity at concentration sample 45 mg/g. Protein hydrolysate of dried giant squid tunic with a higher concentration sample has a lower radical scavenging activity than duck feet gelatin hydrolysate obtained from the Esperase enzyme.

The highest IC_{50} value for ABTS scavenging activity for duck feet gelatin hydrolysate produced from Alcalase and Esperase is at enzyme-substrate ratio (1:20) which are 0.45% and 0.54% and respectively. Meanwhile Flavourzyme, Neutrase and Protamex generate a bioactive peptide that has the highest IC_{50} at enzyme-substrate ratio (1:15) which are 0.74%, 0.57% and 0.60% accordingly. Hydrolysates reducing activity was affected by the enzyme type since there was a verified significant difference between the values in the same period of reaction (Latorres *et al.*, 2017). Alcalase are more likely to cleave protein at the carboxy side of hydrophobic residues (Toldra *et al.*, 2017). Esperase is a serine-type endoprotease that shown a high proteolytic activity on fish and cephalopod waste substrates (Vazquez *et al.*, 2017). Meanwhile, Flavourzyme and Protamex are mixtures of endopeptidase and exopeptidase that work faster in peptide chain compare to Alcalase (Piotrowicz & Mellado. 2015). While Neutrase has broad specificity and preferably for N-terminus, Leu and Phe (Toldra *et al.*, 2017).

CONCLUSION

In conclusion, the type of enzyme and enzyme-substrate ratio of duck feet gelatin hydrolysate play a significant role in antioxidant activity. This activity includes the DPPH radical scavenging activity and ABTS radical scavenging activity. Duck feet gelatin is a potential raw material that can be used to produce bioactive peptide hence reducing the waste product in the environment. Overall, this peptide has the potential to be developed into new biomaterials.

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