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Antihypertensive and Antiproliferative Activities of Bioactive Peptide from Pekin Duck Feet Gelatin Hydrolysed by Different Enzymes

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Pekin duck (*Anasplatyrhyncosdomestica*) is one of the famous duck breed. Gelatin that was extracted from Pekin duck feet has shown a potential raw material for production of bioactive peptide that has multiple effects in the physiological function of organism for example as antihypertensive and antiproliferative agents. Pekin Duck feet gelatin was hydrolyzed by using five commercial enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) to identify the bioactivities of derived peptides. All the five enzymes were studied under three different enzyme-substrate ratio (1:10, 1:15, 1:20) with every enzyme optimum temperature, time and pH.ACE is the angiotensin-I-converting enzyme that regulates the blood pressure and heart function. The optimum condition to produce ACE inhibitory hydrolysate is at enzyme-substrate ratio (1:10).Hydrolysate from the peptide that was produced from Neutrase, Acalase, Protamex, Flavourzyme and Esperase has ACE inhibitory activity of 1.54 mg/ml, 1.71 mg/ml, 2.37 mg/ml, 3.92 mg/ml and 4.43 mg/ml accordingly. Meanwhile, the IC_{50}value forantiproliferative activity of bioactive peptide are 26.40 µg/ml, 40.64µg/ml, 48.23µg/ml, 60.83µg/ml and 71.93µg/ml for Esperase, Protamex, Flavourzyme, Neutrase and Alcalase respectively.Pekin duck feet gelatin hydrolysate shows the highest antiproliferative activity at enzyme-substrate ratio (1:20). The ability of compound to inhibit the growing process of cell is known as antiproliferativeactivity.This finding shows that gelatin hydrolysate from waste product such as duck feet can be an alternative ingredients for food and pharmaceutical industries for antihypertensive and antiproliferative activities naturally.

Keywords: gelatin hydrolysate, duck feet, antihypertensive, antiproliferative, enzymatic hydrolysis

INTRODUCTION

The primary factor of death and disability all over the world as well as Malaysia is cardiovascular diseases involving both genders (Nurulhuda et al. 2020).Blood pressure level that is higher than 120 mmHg systolic and above 80 mmHg diastolic is defined as hypertension (Abdullah et al. 2016).In the both developed and developing countries, hypertension has been recognized as the endanger element for cardiovascular disease (CVDs). It is one of the prevalent components in the worldwide chronic disease. (Ghanbari et al. 2015). It is usually recognized when blood flow in excessive high pressure where systolic values is ≥ 140 mmHg and diastolic values is ≥ 90 mmHg caused by lack of dilated blood vessels and decreased blood flow. Renin-angiotensin system (RAS) plays an important role in modulating human blood pressure which is control by two significant proteases, angiotensin-converting enzyme (ACE) and renin (Aluko., 2015). Renin will cleave angiotensinogen

into angiotensin I and ACE will catalyzes the conversion of angiotensin I to angiotensin II, which is a potent vasoconstricter (Pujiastuti et al. 2019). Furthermore, ACE also inactivating the vasodilatingbradykinin which is also involved in nitric oxide synthesis into inactive peptide fragment (Pujiastuti et al. 2019; Abdelhedi&Nasri., 2019).

The mechanism of antihypertensive peptide acting as ACE inhibitor is by dilating the arterial wall and decreasing the volume of fluid. This action will block the production of angiotensin-II while improving the oxygen flow rate to the kidney, heart and live whilst enhancing the function of heart (Ngo et al. 2012). Treatment of hypertension by using synthetic drugs has been reported to cause headache, dizziness, cough, dysgeusia and angioedema toward the patient. Meanwhile food derived ACE inhibitor has no side effects or mild effects to the patient though it may be less effective than synthetic ACE inhibitors (Norris & Fitzgerald., 2013; Bah et al. 2013) (Daliri et al. 2016). In addition, some peptides have been proven to reducing the angiotensin-converting enzymes (ACE) or renin activity thus regulating the renin-angiotensin system (RAS). Endothelial nitric oxide synthase (Enos) pathway also using peptides to enhance the raise of (NO) levels within vascular walls and boosting vasodilation (Aluko., 2015).

The leading death factor caused by non-communicable diseases (NCDs) that resulting in the worldwide extensive number of deaths is cancer. From 18.1 million cancer case that has been reported in 2018, 9.6 million from it ended with death. Breast cancer is the most popular killing causes for woman while lung cancer is the most repeated cases reported among the man (WHO 2018; Bray et al. 2018). The universal method for treating cancer such a chemotherapy and surgery could not stopping the risk of getting cancer for a second time and the rate of success is low (Harris et al. 2013). Recently, natural source has been a popular choice for treating cancer due to the extensive study of its biological activities on human health (Johari et al. 2019).In the drug research and development process, anticancer peptides have been recognized as the innovative method for the specific cancer in the molecular stage. The anticancer peptides have been the assuring molecules of anticancer agents due to their remarkable properties and distinctive mechanism as it will not damage the normal body physiological function (Gaspar et al., 2013; Huang et al. 2015). Derived peptides from aquatics can kill cell with numerous mechanism such as antiproliferative activity, apoptosis and cytotoxicity (Yaghoubzadeh et al., 2019).

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 extraction of gelatin from Pekin duck feet by using 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%) (Muhammad et al.2017; Abedinia et al., 2017). Previous study from Kuan et al. (2017) focusing on physicochemical properties of duck feet gelatin and less on peptide bioactive compound. Nevertheless, there is little information about antiproliferative peptides from duck feet gelatin. Therefore, the main objectives of this study was to investigate antihypertensive activities and the antiproliferative activities of duck feet gelatin hydrolysate prepared with five different type of enzyme (Alcalase, Esperase, Flavourzyme, Neutrase and Promex) with three different enzyme-substrate ratio treatment (1:20, 1:15 and 1:10) on the MCF-7 (breast cancer) cell line.

MATERIALS AND METHODS

Preparation of Duck Feet Gelatin Hydrolysate

The extraction of Pekin duck feet gelatin has been done by using method described by(Kuan et al. 2017). Duck feet gelatin was hydrolyzed by using five different commercial enzymes which were Alcalase, Esperase, Flavourzyme, Protamex and Neutrase. Sample was hydrolyzed for 6 h 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 were at pH 7 at temperature 50°C and Neutrase at pH 6 and at temperature 50°C. Meanwhile optimum condition for Protamex were at pH 7 and at temperature 60°C and for Esperase the optimum condition were at pH 8 and at temperature 60°C (Lee et al., 2012; Hwang et al. 2010). 1 N NaOH and 1N HCl solution was added to the reaction in order to keep the pH constant. The enzymes were inactivated by heating at 100 °C for 10 min, and the samples were centrifuged (Sigma Sartorius, Germany) at 3000 g for 20 min. The supernatants which comprised the hydrolysates was lyophilized (Freeze dryer Christ alpha 1-4 LD plus) and stored at −80 °C for further assays.

ACE Inhibitory Activity

Cushman and Cheung (1971) method with slight modification was used to prepare ACE inhibitory activity assay. Aangiotension converting enzyme (ACE) extracted from rabbit lung (A6778; Sigma), was prepared with 300 mmol sodium tetraborate buffer (8 mU/50 mL). The synthetic substrate that was used was Hippuryl-L-histidyl-L-leucine (HHL). 50 mL of ACE solution was added to 30 mL of hydrolysate (5 mg/ml). 50 mL of 5 mmol HHL was added to the solution before the reaction started. Solution was incubated at 37°C for 30 min. 380 mL of 1.0 N hydrochloric acid (HCl) was added to terminate the reaction. After that, 1.5 mL of ethyl acetate was added to extract the resulting hippuric acid. The solution was centrifuged at 3600g for 5 min. 1 mL of upper layer was transferred into a microcentrifuge tube and heated in the oven at 100°C for 1 h. After that, 1.0 mL of distilled water was added to dissolve the hippuric acid produced and the solution was read by using spectrophotometer (ThermoSpectronicGenesys 20) at absorbance 228 nm. ACE inhibitory activity of the peptide was calculated using the absorbance of hippuric acid liberated from HHL by ACE. The IC_{50}value was defined as the concentration of peptide (mg/mL) required to inhibit 50% of ACE activity.

Antiproliferative Activity

Antiproliferative activity of Pekin duck feet gelatin hydrolysate was performed according to Oladimenji et al. (2016) with slight modification. Breast cancer cells (MCF-7) with the concentration of 5 × 10^3 cells per well were plated in a 96-well plate and incubated for 24 h. After that, 10 mg/ml of compound with different serial concentrations were added into the plate. The plate was incubated for 72 h in 37 ºC at 5% CO_2. Next, 20 µL of thiazolyl blue tetrazolium bromide (MTT) reagent (5 mg/mL) was added into each well. 150 µL of an absolute DMSO was added after 4 h of incubation. Cisplatin was used as positive control and untreated cell was used as a negative control. The cytotoxicity values were calculated after measuring the absorbance at 570 nm and 630 nm using a microplate reader Multiskan Go Thermo Scientific (Waltham, Massachussetts, United States). The hydrolysate concentration which gives 50% of growth inhibition is referred as IC_{50}. The experiment was repeated three times and the $IC_{50}$of inhibition was calculated using the following formula:

IC_{50}Inhibition (%) = $\frac{(Abs\ control - Abs\ treated)}{Abs\ control}$ x 100%

Statistical Analysis

Each sample was conducted with triplicate. Comparison of means among sample was conducted using Duncan's multiple range tests by one-way ANOVA at a significant level of $p < 0.05$. All data were analyzed using SPSS (Statistical Package for Social Science) software version 25.0 (SPSS Inc., Chicago, II, U.S.A)

RESULTS

Antihypertensive Activities

Table 1 below shows the IC_{50}value for ACE inhibitory 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 5 mg/ml respectively. The higher percentage of ACE activity, the lower the IC_{50}value of the hydrolysates indicates higher effectiveness. The IC_{50}value of five different enzymes with three different enzyme substrate ratios, ranged from 1.51-4.54 mg/ml.

Table 1: IC_{50}value for ACE inhibitory activity of duck feet gelatin hydrolysate hydrolyse with five different enzymes with three diffeIC50 rent enzyme-substrate ratios

Enzyme Name	$_{50}$value (mg/ml)		
	1:20 (%)	1:15 (%)	1:10 (%)
Alcalase	2.04± 0.06^{c}	1.90 ± 0.06^{b}	1.71 ± 0.03^{a}
Esperase	4.37 ± 0.02^{a}	4.54± 0.06^{b}	4.43 ± 0.06^{a}
Flavourzyme	4.52 ± 0.08^{c}	3.52 ± 0.26^{a}	3.92± 0.17^{b}
Neutrase	2.25 ± 0.07^{b}	1.76 ± 0.24^{a}	1.54 ± 0.11^{a}
Protamex	2.98 ± 0.17^{b}	3.03 ± 0.11^{b}	2.37 ± 0.08^{a}

Values are expressed as mean±standard deviation. Mean with different letters were significantly different at the level of P <0.05 in the same row.

Antiproliferative Activities

Table 2 below presents the antiproliferative activity for five enzymes (Alcalase, Esperase, Flavourzyme, Neutrase and Protamex) with three different enzyme-substrate ratio (1:20, 1:15, 1:10) at concentration sample 10 mg/ml respectively. The cytotoxicity effect of duck feet gelatin hydrolysate against breast cancer cell line (MCF-7) was determined using MTT assays. The positive control that has been used for this assay was cisplatin.

Table 2: IC_{50}value for antiproliferative activity of duck feet gelatin hydrolysate hydrolyse with five different enzymes with three different enzyme-substrate ratios

Enzyme Name	IC_{50}value (µg/ml)		
	1:20 (%)	1:15 (%)	1:10 (%)
Alcalase	71.93 ± 0.06[c]	54.33 ± 0.45[a]	66.66 ± 0.41[b]
Esperase	26.40 ± 0.55[a]	41.93 ± 0.07[b]	67.52 ± 0.51[c]
Flavourzyme	48.23 ± 0.30[a]	51.26 ± 0.27[b]	55.23 ± 0.38[c]
Neutrase	60.83 ± 0.35[a]	73.41 ± 0.19[b]	75.70 ± 0.36[c]
Protamex	40.64 ± 0.41[c]	29.64 ± 0.48[b]	27.96 ± 0.13[a]

Values are expressed as mean±standard deviation. Mean with different letters were significantly different at the level of P <0.05 in the same row.

DISCUSSION

Antihypertensive Activities

Among all the three treatment of Neutrase enzyme on duck feet gelatin, the enzyme-substrate ratio 1:10 produced the bioactive peptide with the highest ACE inhibition activity. The IC_{50}values of the hydrolysates gradually decreased as the enzyme-substrate ratio decreased from 1:20 (2.25 mg/ml) to 1:15 (1.76 mg/ml) and lastly 1:10 (1.54 mg/ml). Chicken collagen hydrolysate that was hydrolyzed with Neutrase enzyme that produced hydrolysate with low molecular weight fraction (1200- 450 Dalton) has IC_{50}value of 59.7 ug/ml (Soladoye et al. 2015).

Alcalase enzyme produced hydrolysate that has increasing ACE inhibition activity as enzyme-substrate ratio decreased. The IC_{50}value of enzyme-substrate ratio (1:20) was 2.04 mg/ml followed by 1.90 and 1.71 mg/ml from enzyme-substrate ratio (1:15) and (1:10) accordingly. Intarasirisawat et al.(2013) and Abdelhedi et al., (2019) has reported that bioactive peptide from *Katsuwanapelamis* that was produced by Alcalase enzyme has the IC_{50} value of 2.5 mg/ml which was higher than IC_{50}value of duck feet gelatin hydrolysate. Duck feet gelatin hydrolysate was more effective in inhibiting the ACE activity compare to the *Katsuwanapelamis* hydrolysate. The IC_{50} value forhydrolysate of chicken leg bone protein produced by Alcalase enzyme was 1.96 mg/ml and 0.94 mg/ml at 4 and 8 hours hydrolysis time accordingly (Cheng et al., 2008; Bhat et al. 2015). The IC_{50}value of duck feet gelatin hydrolysate was 1.71 mg/ml at 6 hours hydrolysis time. A longer hydrolysis time has a significant effect towards ACE inhibitory activity (Slizyte et al. 2016; Abdelhedi & Nasri., 2019). As the time of the hydrolysis process increases, the IC_{50} value decreases indicating the ACE inhibition activity of hydrolysates has been more efficient.

The 1:10 enzyme-substrate ratio for enzyme Protamex has the highest ACE inhibition activity of all three treatments. The IC_{50} value of enzyme-substrate ratio 1:10 was 2.37 mg/ml followed by 3.03 mg/ml and 2.98 mg/ml for enzyme-substrate ratio 1:15 and enzyme-substrate ratio 1:20 respectively. Mud crab protein hydrolysate that was prepared with Protamex at optimum condition was 1.96 mg/ml at concentration sample 10 mg/ml (Harun et al., 2017). Fish protein hydrolysate from defatted salmon backbone that was produced from Protamex has IC_{50} value of 3 mg/ml at hydrolysis time 120 minute (Slizyte et al. 2016).

ACE peptide is normally made of lower molecular weight peptides which are < 10 kDa and made of 3-20 amino acids (Harun et al., 2017). Hydrolysis of the gelatin using alkaline protease such as Alcalase has been proved to produce peptide with the highest ACE inhibitory activity (Bao et al., 2016). This correlates with this study where Alcalase enzyme has the lowest IC_{50}value (2.04) at its optimum enzyme-substrate ratio (1:20). Interestingly, Neutrase has produced duck feet gelatin hydrolysate with the highest ACE inhibition activity at enzyme-substrate ratio (1:15) and (1:10) with the IC_{50}value of 1.76 and 1.54 respectively. Duck feet gelatin hydrolysate that was produced by Neutrase enzyme has the highest molecular weight peptides due to the enzyme nature which is known as endopeptidase. Endopeptidase enzyme is the enzyme which does not has specific enzyme cutting site (Yu et al., 2018).

Antiproliferative Activities

Amongst of samples, gelatin hydrolysates obtained from enzyme Esperase, with enzyme-substrate ratio 1:20 (26.40 µg/ml) presented the lowest IC_{50} value in comparison with other hydrolysates obtained with the same enzyme with different treatment. Another two treatments has the IC_{50} value of 41.93µg/ml and 67.52µg/ml from enzyme-substrate ratio 1:15 and and 1:10 respectively. Squid gelatin hydrolysate that was produced from Esperase enzyme hasIC_{50} value of 0.13 mg/ml (Aleman et al., 2011; Chalamaiah et al.2017). Interestingly, gelatin hydrolysates obtained from enzyme Protamexfor the enzyme-substrate ratio (1:20), (1:15) and (1:10) showed the IC_{50} values of 40.64µg/ml, 29.64 µg/ml and 27.96 µg/ml respectively. Thus, the enzyme-substrate ratio (1:10) has the lowest IC_{50} value compare to other hydrolysates produced with Protamex enzyme. Amongst of all sample tested, Esperase (1:20) and Protamex (1:10) has produced the duck feet gelatin hydrolysates which has the capability effect ofhighest cytotoxicity against breast cancer (MCF-7) cell line.

The lowest IC_{50} value for Flavourzyme was at enzyme-substrate ratio 1:20 (48.23 ug/ml), followed by enzyme-substrate ratio 1:15 (51.26 ug/ml) and enzyme-substrate ratio 1:10 (55.23ug/ml) accordingly. Rainbow trout skin hydrolysate that was hydrolyzed with Flavourzyme enzyme has IC_{50} value of 727.4 ug/ml with molecular weight less than 3 kDa when treated at HCT-116 cell line (Yaghoubzadeh et al. 2019). Flavourzyme produced the highest activity of anticancer activity from duck feet gelatin hydrolysate at enzyme-substrate ratio 1:20.

The initiation, promotion and progression stages of cancer were inhibited by the action of anticancer bioactive peptide (De Mejia & Dia., 2010; Lafarga et al., 2017). The death of cancer cell was induced by various mechanisms by anticancer peptides such as inhibiting angionegenesis, apoptosis and affecting the tubulin-microtubule equilibrium (Li et al. 2015). The most efficient way for the body to regulate cell death and division is by undergoing apoptosis process, the safely regulated and programmed of death cell (Indran et al. 2011; Nwachukwu & Aluko., 2019). Disrupting the plasma membrane and mitochondrial pathway are two different mechanism for anticancer peptides to promote the cell death (Wang et al. 2017). Characteristics of bioactive peptide and target membrane affect the mechanism of the membranolytic activity that can happen through membrane dissolution, pore formation in the lipid and the thinning of membrane layer (Riedl et al. 2015; Wang et al.2017).

The structural characteristics of food derived peptides such as amino acid length, sequence, composition, overall charge affect the peptide anticancer activity (Chi et al., 2015). Peptides that were derived from food that exhibit anticancer properties usually made of short amino acid sequence that were ranged between 3 to 25 residues (Pan et al. 2016). Besides that, Pan et al. (2016) stated that amino acid with hydrophobic properties has a crucial role for peptide anticancer activity. This is due to the anticancer peptides that form stronger interaction with cancer cell membrane bilayer hence causes selective and higher cytotoxicity activity (Chi et al. 2015).

CONCLUSION

The present study explore some physiological function derived from waste product such as duck feet gelatin hydrolysate. Alcalase, Esperase, Flavourzyme, Neutrase and Protamex were used for hydrolysis at their optimum temperature, time, pH and different enzyme-substrate ratio. Neutrase (1:10) and Alcalase (1:10) has been the most effective enzymes to produce hydrolysate with high ACE inhibition activity. Meanwhile Esperase (1:20) and Protamex (1:10) induced high toxicity against breast cancer cell line (MCF-7). This study showed weak relationship of peptide bioactive properties such as ACE inhibition activityand antiproliferative activity with enzyme-substrate ratio. However, ACE peptide of Alcalase and antiproliferative peptide of Protamex with enzyme-substrate ratio has been comparatively noted.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

FA performed the experiment, analysed the data and wrote the manuscript. NS, NH and AWS supervised, design the experiment and reviewed the manuscript. All authors read and approved the final version.

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