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Effect of degree of hydrolysis (DH) on the functional properties and angiotensin I-converting enzyme (ACE) inhibitory activity of eel *(Monopterus* sp.*)* protein hydrolysate

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Abstract

The objective of this study was to determine the effect of different degree of hydrolysis (DH) of eel protein hydrolysate (EPH) on the functional properties; namely, water holding capacity, solubility, emulsifying properties, foaming properties and angiotensin-I converting enzyme (ACE) inhibitory activity. The parameters of hydrolysis were based on the different temperature, time, alcalase concentration and three different DH; DH 36, DH 48 and DH 69. The highest value obtained for water holding capacity was EPH at DH 48 (8mg/ml), solubility at DH 69 (1.09%), emulsifying stability index (ESI) at DH 69 (113. 70min), foaming capacity at DH 36 (51.18%,) and ACE inhibitory activity at DH 36 (IC_{50} = 2.128mg/ml). The results suggest that EPH is a promising potential food ingredient that can improve the functional properties with the capability of decreasing blood pressure as it acts as a potent ACE inhibitor.

Introduction

Asian swamp eel *(Monopterus albus)*, which is widely distributed in many countries including India, China, Japan, Malaysia, Indonesia, Bangladesh, Thailand and Vietnam, is a native culture to sub-tropical and tropical Asia (Khanh and Ngan, 2010). Interestingly, this kind of species is able to survive for weeks or months in moist conditions without food (Nico *et al*., 2011). Besides its good taste, the market for Asian swamp eel is becoming increasingly important as a remedy in oriental medicine, and, hence, the activities in culturing this species have grown, especially in China, the Philippines and Vietnam (Khanh and Ngan, 2010; Baquiran and Prudencio, 2013). Considering its low cost and easy culturing activities, this species is selected for the extraction of peptides through enzymatic hydrolysis in order to determine its potential functional properties and bioactivity.

Enzymatic hydrolysis is the process of breaking down the peptide bonds of the parent protein using proteases, whereas the degree of hydrolysis is the ratio of the number of peptides cleaved that occur during hydrolysis to the number of peptide bonds contained in the protein mass (Souissi *et al.*, 2007; Nasri *et al*., 2013). Various enzymes are used for protein hydrolysis, among which, alcalase is generally used due to the high degree of hydrolysis that can be obtained in a relatively short time under moderate pH conditions compared to other neutral or acidic enzymes (Ovissipour *et al*., 2009). The degree of hydrolysis (DH) is generally used as a proteolysis monitoring parameter and is the most widely used as an indicator for comparison among different protein hydrolysates (Gimenez *et al.*, 2009). According to Li *et al.* (2013), the degree of hydrolysis could affect the functional properties of the hydrolysates produced.

The enzymatic hydrolysis of protein not only increases the number of peptides cleaved but also produces a smaller peptide size, which can modify the functional characteristics of the protein and improve its functional qualities (Shen *et al*., 2012). Various studies have shown the improvement in functional properties of fish protein hydrolysates including water and oil holding capacity, solubility, emulsifying and also foaming properties (Wasswa *et al*., 2007). According to Bhaskar *et al.* (2008), the choice of enzyme and substrate as well as the degree of hydrolysis used may influence the physicochemical properties including the functional properties of hydrolysate produced.

Angiotensin I- converting enzyme (ACE) plays an important role in the regulation of blood pressure, and the peptides inhibiting the activity of this enzyme may be useful as therapeutic agents to lower high blood

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pressure. Most protein hydrolysates contain such peptides, and some of them have an ACE-inhibitory activity high enough to be useful as functional for food to control mild hypertension (Gildberg *et al.*, 2011). However, synthetic hypotensive drugs, such as captopril, enalapril, and lasinopril, are still widely used to prevent hypertension even though they are reported to have side effects, such as developing a dry cough, taste disturbances and skin rashes, as well as alterations in serum lipid metabolism (Ghassem *et al*., 2011). Therefore, the aim of this study was to produce eel protein hydrolysate (EPH) for the determination of the effect of the degree of hydrolysis (DH) on the functional properties and angiotensin I-converting enzyme (ACE) inhibition activity of the EPH produced.

Materials and Methods

Materials

Fresh eels *(Monopterus albus)* were bought at Kuala Terengganu, Terengganu, Malaysia and eviscerated, filleted and beheaded in order to obtain the flesh. The flesh was frozen until further use at -80ºC. The enzyme used for the hydrolysis was alcalase (2.4 Au/g and density of 1.18 g/ml), a bacterial endoproteinase from a strain of *Bacillus licheniformis*. All chemicals used were of analytical grade.

Sample preparation

The thawed eel flesh was rinsed to remove the water-soluble compounds, minerals, enzymes and pigments, then, it was chopped into small pieces. A Waring blender (model HGB2WTS3, Connecticut, USA) was used at high speed for 60 sec to homogenize the flesh. Then, minced eel flesh was sealed in plastic packs and stored in a freezer at -40ºC until further use.

Chemical composition of eel flesh

The moisture, protein, fat and ash contents of eel flesh were determined according to the AOAC, (2002) with a conversion factor of 6.25 in order to determine the protein content of the eel flesh.

Preparation of eel protein hydrolysate (EPH)

The preparation of EPH was carried out with several calculations to determine the mass of raw materials according to Kristinsson and Rasco (2000). Enzymatic hydrolysis was performed according to the method of Klompong *et al.* (2007) with slight modification. The minced eel flesh with added distilled water was heated at 85ºC for 20 min in order to inactivate the endogenous enzyme. After cooling at specified temperature, 20 g of alcalase enzyme solution (prepared by diluting the required enzyme mass to a final weight of 20 gram with distilled water) was mixed into the eel flesh and the hydrolysis was initiated immediately.

The hydrolysis conditions for DH 36, DH 48 and DH 69 were a temperature of 40ºC, 60°C, 60°C, time of 120 min, 180 min, 300 min, enzyme concentrations of 1.5%, 2%, 20% and pH of 8.5, 9.5 and 10.5, respectively, with pH adjustment using 1N NaOH. The hydrolysis of EPH was terminated by heating the mixture at 85°C for 20 min to inactivate the alcalase activity. The hydrolysate was then centrifuged at 6000 x g for 20min, freeze dried and kept at -80°C prior to analysis.

Determination of degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

The degree of hydrolysis of EPH was determined by using the trichloroacetic acid (TCA) method with slight modification (Klompong *et al*., 2007; Adler-Nissen, 1986). About 20ml of protein hydrolysate was added to 20ml of 20% (w/v) trichlorocacetic acid (TCA) to produce TCA soluble material. The mixture was left to stand for 30 min to allow precipitation. Then it was centrifuged at 7800 x g for 15min. The nitrogen content of hydrolysate and the supernatant of the sample treated with TCA were analysed using the Kjeldahl method. The calculation of the degree of hydrolysis (DH) was conducted as follows:

$$\text{DH (\%)} = \frac{\text{Soluble N in TCA 10\% (w/v)}}{\text{Total N in the sample}} \times 100$$

Water holding capacity of eel protein hydrolysate (EPH)

A 0.5 g sample was dispersed in 50 ml distilled water and kept at room temperature for 30 min. The mixture was then centrifuged at 5000 x *g* for 30 min, the supernatant was filtered and its volume was measured. The difference between the initial volume of distilled water added to the sample and volume of supernatant were determined. The water holding capacity of EPH was expressed as the volume of water absorbed (ml) per weight of sample (g) (Diniz and Martin, 1997).

Emulsifying properties of eel protein hydrolysate (EPH)

The emulsifying activity index (EAI) and emulsion stability index (ESI) of EPH were determined using the method described by Klompong *et al.* (2007) without the pH adjustment. About 10

ml of corn oil was mixed with 30 ml of 1% protein sample. The mixture was homogenized at a speed of 20,000 rpm for 1min. An aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10min after homogenization, and diluted in 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured immediately at 0min (A_0) and 10min (A_{10}) at 500nm using a spectrophotometer (A_{500}). The ESI of EPH was calculated using the formula suggested by Pearce and Kinsella (1978).

$$\text{Emulsifying stability index (ESI) (min)} = \frac{A_0 \times \Delta t}{\Delta A}$$

Where, A_0 is the absorbance at 0 min after homogenization; A_{10} is the absorbance at 10 min after homogenization; Δt is 10 min and ΔA is difference of absorbance at 0 min and 10 min ($A_0 - A_{10}$).

Foaming properties of eel protein hydrolysate (EPH)

The foaming properties of EPH were determined according to the method by Shahidi *et al*. (1995) with some modifications. Approximately 3 g of sample was dispersed in 100 ml of distilled water. Then, the solution was homogenized in a 250 ml beaker to incorporate the air for 1 min at room temperature (Ultraturrax T18, IKA Malaysia). The total volume was measured at 0, 5, 7, 10, 20 and 30 min after whipping. The foaming capacity index was expressed as foam expansion at 0min and the foam stability index was expressed as the foam expansion during 30 min. The foam expansion was calculated according to the following equation:

$$\text{Foam capacity index (\%)} = [(A-B)/B] \times 100$$

Where,
A = volume after whipping (ml) at different times
B= volume before whipping (ml)

Solubility of eel protein hydrolysate (EPH)

About 200 mg of protein hydrolysate was dispersed into 20 ml of distilled water. Then the mixture was stirred at room temperature and centrifuged at 2560 x *g* for 15 min. The supernatant was filtered through a filter paper. The nitrogen content in the total fraction and in the sample was analysed using the Kjeldahl method. The nitrogen solubility index was calculated using the following formula (Sathe and Salunkhe, 1981):

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant} \times 100}{\text{Total protein in the sample}}$$

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

The reaction mixture was made up of 50 µl of 2.17 mM hippuryl-L-histidyl-L-leucine (HHL), 10 µl of 2 mU of ACE and 10 µl of sample (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH, 8.3) giving a total volume of 70 µl. The peptide fraction and HHL were combined and incubated at 37°C for 10 min in 2ml polyethylene micro centrifuge tubes. ACE was also treated in the same way and incubated at 37°C for 10 min before the two solutions (HHL and sample) were mixed together and incubated further at 37°C for 30 min with continuous agitation. After 30 min, 85 µl of 1M HCl was added to terminate the reaction and vortexed. A positive control (HHL and enzyme) and blank (HHL and buffer) were also prepared in the same manner and subjected to HPLC at an absorbance of 228 nm (Wu *et al.*, 2002).

The ACE activity was calculated as follows:

a) ACE activity (%) = (S-B) / (C-D) X 100

Where;
S = Absorbance of sample hydrolysate
B = Sample without hydrolysate (Blank)
C= Control

b)ACE inhibition (%) = 100 – ACE activity (%)

Data analysis

There were two replications for treatments, and the analysis for these treatments was carried out in duplicate. All data are stated as mean ± standard deviation. One-way ANOVA was used to analyse the data obtained using MINITAB Statistical Software version 16.0.

Results and Discussion

Chemical composition of eel flesh

The results for the chemical composition showed that the eel flesh contained 79.12± 0.20% moisture (wet based), 22.30± 1.49% crude protein, 1.04± 0.12% crude fat and 0.72 ± 0.02% ash; these values were quite similar to that reported by Nurhasan et al. (2010) of 77.9% moisture (wet based), 19.7% protein 0.8% fat and 2.4% ash. The high amount of protein in eel flesh is a benefit for hydrolysis as it allows the release of many amino acids needed for improving the functional properties, such as water holding capacity (WHC), emulsifying and foaming properties, solubility and the bioactivity of the eel

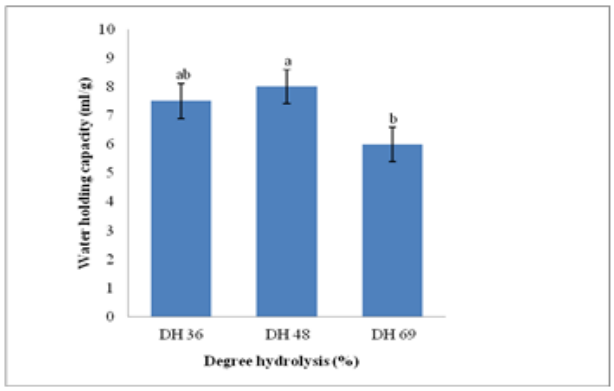

Figure 1. Water holding capacity (WHC) of eel protein hydrolysate (EPH) at different degree of hydrolysis (DH 36, DH 48, DH 69)
*values with the same superscript are not significantly different ($p>0.05$)

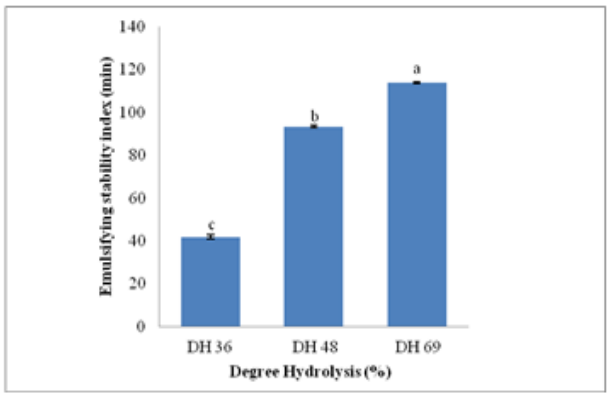

Figure 2. Emulsifying stability index (ESI) of eel protein hydrolysate (EPH) at different degree of hydrolysis (DH 36, DH 48, DH 69)
*values with different superscripts are significantly different ($p<0.05$)

hydrolysate, such as angiotension I-converting enzyme (ACE) inhibitory activity.

Degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

The hydrolysis of the EPH with alcalase resulted in degree of hydrolysis of 36%, 48% and 69% for 120, 180 and 300min of hydrolysis time, respectively. During hydrolysis, the muscle cell membranes tend to round up and form insoluble vesicles, which lead to the removal of membrane structured lipids (Thiansilakul *et al.*, 2007). Generally, the enzymes interact rapidly with the insoluble protein particles and split the loosely bound polypeptide chains in which the more compact core proteins are cleaved slower than the less compact parent proteins (Benjakul and Morissey, 1997).

According to Himonides *et al.* (2011), the pH level of the hydrolysis mixture should be maintained during the entire process as it may influence the properties of the hydrolysates produced. In this study, the pH values of the EPH were kept constant at pH 8.5, 9.5, 10.5 for DH 36, DH 48 and DH 69, respectively. In addition, the hydrolysis time, temperature and enzyme concentration also affect the DH obtained. A study by See *et al.* (2011) on Salmon salar skin using alcalase showed that the DH increased with the increase in enzyme concentration and hydrolysis temperature when the pH was kept constant. Meanwhile, Bhaskar *et al.* (2008) conducted hydrolysis on the Catla catla visceral waste protein and showed DH increases with the increase of hydrolysis time up to its optimum time. These findings are in agreement with the DH obtained in this study where the increase in hydrolysis temperature, time and enzyme concentration increased the DH of EPH.

Water holding capacity of eel protein hydrolysate (EPH)

The ability of protein to bind with water and retain it against a gravitational force within a protein matrix is known as the water holding capacity (WHC) (Foh *et al*., 2010). Figure 1 shows the water holding capacity (WHC) of EPH. Based on the result, the WHC of DH 36 had no significant difference ($p>0.05$) to DH 48 and DH 69, whereas there was a significant difference in WHC ($p<0.05$) between DH48 and DH69. In addition, the results showed that at the highest degree of hydrolysis (DH 69), the WHC of EPH was significantly decreased. This could be explained by the presence of polar groups in the EPH.

Balti *et al.* (2010) stated that the increase in WHC with increasing DH is due to the increase of polar groups, such as –COOH and $–NH_2$, which are released during enzymatic hydrolysis. Therefore, it can be seen that the WHC was increasing from DH 36 to DH 48. A study by Wasswa *et al.* (2007) also showed an increasing WHC with an increase of DH from 5% to 14.9% of grass carp skin hydrolysate. In addition, a study conducted by Slizyte *et al*. (2009) showed a higher WHC with a short hydrolysis time. However, in this study, the breakdown of peptide bonds releasing polar groups might be lower than the peptides releasing non-polar groups as the hydrolysis continued up to 300 min, which contributed to the decrease of WHC in EPH at DH 69.

Apart from the presence of peptides and the polar groups, the molecular size of the hydrolysate also affects its WHC. A study by Razali *et al*. (2015) showed a high WHC at smaller molecular weights of cobia skin gelatin hydrolysate. Hence, the EPH of DH 36 and DH 48 may contain a higher number of low molecular weight peptides resulting in higher WHC compared to the EPH of DH 69.

(a) (b)

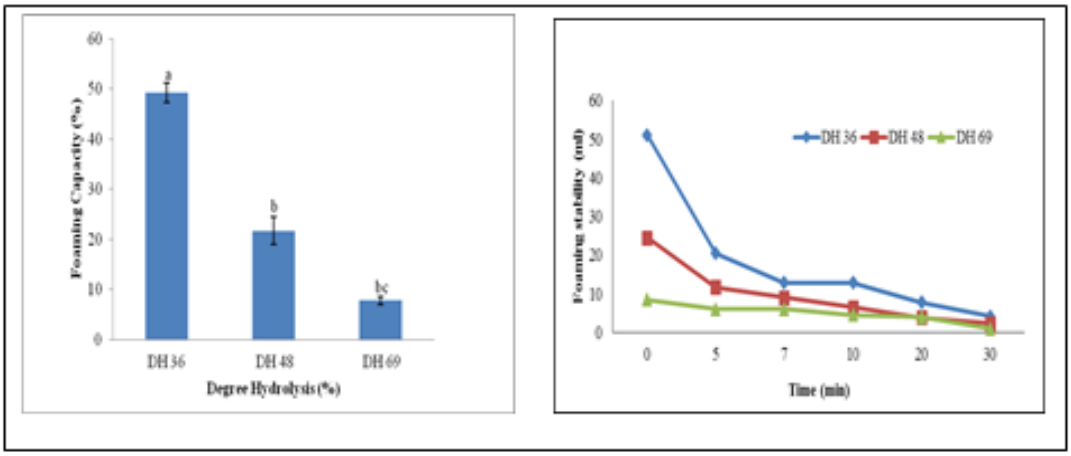

Figure 3. (a) Foaming capacity and (b) Foaming stability of eel protein hydrolysate (EPH) at different degree of hydrolysis (DH 36, DH 48, DH 69)

Emulsifying properties of eel protein hydrolysate (EPH)

The emulsifying properties of hydrolysate are directly related to the effectiveness of the peptides in reducing interfacial tension between hydrophobic and hydrolytic components in food products (dos Santos *et al.*, 2011). The emulsifying stability index (ESI) of EPH increased with the increase of DH (DH 36, DH 48 and DH 69), as shown in Figure 2 in which there was a significant difference ($p<0.05$) between all DH of EPH. The result indicated that the ESI of EPH was significantly affected by the degree of hydrolysis.

The increasing value of ESI might be due to the different pH levels during hydrolysis. According to Taheri *et al.* (2013), in highly alkaline conditions, the presence of higher negative charge peptides leads to more unfolding polypeptides. As a result, the exposure of hydrophilic and hydrophobic peptide residue is higher, and, hence, promoting significant interactions at the oil-water interface. This could be explained in that a higher ESI of EPH was obtained as the DH increased in which the pH used to hydrolyze the eel flesh was 8.5, 9.5 and 10.5 to obtain DH 36, DH 48 and DH 69, respectively.

On the other hand, the difference in ESI between the different DH of EPH probably occurred because of the different molecular size peptides contained in the hydrolysate. The result obtained by Chi *et al*. (2014) on the Spanish mackerel skin collagen hydrolysate showed an increase of ESI with the increase in molecular weight (5.04 kDa to 47.82 kDa). The study of eel protein hydrolysate showed an increasing ESI in the DH of EPH, which may be due to the higher number of larger peptides in higher DH. The finding also supports the probability of the water holding capacity as mentioned before.

Foaming properties of eel protein hydrolysate (EPH)

Good foaming properties show the excellent capability of protein in migrating rapidly to the air-water interface where it will unfold and rearrange at the interface (Klompong *et al*., 2007). The foaming capacity and foaming stability of EPH at different DH (DH 36, DH 48, DH 69) are shown in Figure 3(a) and Figure 3(b), respectively. Figure 3(a) shows that the foaming capacity significantly ($p<0.05$) decreased from DH 36 to DH 69. However, there was no significant difference ($p>0.05$) in foaming capacity between DH 48 and DH 69 of the EPH produced. Meanwhile, Figure 3(b) shows a lower foaming stability at a higher DH of EPH.

The foaming capacity of pink perch hydrolysate also decreased from pH 8 to pH 10 (Naqash and Nazeer, 2013). According to Klompong *et al.* (2007), the reduction of foam capacity in high alkaline condition is due to the ionic repulsion of peptides at the air-water interface. In terms of foaming stability, the result obtained was in agreement with the study conducted by Balti *et al.* (2010) in which the most stable foam was obtained at a lower degree of hydrolysis. The presence of hydrophilic amino acids, such as serine, lysine, arginine and glutamine, found in eel might affect the foaming stability of the EPH (Nurhasan *et al.*, 2010). In addition, the hydroxylation of amino acids increase the number of hydrogen bonds, which results in a dense protein network and influence foam stabilization (Naqash and Nazeer, 2013). Based on the data obtained and discussion from other studies, the increase in hydrophilic amino acids and hydrolxylation of amino acids might occur during the hydrolysis of eel protein at different degree of hydrolysis and influence the foam stability of EPH produced.

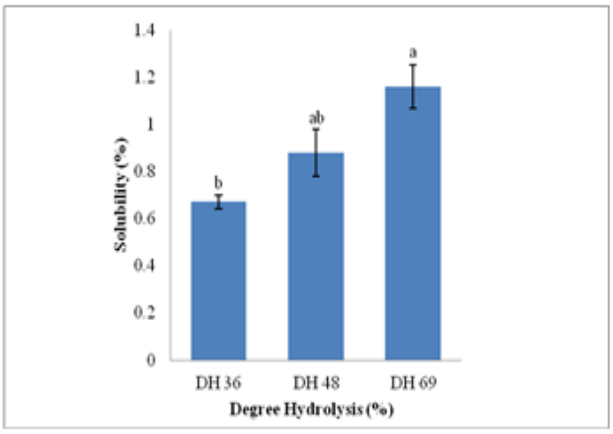

Figure 4. Solubility of eel protein hydrolysate (EPH) at different degree of hydrolysis (DH 36, DH 48, DH 69)

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

Solubility of eel protein hydrolysate (EPH)

The results in Figure 4 show an increasing solubility with significant difference ($p<0.05$) between DH 36 and DH 69 while no significant difference ($p>0.05$) between DH 36 and DH 48 as well as DH 48 and DH 69 of EPH produced. The same trend was also reported in the study by Gbogouri *et al.* (2004) and Klompong *et al.* (2007) where the solubility of respective salmon by-products and yellowstripe trevally hydrolysates increased with the increase in DH. In general, the degradation of protein to smaller peptides leads to more soluble products (Klompong *et al*., 2007). Additionally, enzymatic hydrolysis can produce peptides with lower molecular size, that are more hydrophilic and more solvated in aqueous solution (Li *et al*., 2013).

In contrast to the emulsifying properties, peptides with low molecular weight (1 – 20 kDa) showed greater solubility (Li *et al*., 2013). The EPH with DH 69 may contain a higher number of larger molecular size peptides based on the results of WHC and ESI; however, the presence of hydrophilic, polar amino acids, such as serine, glutamine, asparagine and threonine, in EPH could help the solubility efficiency of the hydrolysate (Nurhasan *et al*., 2010). A study conducted by dos Santos *et al*. (2011) showed higher solubility and content of hydrophilic, polar amino acids of bluewing searobin hydrolysed using alcalase as compared to the hydrolysate produced from flavourzyme. Based on Figure 4, the hydrolysis of eel flesh may produce a higher number of hydrophilic polar group peptides as the DH increases resulting in the higher solubility of EPH.

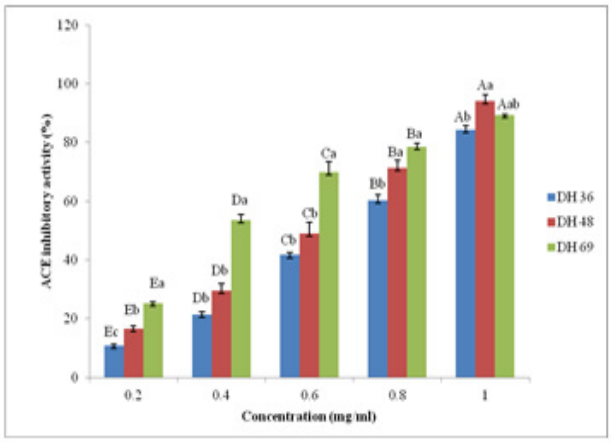

Figure 5. Angiotensin I-converting enzyme (ACE) inhibitory activity of eel protein hydrolysate (EPH) at different concentration (A, B, C, D, E) of the same degree of hydrolysis (DH) and at same concentration of the different degree of hydrolysis (DH) (a, ab, b)

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

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

Angiotensin I-converting enzyme (ACE) inhibitory activity is important to determine the inhibition rate of EPH towards ACE, the enzyme involved in the regulation of blood pressure (Li *et al*., 2015). Figure 5 shows that the ACE activity significantly ($p<0.05$) decreased with the increase in concentration in all DH of EPH (DH 36, DH 48, DH 69). The results obtained showed that DH 69 was the highest inhibitory activity at all concentrations except for the concentration of 1mg/ml followed by DH 48 and DH 36. IC_{50} was used to compare the inhibition potential of EPH on different DH values. IC_{50} value was defined as the concentration of peptide sample required to inhibit 50% of the ACE activity (Cinq-Mars and Li-Chan, 2007). The best inhibition activity would be the hydrolysate that can inhibit ACE at the lowest concentration. In this study, the highest IC_{50} value was observed at DH 36 (2.128 mg/ml) followed by DH 48 (3.045 mg/ml) and DH 69 (3.337 mg/ml). Hence, the findings showed that EPH at DH 36 was able to inhibit ACE activity at the lowest hydrolysate concentration even though DH 69 possessed the highest inhibitory activity.

The presence of tyrosine, proline or phenylalanine at the C-terminal and the branched-chain aliphatic amino acids at the N-terminal are competitive inhibitors, which bind with ACE and inhibit the enzyme activity (Wijesekara and Kim, 2010). The eel flesh, as reported by Nurhasan *et al*. (2010) contained all of the amino acids mentioned. The hydrolysis process might release a greater amount of these amino acids, which contribute to the ACE inhibitory activity.

In addition, the molecular weight of peptides was also one of the factors that influence ACE inhibitory activity. The study on *Channa striatus* myofibrillar protein hydrolysate showed increased inhibitory activity as the molecular weight of peptides decreased (Ghassem *et al.*, 2011). The ability of low molecular weight peptides to inhibit ACE activity was also reported on peptides derived from various fish species, such as bonito, yellowfin sole, Alaskan pollock and sea bream (Raghavan and Kristinsson, 2009). Based on the IC_{50} value of EPH, which showed the highest at DH 36, there might be a higher number of low molecular weight of peptides contained in hydrolysate at this DH value compared to DH 48 and DH 69. Therefore, at DH 36, the ACE activity could be inhibited even at a low concentration of EPH.

Conclusion

In conclusion, the enzymatic hydrolysis of eel protein at different DH had a significant ($p<0.05$) effect on the water holding capacity, foaming properties and ACE inhibitory activity of EPH produced. EPH at DH 36 showed the highest value of foaming properties and IC_{50} of ACE inhibitory activity whereas EPH at DH 69 was the highest for emulsifying stability and solubility. In terms of water holding capacity, the EPH at DH 48 showed the highest compared to DH 36 and DH 69. The results suggest that eel protein hydrolysate can be used in food ingredients as it improves the functional properties and may be used as a potent ACE inhibitor. It is recommended that a study on amino acid composition and molecular weight of EPH be conducted to prove that these properties influence the functional and bioactive properties of EPH produced.

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