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Angiotensin converting enzyme (ACE), antioxidant activity and functional properties of shortfin scad (*Decapterus macrosoma*) muscle protein hydrolysate at different molecular weight variations

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ABSTRACT

The purpose of this research is to determine angiotensin converting enzyme inhibitory activity (ACEI), antioxidant activity, and functional properties (emulsion and solubility) of shortfin scad muscle protein hydrolysate (SSMPH) fractions with different molecular weights variations. Using the ultrafiltration membrane, the hydrolysate was prepared enzymatically and fractionated (< 3, < 5, < 10 kDa). The results obtained show that SSMPH at < 3 kDa possessed the greatest ACE inhibitory activity (77.04%). ACEI activity was shown to decrease as the SSMPH molecular weight increased. The SSMPH samples were prepared at 10 mg/ml for antioxidant activity and functional activity. The DPPH scavenging activity and solubility of < 3 kDa fraction was significantly higher than others fractions. The emulsion activities index (EAI) of < 3 kDa fraction are not significantly different from the others fractions. There was no significantly difference in emulsion stability index of < 5 kDa fraction than that of < 10 kDa fraction. In conclusion, low molecular weight sizes of SSMPH showed good potential bioactivity and functional properties. Therefore, their properties may vary in numerous applications, especially in the food and nutraceuticals industries.

1. Introduction

Hypertension is a cardiovascular risk factor and well recognized as "silent killer" due to its long-term asymptomatic effect in adults (Je et al., 2005). The hypertension and congestive heart failure can be preventing by angiotensin I-converting enzyme inhibitors implementation (Ma et al., 2010). Recently, research interest has focused on hydrolysates and peptides angiotensin converting enzyme (ACE) inhibitors from animal and vegetal sources. Animal sources include the proteins of eel (Azemi et al., 2016; Baharuddin et al., 2016) and tilapia (Roslan et al., 2014). While, plant sources include proteins from soy protein (Roblet et al., 2012) and peanuts (Jamdar et al., 2010).

Different types of animal and plant hydrolysate proteins acquired through enzymatic hydrolysis have been revealed to demonstrated antioxidant activity, including soy protein (Oliveira et al., 2014), rapeseed (He et al., 2012), and barley glutelin (Xia et al., 2012). In addition, protein hydrolysate with antioxidant activity were obtained from different marine organisms, such as cobia (Razali et al., 2015), mackerel (Chi et al., 2014) and golden apple snails (Hamid et al., 2015). Although no information indicates that the molecular weight (MW) range is directly related to peptides activity, a small molecular size peptide always related to high antioxidant activity. Peptide with high antioxidant activity usually were found in molecular weight ranged from 500 to 3000 Da (Je et al., 2007).

Protein hydrolysates are source of bioactive peptides that are inactive in the intact protein but become activate after hydrolysis process. The size of molecular weight peptide is vital for the bioactivity properties such as antioxidant activity and Angiotensin converting enzyme inhibitory activity. Study has found that the high bioactivity of protein hydrolysate fractions were made up of low molecular weight peptides (Azemi et al., 2016; Baharuddin et al., 2016). This is because low molecular weight peptides have been found to have greater antioxidant activity and interact more efficiently with radicals (Azemi et al., 2016).

Study by Razali et al. (2015), found that the 3 kDa fraction had the greatest reducing power but at this low molecular weight fraction does not affect the functional properties. This is because protein hydrolysate fraction with low molecular weight resulted in low value of functional properties. In addition, the 3 kDa fraction's smaller peptides could not maintain a well-ordered molecular orientation at the interface due to hydrolysis and filtering, resulted in shorter peptide sequence. Hence, the adsorption rate, together with the ability to unfold and reorient at the interface, remain the most important factors for foam formation

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(Azemi et al., 2016). Moreover, low molecular weight hydrolysate fractions also results in less emulsifying stability due to a reduction in the hydrophobic/hydrophilic balance (Razali et al., 2015).

Shortfin Scads (*Decapterus macrosoma*) is a tropical fish commonly found in Malaysia. Production of muscle protein hydrolysate from shortfin scad (*Decapterus macrosoma*) can lead to the introduction of new value-added products from underused fish species). Hence, the aim of this research is to elucidate the ACE inhibitory activity (ACEI), antioxidant activity and functional properties of fractionated shortfin scads muscle protein hydrolysate (SSMPH) at different molecular weight (< 3 kDa, < 5 kDa, < 10 kDa) as a new potential of natural food ingredients and food supplement.

2. Material and methods

2.1. Materials

Shortfin scad (*Decapterus macrosoma*) was bought from local market Kuala Terengganu, Terengganu. The hippuric acid (HA), Hippuryl-l-histidyl-l-leucine (HHL), alcalase, captopril, and an ACE from rabbit lung were bought from a Sigma–Aldrich, USA.

2.2. Sample preparation

The shortfin scad was washed under tap water and cut into small pieces prior storage in freezer (−40 °C). The shortfin scad pieces were thawed in a chiller overnight before use. The parts of shortfin scad were thawed overnight before use in a chiller.

2.2.1. Preparation of shortfin scad muscle protein hydrolysate (SSMPH)

The SSMPH was prepared following Razali et al. (2015) and Baharuddin et al. (2016) with some modification. Approximately 44 g of homogenized fish muscle were mixed with 44 g of distilled water (dH_2O). The mixture was heated to 85 °C and stirred for 20 min to inactivate the endogenous enzyme. Hydrolysis was initiated immediately under controlled conditions at 50.11 °C for 84.02 min, pH of 7.89, and enzyme to substrate ratio of 2.26%. The 1N hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH) was used to adjust the pH mixture to the desired value. Then, the mixture was heated (85 °C for 20 min) and centrifuged (6000 rpm, 20 min) (CR 22N, Hitachi, Japan) to deactivate the enzyme activity after hydrolysis process. The supernatant was filtered and the collected supernatant was then fractionated into different molecular weight cut-off (MWCO) (10, 5 and 3 kDa) using vivaspin ultrafiltration membranes (Sartorius, Germany). Four fractions were obtained as: MW > 10 kDa, 5 < MW < 10 kDa, 3 < MW < 5 kDa, and MW < 3 kDa. Then, the SSMPH at each fraction of molecular weights (MW) were freeze-dried and used for further analysis. However, the sample chosen in this study were < 10 kDa, < 5 kDa, and < 3 kDa fraction.

2.2.2. Angiotensin converting enzyme (ACE) activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

SSMPH's ACEI activity is evaluate the released hippuric acid (HA) from histidyl-l-leucine (HHL). The determination of ACEI activity assay was conducted following Azemi et al. (2016). A total of 70 µl solution mixture was formed by combining 10 µl ACE (2 mU), 50 µl HHL (2.17 mM) and 10 µl SSMPH (1 mg/ml) solution (contains 100 mM borate buffer and 300 mM NaCl, pH 8.3). ACE was incubated (37 °C, 10 min) with continuous stirring of the SSMPH and HHL. After 30 min, HCl (1M) (85 µl) was added to the solution mixture and vortexed. Then, approximately 500 µl of ethyl acetate was added and centrifuged (5000 rpm, 10 min). Approximately 200 µl of solution mixture from the bottom layer was pipetted into a test tube and oven dried (80 °C) after 1 ml of dH2O was added. The spectrophotometer (Cary WinUV, USA) was then used to determine absorbance of mixture at 228 nm. The blank (HHL and buffer) and positive control (HHL and enzyme) were done using the same method. As a positive control, captopril (1 mg/ml) was implemented. Consequently, the ACEI activity was determine using the equation:

$$\text{ACEI activity } (\%) = (A_c - A_s) / (A_c - A_b) \times 100$$

Where,

A_b = Blank absorbance,absorbance, A_c = Control absorbance, A_S = Sample absorbance

2.2.3. DPPH radical-scavenging activities of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

Radical scavenging activity of fractionated SSMPH 1,1-diphenyl -2-picrylhydrazyl (DPPH) was carried out using the Razali et al. (2015) method. About 1 ml of DPPH (0.1 mM) solution was pipetted into 3 ml of sample (10 mg/ml) before incubated at room temperature for 30 min. The spectrophotometer is used to measure the absorbance at 517 nm. Distilled water was applied as a control, while a positive control was applied to 1% (w/v) of Butylated hydroxytoluene (BHT). SSMPH's capacity to scavenge DPPH radicals has been determined using the following equation:

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

Where:

A_0 = the control absorbance; A_1 = the mixture − containing sample absorbance.

2.2.3.1. Reducing power of fractionated shortfin scad muscle protein hydrolysate (SSMPH).

The reducing power of fractionated SSMPH was determined following Razali et al. (2015). About 1.0 ml (10 mg/ml) sample was added to 2.5 ml of 1% (w/v) potassium ferricyanide ($K_3Fe(CN)_6$) and 2.5 ml of phosphate buffer (0.2M, pH6.6). The mixture was incubated (50 °C, 20 min) and mixed with 2.5 ml trichloroacetic acid (TCA) (10%, w/v). The mixture was then centrifuged (3000 rpm, 10 min) and added 0.5 ml of 1% ferric oxide ($FeCl_3$) with 2.5 ml dH_2O. The absorbance of a blank sample at 700 nm was obtained using a spectrophotometer. The 1% (w/v) of BHT was acted as positive control.

2.2.4. Chelating effects of the ferrous ion of fractionated shortfin scad muscle protein hydrolysate (SSPMH)

The chelation of Fe^{2+} of fractionated SSMPH was measured following Razali et al. (2015). 10 mg/ml sample in 0.5 ml methanol was added with 2.5 µl of $FeCl_2$ (2 mM). After mixing with 0.1 ml of ferrozine (5 mM), reactions were initiated and incubated for 10 min at room temperature. The spectrophotometer (Cary WinUV, USA) measured Fe^{2+}-ferrozine complex absorbance at 562 nm. Then, 1% (w/v) of BHT was used as a positive control. SSMPH's capability to scavenge chelating metal activity has been calculated as follows:

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

Where:

A_0 = the control absorbance; A_1 = the mixture − containing sample absorbance

2.2.5. Emulsifying properties of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

For emulsion activity index (EAI) and emulsion stability index (ESI) of each SSMPH fraction were determined as described by Razali et al. (2015). Approximately 5 ml of each fraction of the SSMPH solution (10 mg/ml) with 5 ml of oil was added. A homogenizer was used to homogenize the mixture (18,000 rpm, 1 min). Then, the emulsion was centrifuged (1180 rpm, 5 min). The EAI was calculated using the

following equation:

EAI (%) = Emulsified layer height x 100 Total content height

For emulsion stability index (ESI), the samples were homogenized and heated in water bath (55 °C, 30 min). Then, the sample was centrifuged (2000 rpm, 5 min) to form two layered emulsions. The value of ESI was calculated as follows:

ESI (%) = Emulsified layer height after heating x 100
Total content height before heating

2.2.6. Solubility of fractionated shortfin scad muscle protein hydrolysate (SSPMH)

Protein solubility of each SSMPH fraction was performed using the method by Baharuddin et al. (2016). About 200 mg SSMPH was placed in 20 ml dH_2O and at room temperature stirred for 30 min. The mixture was centrifuged at 8000 rpm (4 °C) for 15 min. Using the Kjeldahl technique, protein content in the supernatant was determined after sufficient dilution. The following equation was used to calculate for the solubility of proteins:

Protein solubility (%) = Protein content in supernatant x 100
Total protein content in sample

2.3. Statistical analysis

All data measurement were taken in triplicates. The mean values ± standard deviation (SD) were calculated. In MINITAB 14, statistical analysis was conducted using variance analysis (ANOVA) to determine whether the difference was significant at $p < 0.05$.

3. Results and discussion

3.1. Bioactive properties

3.1.1. Angiotensin converting enzyme (ACE) activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

The inhibition activity of fractionated SSPMH against ACE is illustrated in Fig. 1. The strongest ACEI activity was found in < 3 kDa SSPMH fraction (77.40%), followed by < 5 kDa (62.18%) and < 10 kDa (32.53%) fraction. However, the positive control captopril showed the highest (95.19%) ACEI activity compared to all fractions. The figure demonstrates that SSMPH's ACEI activity between all molecular weight fractions and captopril was significantly different ($p < 0.05$).

Based on the finding, the use of alcalase is efficient in the production of ACEI peptides. The findings of this research were endorsed by Ghassem et al. (2011) who indicated that alcalase produces a comparatively shorter peptide sequence that give better to ACE inhibitory activity and peptides that are also generally integrated in peptide low molecular weight (3 kDa). In addition, the result obtained in this study was in agreement with the study done by Jung et al. (2006) who discovered that protein hydrolysate with low molecular weight in yellowfin sole had greater ACE inhibition activity than high molecular weight fractions. Hence, current findings showed that ACE inhibition activity was greater at low molecular weight (< 3 kDa). This proved that the ACEI activity of hydrolysate peptides is influenced by different molecular masses (Lodish et al., 2000).

Table 1
Antioxidant activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH).

Fraction	Metal chelating (%)	DPPH radical scavenging activity (%)
< 10 kDa	43.32 ± 2.50[b]	2.68 ± 1.31[d]
< 5 kDa	49.63 ± 0.678[a]	30.75 ± 1.73[c]
< 3 kDa	51.54 ± 2.68[a]	59.93 ± 2.57[b]
BHT	49.48 ± 0.676[a]	70.54 ± 1.69[a]

Antioxidant activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH) at different molecular weight fractions (< 3 kDa, < 5 kDa, < 10 kDa). BHT used as a positive control. The values are reported as mean ± SD of triplicate determination (n = 3). Value with different superscript letter [(a-d)] within columns indicates significant different ($p < 0.05$).

3.1.2. DPPH radical scavenging activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

The DPPH radical scavenging activity of fractionated SSMPH at different molecular weight was determined at a 10 mg/ml concentration with BHT as a positive control. Table 1 shows that the hydrolysate fraction with MW < 3 kDa exhibited the highest DPPH scavenging activity, followed by < 5 kDa and < 10 kDa. Fraction < 10 kDa showed very low DPPH scavenging activity and there was a significant difference between all fractions in DPPH radical scavenging activity ($p < 0.05$). The composition, sequence and hydrophobicity of amino acids are generally associated with the radical scavenging activity of fish protein hydrolysates (Bougatef et al., 2010; Chalamaiah et al., 2012).

The results obtained was in agreement with a study by Razali et al. (2015), who reported that the 3 kDa had the greatest percentage of DPPH radical inhibition of all three fractions of cobia skin gelatin hydrolysate, however BHT had the highest percentage of DPPH radical inhibition among all tested samples. DPPH is a stable free radical with a

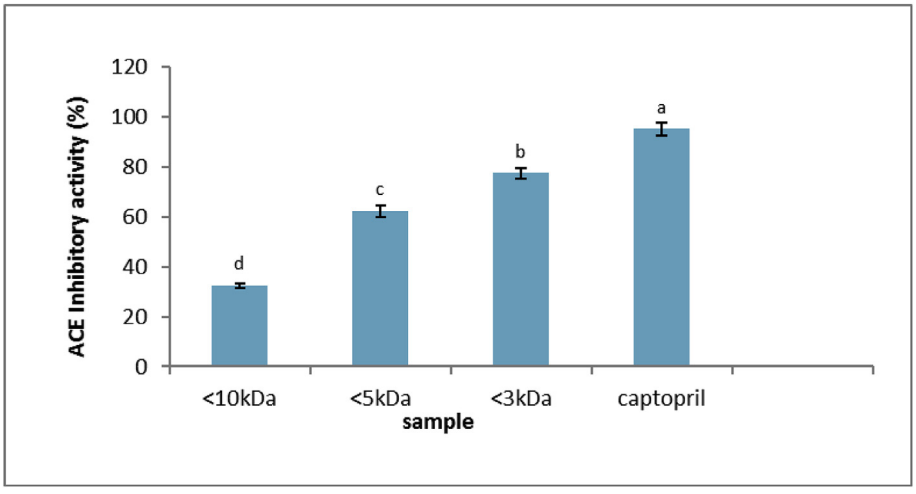

Fig. 1. Angiotensin Converting Enzyme (ACE) activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH).

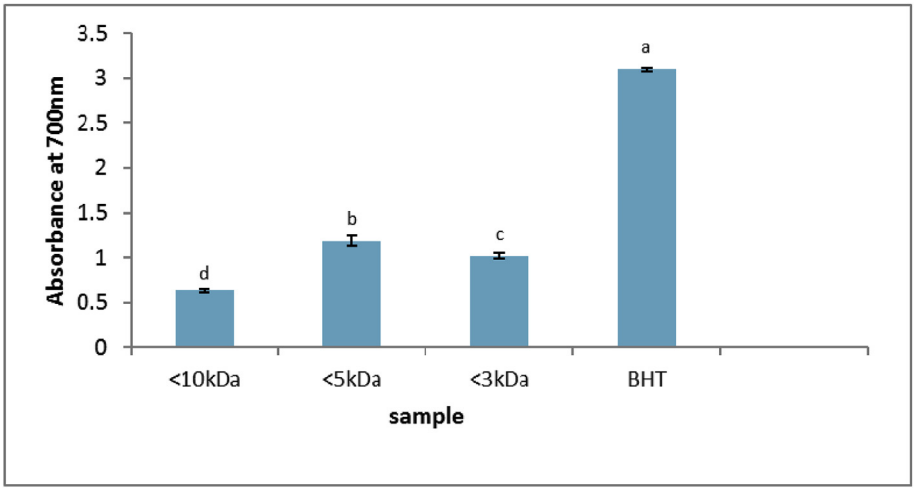

Fig. 2. Reducing power activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH).

maximum absorption rate of 517 nm. If DPPH radicals meet a proton-donating substrate like an antioxidant, radicals would be scavenged and absorbance reduced (Bougatef et al., 2010). As a measure for radical-scavenging activity, the reduction in absorbance is taken. The SSMPH contains hydrogen donors that stabilize by reacting with free radicals because amino acids with hydrophobic side chain residues can encourage electron transfer from peptides to radical DPPH (Qin et al., 2011).

3.1.3. Reducing power activity of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

Fig. 2 illustrates that hydrolysate fraction with < 5 kDa had the strongest reducing power activity followed by < 3 kDa and < 10 kDa fractions. The fractionated hydrolysate and positive control (BHT) showed significant difference in reducing power of all fractions ($p < 0.05$). In addition, Moure et al. (2006) found that peptides size and concentration affect the reducing power. The results of this finding were in the same agreement with Qin et al. (2011), which discovered that LMW of purple sea urchin gelatin hydrosylate had greater reducing power. Thus, potassium ferricyanide is frequently used to measure the reducing power of a hydrolysate fraction's antioxidant activity for which higher absorbance indicates higher reducing power.

3.1.4. Chelating effects of the ferrous ion of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

The chelating effects on the ferrous ion of fractionated SSMPH at different molecular weights were determined at 10 mg/ml. Table 1 shows that < 3 kDa fraction possessed the highest chelating activity, followed by < 5 kDa and < 10 kDa fraction. The < 10 kDa is significant different with the < 5 kDa and < 3 kDa ($p > 0.05$), while BHT as positive control.

Based on the results obtained, the low molecular weight of SSPMH compete the ferrozine-Fe^{2+} complexes. The chelating activity of small peptides in hydrolysates could improve tissue capacity to lower the rate of deteriorating lipid oxidation metal-catalyzed. Then, small peptides can also act as useful agents to avoid metal ion-dependent oxidative damage to food lipids and thus act as food preservatives (Ajibola et al., 2011). The outcome was similar to that of the investigation from Taheri et al. (2014) who examined various salted herring saline solution hydrolysate and discovered low molecular weight (1 kDa) increased chelating activity more than high molecular weight. However, the result obtained differ from those of He et al. (2012), who stated that strong chelating capacity were exhibited by HMW (5–10 kDa) compared to low molecular weight (3 kDa). In addition, the effectiveness of protein hydrolysate has appeared depending on the peptide/amino acid composition of the soluble protein fraction. Besides, they also dependent on the sequence and protein configuration (Elias et al., 2008). Thus, when high molecular weight fractions of SSMPH change the conformation and structure of water-soluble peptides, the ability to chelate iron has been slightly reduced.

3.2. Functional properties

3.2.1. Emulsion activity index (EAI) of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

Table 2 shows the EAI for SSMPH fractions. From the result, the EAI of SSMPH had no significant differences ($p > 0.05$) between < 3 kDa fraction, followed by 5 kDa and 10 kDa fraction. The EAI of fractionated SSMPH not affected by molecular weight. In addition, Kristinsson and Rasco (2000) indicated that there is no clear relationship between peptide size and emulsification, showing that the physical and chemical properties of the peptide may play an important role in functional properties.

The findings obtained was in line with a study done by Razali et al. (2015) that observed cobia skin gelatin hydrolysate, showed no significant differences (43, 43.7, and 45%), for 10 kDa, 5 kDa and 3 kDa, respectively. But, EAI increase as SSMPH peptides decrease. This is because low molecular weight peptides can exhibit superior emulsifiers, proteins and/or peptides, which soon enter the interface of water/oil that promptly open and reposition the interface (Kotlar et al., 2013). However, because of the lack of unfolding and reorientation at the interface as large peptides, small peptides diffuse and absorb quickly at the interface, they are less efficient in reducing interfacial tension (Pacheco-Aguilar et al., 2008).

Table 2
Functional properties of fractionated shortfin muscle protein hydrolysate (SSMPH).

Fractions	Emulsion activity index (EAI) (%)	Emulsion stability index (ESI) (%)	Solubility
< 10 kDa	35.42 ± 3.24^{a}	42.33 ± 0.60^{a}	1.77 ± 0.34^{b}
< 5 kDa	37.70 ± 7.51^{a}	42.87 ± 5.09^{a}	1.99 ± 0.33^{ab}
< 3 kDa	44.63 ± 5.56^{a}	34.40 ± 1.86^{b}	3.15 ± 0.90^{a}

Functional properties of fractionated shortfin scad muscle protein hydrolysate at different molecular weights (< 3 kDa, < 5 kDa, < 10 kDa). The values are reported as mean ± SD of triplicate determination (n = 3). Value with different superscript letter $^{(a-b)}$ within columns indicates significant difference ($p < 0.05$).

3.2.2. Emulsion stability index (ESI) of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

The SSMPH fraction the emulsion stability index (ESI) is shown in Table 2. The 5 kDa fraction showed the greatest ESI followed by 10 kDa and 3 kDa fraction. Meanwhile, the 10 kDa was not significant different ($p > 0.05$) from the 5 kDa fraction, although there are significant differences between 5 kDa and 3 kDa fraction in emulsion stability index for SSMPH ($p < 0.05$). Based on the results obtained, 5 kDa and 10 kDa have higher ESI compared 3 kDa. This can be related to higher molecular weight, the activation energy barrier prevents protein migration from occurring in a diffusion-dependent manner, leading to the accumulation of proteins in the aqueous phase, thus increasing the mixture's emulsion stability (Thiansilakul et al., 2007). Moreover, high molecular weight peptides or more hydrophobic peptides lead to emulsion stability (Li et al., 2015). While small peptides and amino acids lack openings and interface reorientation resulting in low effectiveness in interface tension reduction (Foh et al., 2010).

These findings are similar to the findings of Taheri et al. (2014) that stated herring brain fish hydrolysate reduced in the ESI due to lower peptide size (< 1 kDa). While LMW peptides may rapidly migrate to the interface, the peptide hydrophobic/hydrophilic balance is inadequate to stabilize emulsions (Deng et al., 2011). Findings have shown that the size and sequence of peptides in hydrolysis are crucial in increasing the SSMPH ESI at the HMW. Optimum molecular size for peptides function as an effective emulsifier. As peptide size increases, the oil-water interface is more flexible and arranged (Pacheco-Aguilar et al., 2008).

3.2.3. Solubility of fractionated shortfin scad muscle protein hydrolysate (SSMPH)

Table 2 indicates that the highest solubility was recorded in < 3 kDa fraction, followed by < 5 kDa and < 10 kDa fraction. There was significant difference between 10 kDa and 3 kDa fraction ($p < 0.05$), but no significant differences between 3 kDa and 5 kDa fraction in terms of solubility. A slightly modified molecular weight protein chain within SSMPH highly encourages solubility in an aqueous medium by the effect of hydrophilic/hydrophobic residues (Liu et al., 2015). In addition, protein hydrolysis separates insoluble protein aggregates, produces smaller peptides, increases hydrophilic exposure, and facilitates hydrophilic amino acids interactions in an aqueous environment.

The findings collected are consistent with previously reported outcomes from Ktari et al. (2012), which indicated that protein hydrolysates from the muscle of zebra blenny exhibited greater solubility due to their LMW. In addition, the peptides must be able to form hydrogen bonds between their hydrophilic polar amino acid side groups and the water molecules to efficiently bind to water molecules. Thus, lower protein hydrolysis peptides have a proportionately higher polar residue with increased hydrogen bond ability (Kristinsson and Rasco, 2000). The food that contains high solubility and protein hydrolysates will give a good appeal and 'smooth' feel to the mouth (Tanuja et al., 2012). SSMPH is therefore more soluble at low molecular weight because the amount and size of peptide chains also affects solubility (Mahmoud, 1994).

4. Conclusion

The present study successfully determined the bioactivity and functional properties of SSMPH. For ACEI activity, SSMPH peptides at < 3 kDa indicated the highest ACEI activity while decreased as SSMPH molecular weight increased. Then, < 3 kDa SSMPH fraction exhibited significantly higher DPPH scavenging activity than the other fractions. Furthermore, the lower molecular weight fraction do not necessarily exhibit the highest antioxidant activity and functional characteristics of SSMPH.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101254.

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