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Bioactivity of enzymatically prepared eel (*Monopterus* sp.) protein hydrolysate at different molecular weights

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Abstract

This study determined the antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activity of fractionated (3, 5, 10kDa) eel protein hydrolysate (EPH), which was prepared enzymatically using alcalase. The bioactivities observed were reducing power, metal chelating activity, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, hydroxyl radical scavenging activity, and ACE inhibitory activity. The results showed that the EPH at 5 kDa had significantly ($p<0.05$) higher reducing power, metal chelating activity and hydroxyl radical scavenging activity compared to the 3kDa and 10kDa EPH fractions. Meanwhile, the 10 kDa EPH fraction showed significantly ($p<0.05$) higher DPPH scavenging activity compared to the 3 kDa and 5 kDa fractions. In terms of ACE inhibitory activity, EPH at 3 kDa showed the highest inhibition ability (71.90%) significantly ($p<0.05$) and the ACE inhibitory activity decreased with the increase in the molecular weight (MW) of the EPH. The results obtained showed that EPH possessed potential bioactivity and antioxidant and antihypertensive properties, and that the activity was highly affected by the molecular size in hydrolysate production using alcalase. The EPH produced also showed the potential for the use of EPH in the food and nutraceutical industry.

Introduction

Generally, bioactivity refers to the specific effect or a reaction that occurs in living organisms upon the exposure to a substance. A substance with biological activity is called a bioactive compound (Guaadaoui *et al*., 2014). Bioactive compounds in native proteins are unable to perform bioactivity unless they are released by proteolysis (*in vivo* digestion) or hydrolysed (in vitro using enzymes) to demonstrate biological functions, such as anti-oxidation, anti-proliferation, anti-hypertension or anti-inflammation (Suarez-Jimenez *et al*., 2012; Chakrabarti *et al*., 2014)

In recent years, lipid peroxidation has become the biggest concern in the food industry as it contributes to the undesirable off-flavours and potential toxicity. Many synthetic antioxidants, such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), tert-Butylhydroquinone (TBHQ) and Propyl Gallate (PG) are used to prevent oxidation. However, due to health hazards, the use of synthetic antioxidants is restricted (Centenaro *et al.*, 2011). Therefore, the effort to find natural antioxidants from natural sources, such as plants and marine living organisms is increasing including research on fish protein hydrolysates. Peptides derived from fish proteins by enzymatic hydrolysis have shown the ability to exert significant antioxidative activities in different oxidative systems (Klompong *et al*., 2007; Je *et al.*, 2009; Batista *et al.*, 2010).

Another great contemporary concern is high blood pressure (hypertension), as it accounts for about 12.8% of total deaths, worldwide, as reported by the World Health Organization (WHO, 2015). Angiotension I-converting enzyme (ACE) leads to an increase in blood pressure by producing the vasoconstrictor peptide angiotensin II and by degrading the vasodilator peptide bradykinin (Je *et al*., 2009). Hence, ACE Inhibitors are used as therapeutic agents against hypertension. However, the synthetic hypotensive drugs (e.g. captopril, enalapril and lasinopril) have many side effects, such as dry cough, taste disturbances and skin rashes (Ghassem *et al*., 2011). Therefore, naturally safe alternative products with high ACE-inhibitory activity are being sought for the prevention and remedy of hypertension (Nasri *et al.*, 2013). The study of the ACE inhibitory activity of peptides has been widely conducted and has been reviewed by Wijesekara and Kim (2010). Different peptide sequences have been found from different fish sources; for example, Channa striatus hydrolysate (Val-Pro-Ala-Ala-Pro-Pro-Lys) (Ghassem *et al.*, 2011), lizard fish hydrolysate (Ser-Pro-Arg-Cys-Arg) (Wu *et al.*, 2012) and Alaska Pollack hydrolysate

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(Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala) (Je *et al*., 2004).

Hydrolysis of fish proteins by different enzymes, such as alcalase, neutrase, papain, trypsin and bromelain is the most applied technique to break down the complex proteins into smaller chain peptides containing 2-20 amino acids (Chalamaiah *et al*., 2012). Alcalase has been shown to have high proteolytic activity, compared to acid and neutral enzymes (Klompong *et al*., 2007). The enzymatic treatments of protein are able to generate peptides and amino acids, which can modify the biological and functional characteristics of the protein, thus improving their quality and offering interesting opportunities for food applications (Balti *et al*., 2010). A great deal of interest has been expressed regarding marine derived bioactive peptides because of their numerous beneficial effects on health (Wijesekara and Kim, 2010). Eels are considered to be one of the marine organisms that have the potential to be explored as they are a valued remedy in oriental medicine (Khanh and Ngan, 2010). Besides that, it is widely distributed in many countries from India to China, Japan, Malaysia, Indonesia, Bangladesh, Thailand and Vietnam (Froese and Pauly, 2008). As the market of eels getting high demand, many countries such as China, Philippines and Vietnam start the eel culture activities while in Malaysia, the activities are still unsuccessful (Subasinghe and Hasan, 2010; Khanh and Ngan, 2010; Baquiran and Prudencio, 2013).

In particular, the peptide molecular weight (MW) has a great impact on the characteristics of the hydrolysate produced, hence, pressure-driven membrane separations can be used as a second control step to increase their specific activity (i.e. per gram of peptide) (Bourseau *et al*., 2009). In addition, short peptides, below 3 kDa or 4 kDa, usually harbour bioactive properties and the peptide size is a physicochemical parameter (regardless of the specific peptide chains) in controlling the peptide activities (Picot *et al*., 2010). Therefore, the objective of this study was to determine the antioxidant activity and ACE inhibitory activity of fractionated (3 kDa, 5 kDa, 10 kDa) eel protein hydrolysate (EPH) in order to investigate its potential applications, especially in the food industry.

Material and Methods

Materials

Eels were purchased from the local market in Kuala Terengganu, Malaysia and brought to the laboratory alive. Fresh eels were eviscerated, filleted and beheaded in order to obtain their flesh, which was homogenized and frozen until further use at -80ºC. The enzyme alcalase (2.4Au/g and density of 1.18g/ml), Angiotensin Converting Enzyme (ACE) from rabbit lung, hippuric acid (HA) and substrate peptide (hippuryl-L-histidyl-L-leucine, HHL) were purchased from Sigma-Aldrich company ltd. All the other chemicals used were of analytical grade.

Preparation of eel protein hydrolysate (EPH)

The eel flesh was thawed overnight in a chiller at 4ºC prior to use. The hydrolysis was conducted according to the method of Jamil *et al.* (2016) with slight modification. About 44g of eel was added to 99 ml of distilled water. Then, the mixture was heated at 85ºC for 20min in a water bath to inactivate the endogenous enzyme. After cooling to a specified temperature (56ºC), 20g of the alcalase solution (prepared by diluting 1.8% enzyme to a final weight of 20g with distilled water) was mixed into the mixture and the hydrolysis was initiated immediately. The pH of the hydrolysis mixture was maintained and adjusted to pH 9 by manual adjustment using 1N NaOH. After 2hours, the hydrolysis process was terminated by heating the mixture at 85 ºC for 20min to inactivate the alcalase activity. The mixture was cooled and centrifuged at 3500 rpm (Gyrozen 158R, Korea) for 30 min at 4ºC.

The supernatants were collected and fractionated at different molecular weights (3, 5, 10 kDa) using a Vivaspin ultrafilter membrane (Sartorius, Germany). The hydrolysate was fractionated through a 10 kDa molecular weight cut-off (MWCO) by centrifuging at the same speed and time as mentioned, and the resultant fraction was then fractionated through a 5 kDa molecular weight cut-off (MWCO) before further fractionation through a 3 kDa molecular weight cut-off (MWCO) membrane. Then, the eel protein hydrolysate (EPH), at different molecular weights, was freeze-dried, weighed and stored at -80ºC for further analysis.

Reducing power activity of fractionated eel protein hydrolysate (EPH)

The reducing power activity of fractionated eel protein hydrolysate (EPH) was determined according to Razali *et al.* (2015). Approximately 2.5ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of 1% weight/volume potassium ferricyanide (K3Fe(CN)6) was added to 1.0ml of 10mg/ml sample. The resulting mixture was incubated at 50ºC for 20min, followed by the addition of 2.5ml of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 3000rpm for 10min to collect 2.5ml of the supernatant to be mixed with 2.5ml distilled water and 0.5ml of 0.1%

ferric chloride ($FeCl_3$). The absorbance was then measured at 700 nm by spectrophotometer (Cary WinUV, USA) against a blank sample. The butylated hydroxytoluene (BHT) was used as positive control. Higher absorbance indicates higher reducing power.

Metal chelating activity of fractionated eel protein hydrolysate (EPH)

The chelation on Fe^{2+} of fractionated eel protein hydrolysate (EPH) was estimated using the method of Razali *et al*. (2015). About 2.5 µl of 2 mM $FeCl_2$ was added to a 10 mg/ml sample in 0.5 ml methanol. The reaction was started by the addition of 0.1 ml of 5mM ferrozine and incubated at room temperature for 10min. The absorbance of Fe^{2+}-ferrozine complex was then measured at 562 nm by using a spectrophotometer (Cary WinUV, USA). The butylated hydroxytoluene (BHT) was used as positive control. The ability of EPH to scavenge metal chelating radical activity was calculated as follows:

$$\text{Metal chelating activity (\%)} = ([A_0 - A_1]/ A_0) \times 100$$

Where:

A_0 = the absorbance of the control

A_1 = the absorbance of the mixture containing sample

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of fractionated eel protein hydrolysate (EPH)

The 1, 1- diphenyl- 2-picrylhydrazyl (DPPH) radical scavenging activity of fractionated eel protein hydrolysate (EPH) was determined according to Razali *et al.* (2015). Approximately, 1ml of 0.1mM DPPH solution in 99.5% of ethanol was mixed with 3ml of 10mg/ml sample. The mixture was incubated at room temperature for 30min and the absorbance was measured at 517nm by using a spectrophotometer (Cary WinUV, USA). Distilled water was used as a control and tested in the same manner as the sample. The butylated hydroxytoluene (BHT) was used as positive control. The ability of EPH to scavenge DPPH radicals was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = ([A_0 - A_1]/ A_0) \times 100$$

Where:

A_0 = the absorbance of the control

A_1 = the absorbance of the mixture containing sample

Hydroxyl radical scavenging activity of fractionated eel protein hydrolysate (EPH)

The radical hydroxyl radical scavenging activity of fractionated eel protein hydrolysate (EPH) was determined using the method described by Kunchandy and Rao (1990) with modification. The reaction mixture contained 1ml of 0.75mM 1, 10-phenanthroline, 1ml of 0.75mM $FeSO_4$, 2ml phosphate buffer (pH 7.4), 1ml of 0.01% H_2O_2 and 1ml of 10mg/ml sample. The reaction started with the addition of H_2O_2. The mixture was incubated at 37°C for 60 min before being measured by spectrophotometer (Cary WinUV, USA) at 536 nm. The butylated hydroxytoluene (BHT) was used as positive control. The hydroxyl radical scavenging activity of the fractionated EPH was determined using the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where,

A_0 = the absorbance of control

A_1 = the absorbance of the mixture containing sample

Angiotensin-I-converting enzyme (ACE) inhibitory activity of fractionated eel protein hydrolysate (EPH)

The angiotensin-I-converting enzyme (ACE) inhibitory activity of fractionated eel protein hydrolysate (EPH) is based on the amount of hippuric acid (HA) released from hippuryl-L-histidyl-L-leucine (HHL) by the action of ACE. The ACE inhibitory activity assay was performed according to the method of Wu *et al.* (2002) with some modification by Kasase (2009). The reaction mixture was made up of 50 µl of 2.17 mM HHL, 10 µl of 2 mU of ACE and 10 µl of sample fractions (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH 8.3) giving a total volume of 70 µl. ACE was incubated at 37°C for 10min before the two solutions (HHL and sample) were mixed together and incubated at 37°C for 30min with continuous agitation. After 30 min, 85 µl of 1M HCl was added to terminate the reaction and vortexed. Then, 500 µl of ethyl acetate was added to extract the hippuric acid and vortexed. Through evaporation, a 200 µl aliquot of the extract was removed. The residue was dissolved in 1 ml distilled water and vortexed, before reading, the absorbance was measured at 228 nm using a spectrophotometer (Cary WinUV, USA). A positive control (HHL and enzyme) and blank (HHL and buffer) were also prepared in the same manner. The ACE inhibition activity of fractionated EPH was

determined using the following equation:

ACE inhibition activity (%) = $(A_c - A_s / A_c - A_b) \times 100$

Where,

A_b = the absorbance of the blank

A_c = the absorbance of the control

A_s = the absorbance of the mixture containing sample

Data analysis

One-way analysis of variance (ANOVA) was conducted and all data were presented as mean ± standard deviation using MINITAB Statistical Software version 14.0 and Microsoft Excel. All the results were determined in triplicate.

Results and Discussion

Reducing power activity of fractionated eel protein hydrolysate (EPH)

The reducing power activity (RPA) of fractionated eel protein hydrolysates (3, 5 and 10 kDa) was determined at a concentration of 10mg/ml. The EPH at all fractions showed the ability to reduce the ferric cyanide complex. Figure 1 shows that the highest RPA was at fraction 5 kDa (0.403) followed by 10 kDa (0.388) and 3 kDa (0.278). However, there was no significant difference between 5 kDa and 10 kDa. The RPA of the fractionated EPH was significantly lower than that for the positive control, BHT ($p<0.05$). Based on the result obtained, the ability to reduce the ferric cyanide complex increased with the increase in molecular size of the EPH. During hydrolysis, the native protein cleaved and exposed more electron donating sites. The hydrolysate was further fractionated; hence, its specificity towards bioactivity increased (Bourseau *et al*., 2009). EPH with higher molecular weight (MW) might obtain more electron donating substances collected during fractionation compared to EPH with a lower molecular weight. The result indicated that different MW significantly affected the reducing power of EPH ($p<0.05$).

The reducing power assay was used to evaluate via spectrophotometry the ability of the protein hydrolysates to reduce iron (III) to iron (II) by donating electrons (Yildirim *et al*., 2001; Centenaro *et al*., 2011). According to Elavarasan et al. (2014), electron-donating substances (reductants) reduce the ferric cyanide complex to ferrous cyanide complex, which could be monitored by measuring the formation of the Prussian blue colour using a spectrophotometer at 700nm. Higher absorbance indicates higher reducing power (Centenaro *et al*., 2011).

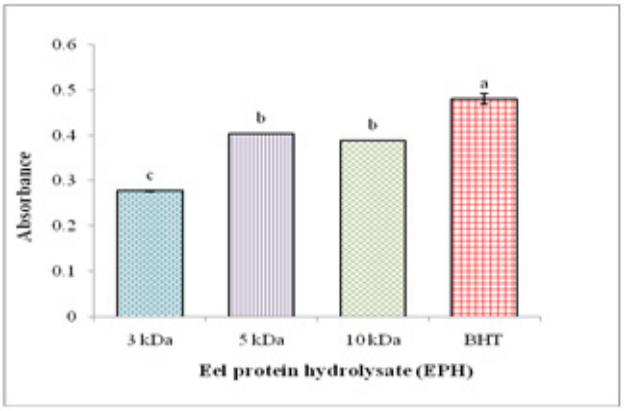

Figure 1. Reducing power of fractionated eel protein hydrolysate (EPH) at 10mg/ml concentration

*values with the same superscript are not significantly different ($p>0.05$)

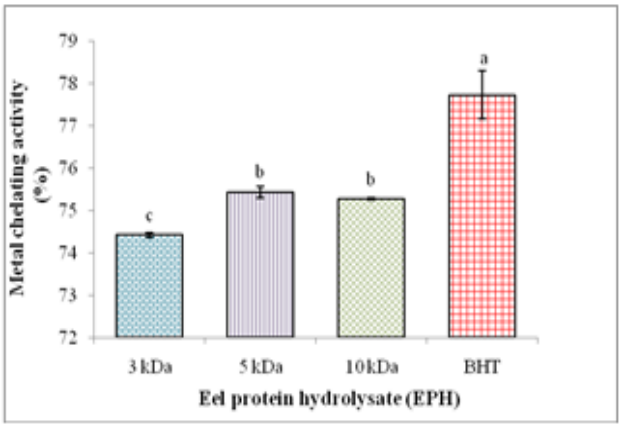

Figure 2. Metal chelating activity of fractionated eel protein hydrolysate (EPH) at 10 mg/ml concentration

*values with the same superscript are not significantly different ($p>0.05$)

Metal chelating activity of fractionated eel protein hydrolysate (EPH)

The metal chelating activity of fractionated eel protein hydrolysates (3, 5 and 10 kDa) was determined at a concentration of 10mg/ml. Figure 2 shows that fraction 5 kDa (75.44%) had the highest chelating activity, followed by 10 kDa (75.27%) and 3 kDa (74.25%). The result showed a significant difference between 3 kDa peptide and 5 kDa peptide ($p<0.05$), while no significant difference was observed between the 5 kDa peptide and 10 kDa peptide. Although the chelating activity of fractionated EPH was significantly lower than BHT ($p<0.05$), the EPH at all fractions showed the ability to disrupt the ferrozine-Fe^{2+} complexes. The fractionation of EPH yielded in the collection of hydrolysate containing chelating agents. However, when the EPH was fractionated at 3 kDa, the hydrolysate containing chelating agents was reduced. The result was in agreement with Razali *et*

al. (2015), who found that the chelating activity of the cobia skin gelatin hydrolysate (CSGH) was the highest at 5 kDa peptide. The study by He *et al*. (2012) also showed higher chelating activity for higher molecular weight peptides (5–10 kDa) compared to lower molecular weight peptides (<3 kDa).

The chelating of ferrous ions was used to determine the ability of hydrolysates in the metal chelating activity. The chelation occurs when the formation of ferrozine-Fe^{2+} complexes is disrupted with the presence of chelating agents resulting in the reduction of the red colour of the complexes (Yamaguchi *et al*., 2000). Transition metal ions, such as Fe^{2+} and Cu^{2+}, can catalyse the generation of reactive oxygen species, which oxidizes unsaturated lipids, therefore, the chelation of these metal ions by antioxidative peptides would retard the oxidation reaction (Klompong *et al.*, 2007).

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of fractionated eel protein hydrolysate (EPH)

The DPPH scavenging activity of fractionated eel protein hydrolysates (3, 5 and 10 kDa) was determined at a concentration of 10mg/ml. Figure 3 shows that the highest DPPH scavenging activity of hydrolysate was at 10 kDa (33.74%) followed by 3 kDa (28.94%) and 5 kDa (20.33%) with a significant difference identified between 10 kDa and 5 kDa ($p<0.05$), while there was no significant difference between the 10 kDa and 3 kDa EPH fractions. Generally, lower MW peptides have a stronger antioxidant activity (Qin *et al*., 2011). The trend can be seen in the results obtained for the 3 kDa and 5 kDa EPH fractions in which the 3 kDa EPH had higher DPPH radical scavenging activity than the 5 kDa EPH. The result obtained was not in line with the previous finding by Razali *et al.* (2015) which found that 3kDa cobia skin gelatin hydrolysate had the highest DPPH radical scavenging activity.

In addition, the antioxidant activity can be improved with the presence of hydrophobic amino acids in fish hydroysates (Samarayanaka and Li-Chan, 2008). According to Qin *et al*. (2011), amino acids with hydrophobic side chain residues could facilitate the electron transfer from peptides to the DPPH radical, thus, stabilizing the radical. Based on the result, the scavenging activity decreased from the 10 kDa fraction to the 5kDa EPH fraction. This might be due to the amount of hydrophobic amino acids required to stabilize the DPPH free radical reaction at 10 kDa EPH being higher than the amount of hydrophobic amino acids contained in the 5 kDa EPH. The fractionated EPH showed the ability to perform DPPH radical scavenging assay albeit the activity was significantly lower than BHT ($p<0.05$). As mentioned before, the result obtained in this study was not in line with the previous finding by Razali *et al*. (2015). It might be because the 10 kDa EPH fraction had greater amount of hydrophobic amino acids as compared to 5 kDa and 3 kDa EPH fractions. Meanwhile, the 3 kDa fraction of cobia skin gelatin hydrolysate had greater amount of hydrophobic amino acids as compared to 10 kDa and 5 kDa of the protein fractions.

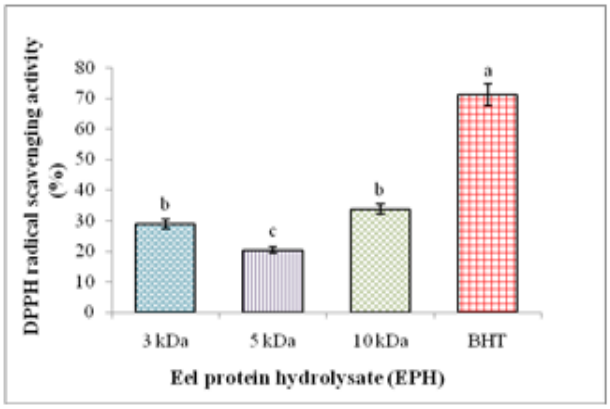

Figure 3. DPPH radical scavenging activity of fractionated eel protein hydrolysate (EPH) at 10 mg/ml concentration
*values with the same superscript are not significantly different ($p>0.05$)

The mechanism of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity has been clearly explained by Nur Alam *et al*. (2013). DPPH is characterized as a stable free radical due to the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of the electrons also gives rise to the deep violet colour, which can be characterized by the absorption band in ethanol solution centred at about 517 nm. When a solution of DPPH is mixed with substrate that can donate an electron, such as protein hydrolysate, it gives rise to the reduced form with the loss of the violet colour and the solution changed to clear yellow.

Hydroxyl radical scavenging activity of fractionated eel protein hydrolysate (EPH)

The hydroxyl radical is a highly reactive oxygen species, which reacts with the polyunsaturated fatty acid of the cell membranes phospholipids causing cell damage (Nur Alam *et al.*, 2013). This damage can contribute to aging, cancer and several other diseases (Arouma, 1998). The hydroxyl radical scavenging activity (HRSA) of fractionated eel protein hydrolysates (3, 5 and 10 kDa) was determined at a concentration of 10mg/ml. Figure 4 indicates that

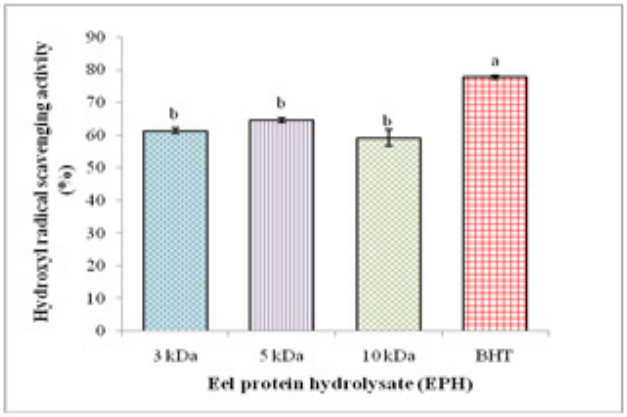

Figure 4. Hydroxyl radical scavenging activity of fractionated eel protein hydrolysate (EPH) at 10mg/ml concentration

*values with the same superscript are not significantly different ($p>0.05$)

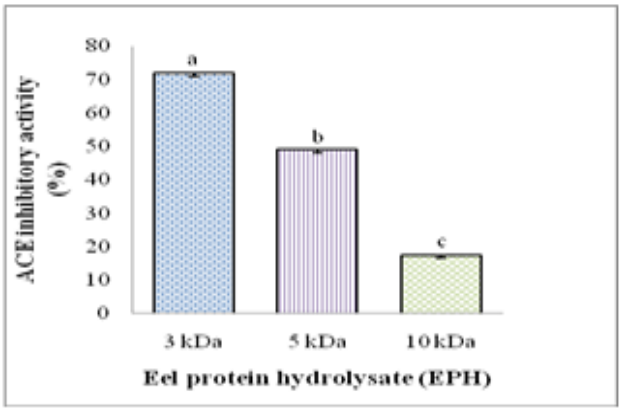

Figure 5. ACE inhibitory activity of fractionated eel protein hydrolysate (EPH) at 1mg/ml concentration

*values with the different superscript are significantly different ($p<0.05$)

the highest HRSA was at 5 kDa (64.69%) followed by 3 kDa (61.36%) and 10kDa (59.18%). However, although there was no significant difference in HRSA for any of the fractionated EPH ($p>0.05$), the HRSA of BHT was significantly higher than that of all the EPH fractions ($p<0.05$). Besides the molecular weight, the difference in radical scavenging activity was affected by the different amino acid compositions, sequence and hydrophobicity of fish hydrolysates (Chalamaiah *et al*., 2015). The EPH at all fractions might contain phenylalanine, a free radical scavenger, as well as cysteine, which can react directly with free radicals by donating sulphur hydrogen to stabilize the radical (Kumar *et al*., 2010).

This result was in line with a study conducted by Zhu *et al.* (2006) on silver carp protein hydrolysate in which lower molecular weight peptides had higher hydroxyl radical scavenging activity compared to higher molecular weight peptides. In addition, the study by Onuh *et al*. (2013) on chicken skin enzymatic protein hydrolysate showing hydroxyl radical scavenging activity reported an increase with a decrease in the fraction of molecular weight (10, 5 and 3 kDa). The high percentage of hydroxyl radical scavenging activity showed by EPH fractions showed its ability to scavenge hydroxyl radical induced damage (Luo *et al.,* 2013).

Angiotensin-I-converting enzyme (ACE) inhibitory activity of fractionated eel protein hydrolysate (EPH)

Figure 5 depicts the inhibitory activity of fractionated EPH against ACE. From the graph, it can be seen that there was a significant difference ($p < 0.05$) on ACE inhibitory activity between EPH at all molecular weight (MW). The 3 kDa EPH fraction showed the highest ACE inhibitory activity (71.90%), followed by 5 kDa (49.05%) and 10 kDa (17.52%). During enzymatic hydrolysis, the proteins cleaved and exposed side chains, which were able to bind with the active site of ACE and inhibit its activity. Peng *et al*. (2009) reported that the antioxidant and ACE inhibitory activities of peptides were highly influenced by peptide MW. In addition, the hydrophobic amino acids contained in the hydrolysate also play an important role in inhibiting ACE activity.

The amino acids at the C-terminal and branched chain aliphatic amino acids at the N-terminal, such as tryptophan, tyrosine, proline or phenylalanine, are suitable competitive inhibitors that bind to the active site of ACE and block its activity (Wijesekara and Kim, 2010). Moreover, it is suggested that the presence of aromatic (Trp, Tyr, Phe) and aliphatic (Ile, Ala, Leu, Met) residues provides optimal sites that increase peptide ACE inhibitor activity (Ghassem *et al*., 2011). Based on the results obtained for the ACE inhibitory activity of EPH at different MW, the amount of hydrophobic amino acids contained in EPH might increase with a decrease in the MW during fractionation of the hydrolysate; therefore, the lowest fraction showed the highest inhibition on ACE activity. The ACE inhibitory activity of low MW peptides was also shown in the study of fish hydrolysates from different sources, such as Channa striatus hydrolysate (0.45 µM, purified from <3 kDa); lizard fish hydrolysate (41.00±1 µM, fractionated from < 5 kDa); and Alaska Pollack hydrolysate (14.70µM, purified from <1kDa), respectively (Ghassem *et al*., 2011; Wu *et al*., 2012; Je *et al*., 2004). This study showed that EPH possessed potential ACE inhibitory activity and is significantly affected by the molecular weight.

Conclusion

In conclusion, the antioxidant activity and angiotensin I-converting enzyme (ACE) inhibitory activity of eel protein hydrolysate (EPH) were observed. The EPH at 5kDa showed the highest reducing power, metal chelating activity and hydroxyl radical scavenging activity compared to the 3 kDa and 10 kDa EPH fractions. Meanwhile, the 10 kDa EPH fraction showed higher DPPH scavenging activity compared to the 3 kDa and 5 kDa EPH fractions. Although EPH at all fractions showed ability as a reductant, metal chelator and radical scavenger, BHT as positive control had the highest antioxidant activity compared to all the EPH fractions. In terms of ACE inhibitory activity, EPH peptides at 3kDa showed the highest ACE inhibitory activity and decreased with the increase of MW of EPH. The results obtained showed that the bioactivity, in terms of antioxidant activity and ACE inhibitory activity, EPH was highly affected by the molecular size in hydrolysate production using alcalase.

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