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Profiling of antioxidative proteolysate enzymatically hydrolysed from stone fish (*Actinopyga lecanora*)

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Abstract

Marine livings represent one of the richest sources of protein with valuable bioactives. The present work explores the antioxidative potential of stone fish, a sea cucumber species typically discarded as by-catch. Stone fish was enzymatically hydrolysed using papain, and the resulting proteolysate exhibited strong antioxidant activity in DPPH$^{\bullet}$ radical scavenging (IC_{50} = 0.49 mg/mL), ABTS$^{\bullet}$ (IC_{50} = 0.36 mg/mL) radical scavenging, and FRAP value (0.29 mM $FeSO_4$) after 8 h of hydrolysis. Fractionation of proteolysate was then performed using three approaches namely ultrafiltration, reversed-phase high performance liquid chromatography, and isoelectric focusing techniques to profile and characterise the antioxidative proteolysate. Results indicated that papain-generated proteolysate from stone fish flesh possessed considerable amount of antioxidative peptides with molecular weight of approximately 2 kDa, low hydrophobicity (< 20%), and pI = 9.

Introduction

Oxidative damage plays a key role in the aetiology of many acute and chronic diseases such as traumatic brain injury, stroke, Alzheimer, cancers, and cardiovascular diseases. Its role in natural physiological processes including aging, bone loss, and menopause in women is strongly significant as well. Studies show that intake of antioxidant supplements or food rich in antioxidants can considerably decrease such damages (Bar-Or *et al.,* 2015; Zaheer and Akhtar, 2017; Neha *et al.,* 2019). The usage of synthetic antioxidants has been recently confined in terms of their effects on inducing DNA damages as well as their toxicity. Due to the current tendency to consume natural products, there is a great demand in finding new antioxidants from natural bio-resources.

Marine organisms represent one of the richest sources of protein, and contain valuable natural bioactives possessing nutraceutical, pharmaceutical, and cosmeceutical potentials. However, due to a lack of exploration, much of their potential remains undiscovered, including that from sea cucumbers. Sea cucumbers are known to have high contents of protein and low fat in their body walls. Their body walls are also rich in nutrients such as minerals and polysaccharides (Bordbar *et al.,* 2011). Among all these specific nutrients, bioactive peptides are mainly responsible to exert radical scavenging activities (Bougatef *et al.,* 2010).

The application of enzymatic proteolysis is commonly known as the most conducive method for generating bioactive peptides which is moderate, safe, and more efficient as compared to the former methods of using organic solvents or toxic chemicals (Zhang *et al.,* 2017). By controlling the parameters such as type of enzymes, temperature, time, and other conditions, various proteolysates can be obtained containing different types of bioactive peptides. These peptides can be further profiled and characterised by a combination of fractionation and purification techniques including ultra-filtration, high-performance liquid chromatography (HPLC), gel filtration, ion exchange separation, capillary electrophoresis, and high-speed counter-current chromatography (HSCCC) which separate peptides based on their differences in size, molecular weight, polarity, charge, binding force, and other characteristics (Zhang *et al.,* 2017).

Nowadays, peptides derived from different species of sea cucumber are widely applied in the production of food products, pharmaceuticals, nutraceuticals, and cosmetics. While the antioxidant

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activity of peptides derived from certain sea cucumbers have been reported previously, there may still be some species that contains substantial amount of antioxidative compounds but remain unisolated and unidentified. Stone fish (*Actinopyga lecanora*), which is classified among the edible species of sea cucumbers, is from those species that remain unutilised and discarded as a by-catch due to unpopularity. In recent years, not much data have been reported on the bioactivity of this marine organism. The first study reporting on the antioxidant potential of stone fish showed that upon enzymatic hydrolysis of its flesh, an appreciable amount of antioxidative protein hydrolysate (proteolysate) was generated (Bordbar *et al.,* 2013). Furthermore, the angiotensin-converting enzyme and antioxidant activities as well as anti-inflammation activity of stone fish have been extensively studied using bromelain enzyme (Auwal *et al.,* 2017; 2018; 2019; Ghanbari and Ebrahimpour, 2018; Ghanbari *et al.,* 2016). However, the papain-generated proteolysate with antioxidant activity from stone fish has not been elucidated. Therefore, the present work focuses on profiling and characterisation of antioxidative proteolysate from stone fish in the search for potentials as a low-cost natural source of functional biopeptide as well as a valuable food ingredient.

Materials and methods

Materials

Papain and 2,4,6-tri(2-pycridyl)-s-triazine (TPTZ) were purchased from Acros Organics (Geel, Belgium). DPPH• reagent (2,2-diphenyl-2-picrylhydrazyl), *o*-phthaldialdehyde, glutathione, and sodium dodecyl sulphate were purchased from Sigma-Aldrich, Inc. (St Louis, MO). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) reagent was purchased from Merck & Co. (Kenilworth, NJ). Coomassie Brilliant Blue R-250 was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Acetonitrile and trifluoroacetic acid (HPLC grade) were purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals used were of analytical grade.

Sample preparation

Five grams of freeze-dried stone fish flesh was autoclaved (121°C, 15 min) to inactivate endogenous enzymes and destroy microorganisms, and then dialysed for 28 h at 4°C in the dark. The dialysed sample was placed in 50 mM phosphate buffer until it reached the reaction temperature. Then, papain was added at 1:100 (w/w) enzyme:substrate ratio. Prior to the selection of papain, a preliminary study was performed on four proteases namely alcalase, bromelain, flavourzyme, and papain to screen for their ability to generate antioxidative peptides from stone fish. Protein hydrolysis was then conducted using papain for 8 h under optimum conditions (pH 6.5, 55°C) as recommended by the enzyme manufacturer. Papain was re-added at the same ratio after 5 h. Samples were withdrawn every hour until the end of hydrolysis, then immediately boiled for 10 min at 100°C to inactivate the papain, cooled in ice bath, and centrifuged for 20 min at 10,000 *g*. The supernatant was collected and kept at -80°C until further analyses.

Determination of peptide content and degree of hydrolysis

The technique described by Church *et al.* (1983) was applied with slight modifications. Briefly, 40 µL of sample was mixed with 300 µL of *o*-phthaldialdehyde (OPA) solution in a 96-well plate, incubated at room temperature for 2 min prior to absorbance reading at 340 nm using a microplate reader (Powerwave X340, Bio-Tek Instruments Inc., Winooski, VT). OPA solution consisted of 6 mM OPA (prior dissolved in 96% ethanol), 50 mM sodium tetraborate decahydrate, 1% sodium dodecyl sulphate, and 0.2% β-mercaptoethanol. Peptide content was calculated using a glutathione standard curve with concentration between 0 - 200 ppm. Distilled water was used as negative control. Degree of hydrolysis (DH) was calculated using Eq. 1:

$$DH\ (\%) = [(A_S - A_C - A_P) / A_T] \times 100 \qquad \text{(Eq. 1)}$$

where, A_S = absorbance of sample at different hydrolysis hour, A_C = absorbance of negative control, A_P = absorbance of protein solution before adding enzyme, and A_T = absorbance after complete hydrolysis (total hydrolysis).

Antioxidant activity measurements

DPPH• assay

The DPPH• radical scavenging activity was measured according to Bersuder *et al.* (1998) with slight modifications. Briefly, 50 µL of sample was pipetted into a 96-well plate, and diluted with equal volume of deionised water. Then, 100 µL of DPPH• reagent (0.20 mM in 80% ethanolic solution) was added. The mixture was incubated for 1 h at room temperature in the dark, and the absorbance was read at 517 nm. Glutathione and distilled water were used as standard (positive control) and blank (negative control), respectively. The difference between

positive and negative controls yielded the final absorbance for control. DPPH• radical scavenging activity was calculated using Eq. 2:

$$\text{DPPH}^{\bullet}\text{ scavenging activity (\%)} = [(A_C - A_S) / A_C] \times 100 \quad \text{(Eq. 2)}$$

where, A_C = absorbance of control, and A_S = absorbance of sample.

ABTS• assay

ABTS• radical scavenging activity was determined based on the procedure described by Re *et al.* (1999) with slight modifications. The ABTS• stock solution (consisting of 7 mM ABTS• dissolved in 2.45 mM potassium persulphate) was prepared and incubated for 16 h at room temperature in the dark prior to use. Sample aliquot of 20 µL was mixed with 980 µL of ABTS• working solution (diluted from stock to reach absorbance = 0.80 ± 0.02), and incubated at 37°C for 10 min in the dark prior to reading at 734 nm. Glutathione and distilled water served as standard (positive control) and blank (negative control), respectively. ABTS• radical scavenging activity was calculated using Eq. 3:

$$\text{ABTS}^{\bullet}\text{ scavenging activity (\%)} = [(A_C - A_S) / A_C] \times 100 \quad \text{(Eq. 3)}$$

where, A_C = absorbance of control, and A_S = absorbance of sample.

Ferric reducing antioxidant power (FRAP) assay

FRAP was determined following the method described by Guo *et al.* (2003). FRAP reagent was freshly prepared on the day of experiment by mixing TPTZ (10 mM, dissolved in 40 mM HCl), $FeCl_3$ (20 mM), and acetate buffer (0.3 M) at 1:1:10 (v/v/v) ratio. pH of the final solution was adjusted to 3.6. Aliquots of 40 µL of sample was added to 1.8 mL of FRAP solution, topped to 2 mL using distilled water, and incubated at 37°C for 10 min. The absorbance was read at 593 nm. $FeSO_4$ and distilled water were used as positive and negative control, respectively. Result was expressed as the antioxidant concentration having the ability to reduce ferric into ferrous, equivalent to that of 1 mM $FeSO_4$.

Amino acid composition

The method of Rozan *et al.* (2000) was followed to compare the amino acid composition of stone fish before and after hydrolysis. Sample was first hydrolysed using 6 N HCl at 110°C for 24 h, followed by vacuum drying (10 µL) for 30 min. Then, the dried sample was mixed with 20 µL of coupling buffer made up of methanol, water, and triethylamine (2:2:1, v/v), gently swirled, and again vacuum dried for 30 min. The sample was then derivatised using 20 µL of methanol, water, triethylamine, and phenylisothiocyanate reagent (7:1:1:1, v/v) at room temperature for 20 min, and finally vacuum dried for another 30 min. The derivatised sample (20 µL) was loaded onto Purospher® STAR RP-18e column (250 × 4.6 mm; Merck & Co., Kenilworth, NJ) attached to a HPLC system (JASCO International Co., Ltd., Tokyo, Japan), and equipped with photodiode array detector (MD-2010), pumps (PU-2080), and a degasser (DG-2080-54). Two mobile phases were used namely buffer A (0.1 M ammonium acetate, pH 6.5) and buffer B (0.1 M ammonium acetate containing acetonitrile and methanol, 44:46:10, v/v, pH 6.5). Separation was performed with a linear gradient of 100 - 0% buffer A for 50 min at 43°C and flow rate of 1 mL/min. Formation of amino acid peaks was monitored using a UV absorption detector at 254 nm. The identification of amino acids was performed by comparing their retention times and peak areas to pure amino acid standards. Calibration curve was employed to quantify the amino acids. Result was analysed using Borwin chromatography software (Version 1.5, JASCO International Co., Ltd., Tokyo, Japan).

Molecular weight fractionation (ultrafiltration)

Ultrafiltration was performed following the method of Shamloo *et al.* (2012). Freeze-dried proteolysate which dissolved in distilled water was ultrafiltrated using Vivaspin-15R ultrafiltration membrane (Sartorius Stedim Biotech GmbH., Goettingen, Germany) at different M_W cut-offs of 2, 5, and 10 kDa to yield four fractions of $M_W > 10$, 5 - 10, and 2 - 5 kDa, and $M_W < 2$ kDa. The yield of each fraction was calculated using Eq. 4:

$$\text{Yield (\%)} = [(\text{Volume of each collected fraction}) / (\text{Volume of total proteolysates})] \times 100 \quad \text{(Eq. 4)}$$

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) using stacking and resolving gels at 4 and 15% concentrations, respectively. Freeze-dried proteolysate was dissolved in deionised water to give a concentration of 10 mg protein/mL. Then, it was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS and 20% glycerol) at 1:1 (v/v)

ratio. Mixture was heated for 3 min at 90°C, followed by loading of 10 µL into individual well. A constant current of 30 mA was passed through the gel for 1 h to allow for complete peptide separation. The gel was carefully retrieved, stained with Coomassie Blue, destained with 200 mL of destaining solution (40% methanol + 30 mL of 6% acetic acid, adjusted to 500 mL with deionised water), and preserved in 10% acetic acid + 5% glycerol solution. The M_W were estimated using standards from Bio-Rad Laboratories, Inc. (Hercules, CA).

Reverse phase high performance liquid chromatography (RP-HPLC)

The ultrafiltrated proteolysate fraction showing the most potent antioxidative property was further fractionated according to their hydrophobicity. An amount of 0.2 mg of freeze-dried ultrafiltrated sample was diluted in 1 mL of deionised water, and filtered through 0.20 µM membrane. Then, 500 µL of sample was loaded on a C-18 column (ZORBAX 300 SB-C18, 250 × 9.4 mm, 5 µm) attached to a semi-preparative HPLC system (1200 Series, Agilent Technologies, Santa Clara, CA), coupled with a multiple wavelength detector and fraction collector. Separation was performed at room temperature at a flow rate of 4 mL/min using an elution gradient as follows: 0 - 5 min, 100% buffer A (deionised water containing 0.1% TFA); 5 - 67.5 min, 0 - 40% buffer B (acetonitrile containing 0.1% TFA). The peak's formation was monitored at 205 nm which corresponded to maximum peptide absorption. Fractions were collected, stored at -80°C, and freeze dried for further analysis.

Isoelectric point focusing fractionation

Following RP-HPLC, antioxidative proteolysate was further separated based on the isoelectric point (pI) of peptides using Agilent 3100 OFFGEL fractionator (Agilent Technologies, Santa Clara, CA). Firstly, immobilised pH gradient (IPG) gel strip was rehydrated using deionised water, and left for 15 min to allow gel swelling. Then, 150 µL of sample (previously diluted in deionised water) was loaded into 12 individual wells segregated on IPG. Separation was conducted for 48 h at a voltage of 500 – 4,000 V and current of 50 µA. Peptide solutions, hold at their respective pIs regions, were retrieved for analysis upon complete separation.

Statistical analysis

All measurements were performed in triplicate, and results were reported in mean ± standard deviation. One-way ANOVA was conducted, followed by Tukey's test to compare means at 5% ($p \leq 0.05$) significance level, using Minitab software version 14 (Minitab Inc., State College, PA).

Results and discussion

DH, peptide content, and antioxidant activity over 8 h of hydrolysis

Hydrolysis of stone fish was performed until 8 h. This duration was selected based on the preliminary study which monitored the antioxidant activity of proteolysate for a prolonged duration of 24 h. In the preliminary study, samples were withdrawn at every 2 h interval, and it was found that, between 10 - 24 h of hydrolysis, the antioxidant activity decreased, recording at least 20% of reduction for all assays, thus reaction was stopped at 8 h.

From Table 1, the highest amount of peptide was found to be 3.50 ± 0.45 mmol obtained at DH = 87.66 ± 0.39%. DH and peptide content increased concurrently from 0 to 8 h of hydrolysis, thus indicating a progressive substrate hydrolysis along with product formation (peptide). Despite overall increment in DH from 0 - 8 h of hydrolysis, the hydrolysis rate/speed decreased up to 5 h, *i.e.*, slower DH increment from one hour to the other during the first 5 h, thus indicating a reduced enzyme activity due to wear and tear after long hour of tedious hydrolysis work. This explained the need of re-adding enzyme at 5 h to speed up reaction rate and achieve maximum hydrolysis in a shorter time. While maximum hydrolysis may not necessarily reflect the highest antioxidant activity in some proteinaceous samples, stone fish showed that antioxidant activity increased concurrently with increasing DH. This type of DH versus hydrolysis time curve is common when looking for antioxidative hydrolysates in other marine products such as sardine (Bougatef *et al.,* 2010) and sea urchin (Qin *et al.,* 2011). Previous studies also revealed that DH of seafood products using different proteases was mainly dependent on hydrolysis conditions, substrate nature, ratio of enzyme/substrate, enzyme activity, and stability (Wiriyaphan *et al.,* 2012; Zhou *et al.,* 2012).

In terms of radical scavenging activity, stone fish proteolysate showed significantly ($p \leq 0.05$) higher DPPH$^{\bullet}$ and ABTS$^{\bullet}$ scavenging activities as compared to the non-hydrolysed sample. The IC_{50} of radical scavenging activity is depicted in Table 1. At the end of hydrolysis, the IC_{50} for DPPH$^{\bullet}$ and ABTS$^{\bullet}$ were 0.49 and 0.36 mg/mL, respectively.

Table 1. Degrees of hydrolysis, peptide contents, and antioxidant activities of papain-digested stone fish proteolysate during 8 h of hydrolysis.

Time (h)	Degree of hydrolysis (%)	Peptide content (mmol glutathione/g dried sample)	DPPH• scavenging activity		ABTS• scavenging activity		FRAP value (mM $FeSO_4$)
			(%)	IC_{50} (mg/mL)	(%)	IC_{50} (mg/mL)	
0	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	-	0.00 ± 0.00[a]	-	
1	14.4 ± 0.38[b]	0.57 ± 0.05[b]	38.99 ± 0.37[b]	1.25[a]	40.23 ± 0.25[b]	1.08[a]	0.00 ± 0.00[a]
2	28.52 ± 0.13[c]	0.85 ± 0.18[c]	56.09 ± 0.24[c]	0.91[b]	51.55 ± 0.14[c]	0.85[b]	0.09 ± 0.02[b]
3	38.92 ± 0.25[d]	1.27 ± 0.20[d]	62.58 ± 0.12[d]	0.77[c]	66.38 ± 0.23[d]	0.71[c]	0.12 ± 0.01[c]
4	43.18 ± 0.12[e]	1.96 ± 0.08[e]	74.96 ± 0.06[e]	0.66[d]	79.49 ± 0.11[e]	0.56[d]	0.16 ± 0.02[d]
5	50.84 ± 0.20[f]	2.17 ± 0.16[f]	77.03 ± 0.31[f]	0.64[d]	80.07 ± 0.13[e]	0.56[d]	0.21 ± 0.01[e]
6	67.27 ± 0.54[g]	3.13 ± 0.14[g]	80.62 ± 0.12[g]	0.52[e]	88.56 ± 0.15[f]	0.43[e]	0.23 ± 0.03[e]
7	84.34 ± 0.45[h]	3.29 ± 0.25[h]	80.58 ± 0.18[g]	0.52[e]	91.04 ± 0.10[g]	0.39[f]	0.25 ± 0.05[e]
8	87.66 ± 0.39[i]	3.50 ± 0.45[i]	85.73 ± 0.18[h]	0.49[f]	93.36 ± 0.14[h]	0.36[g]	0.26 ± 0.08[e]

Values are mean ± SD of triplicates (n = 3). Means with different lowercase superscripts within a column are significantly different at $p \leq 0.05$.

Interestingly, IC_{50} in both assays decreased over time, thus indicating the generation of peptides with higher radical scavenging potency with increasing hydrolysis time. This is in accordance with the higher radical scavenging activities recorded towards the end of hydrolysis. The observed free radical scavenging capacity could likely be resulted from the release of antioxidative peptides via enzymatic cleavage of intact parent protein molecules which enhanced the exposure of effective amino acids. It is suggested that the composition of amino acids, the amino acids sequences at N- and C-terminal, as well as the size of peptides and their solubility play key roles in the radical scavenging efficacy.

FRAP assay was performed to evaluate the ability of stone fish proteolysate in donating electron and reducing Fe^{3+} to Fe^{2+}. The FRAP values showed an increasing trend during the 8 h of hydrolysis. The ferric-reducing ability is related to peptide chain length, molecular weight, amino acid sequence, degree of hydrolysis, and type of protease used. Specifically, the contribution from sulphur-containing and hydrophobic amino acids present within the peptides sequence was elucidated by Udenigwe and Aluko (2011), whereby a positive relationship was established between the presence of these amino acids and ferric-reducing ability. Overall, the results obtained in the present work are in line with the reducing power observed for other marine products such as giant kingfish muscle and skin proteolysate (Nazeer and Kulandai, 2012), and fresh water carp proteolysate (Elavarasan *et al.,* 2014).

Correlation between DH and antioxidant activity

The correlation between DH and antioxidant activity of papain-generated stone fish proteolysate is depicted in Figure 1. A strong correlation ($R^2 > 0.9$) was observed, and this result is in agreement with previous literatures where a noticeable increase in the antioxidant activity was reported with the increase in DH (Wiriyaphan *et al.,* 2012; Elavarasan *et al.,* 2014). Prolonging the hydrolysis time to 8 h, which led to the increase in DH, caused the improvement in antioxidant capacity due to the generation of small size peptides. In addition, the prolonged hydrolysis time may directly cause the exposure of side chain group residues, which likely facilitates the reaction between antioxidative peptides and free radicals and transitional metal ions (Zhong *et al.,* 2007). Although the mentioned factors (generation of small-sized peptides and exposure of active side groups as a result of high DH) may explain the strong correlation between DH and antioxidant activity, it does not necessarily mean that a proteolysate with higher DH will always show stronger antioxidant activity. In general, peptides are potentially better antioxidants than free amino acids. Extensive hydrolysis of proteins will produce higher amount of free amino acids and reduce the overall antioxidant capacity (Zhong *et al.,* 2007).

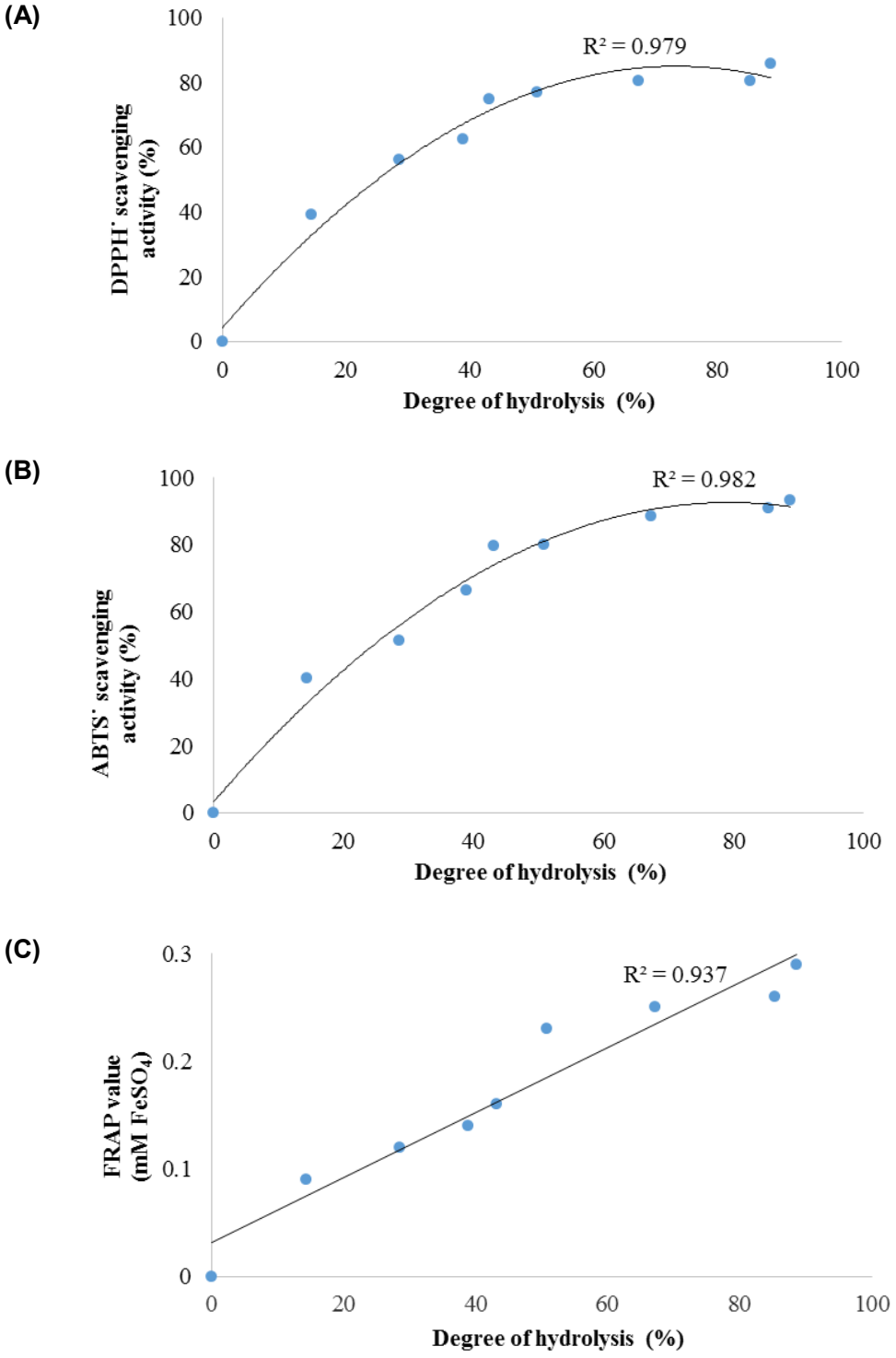

Figure 1. Correlation between degree of hydrolysis with (A) DPPH˙ radical scavenging activity, (B) ABTS˙ scavenging activity, and (C) FRAP values of papain-digested stone fish proteolysate.

Amino acid composition

The amino acid contents of stone fish flesh (crude protein) and papain-digested proteolysate are displayed in Table 2. Hydrolysis significantly decreased the amino acid contents after papain digestion. This could be due to the enzyme nature of papain which acts as an endopeptidase and cleaves peptide bond in the interior of parent protein molecule, thus not producing significant amount of free amino acid after hydrolysis (Hudson, 1992). Similar trend of lowered amino acid content after hydrolysis was observed in sardinella fish using an in-house microbial protease, as well as in potato protein isolate using Alcalase as reported by Chasanah *et al.* (2019) and Pęksa and Miedzianka (2014), respectively. Based on Table 2, a total of 17

Table 2. Amino acid composition (g/100 g dry weight) of freeze-dried stone fish tissue and papain-digested proteolysate.

Amino acid	Stone fish crude protein	Papain-digested proteolysate
Alanine	6.21 ± 0.70[a]	4.83 ± 0.08[b]
Arginine	6.12 ± 0.11[a]	4.25 ± 0.23[b]
Aspartic acid	7.13 ± 0.32[a]	4.73 ± 0.15[b]
Cysteine	0.22 ± 0.15[a]	0.10 ± 0.05[a]
Glutamic acid	9.07 ± 0.24[a]	8.02 ± 0.10[b]
Glycine	13.95 ± 0.35[a]	10.40 ± 0.33[b]
Histidine	1.12 ± 0.57[a]	0.35 ± 0.08[b]
Isoleucine	5.62 ± 0.33[a]	1.18 ± 0.57[b]
Leucine	4.31 ± 0.86[a]	3.54 ± 0.09[a]
Lysine	4.77 ± 0.22[a]	2.60 ± 0.55[b]
Methionine	1.73 ± 0.18[a]	0.38 ± 0.27[b]
Phenylalanine	2.97 ± 0.14[a]	1.52 ± 0.63[b]
Proline	6.01 ± 0.15[a]	3.89 ± 0.12[b]
Serine	2.12 ± 0.05[a]	1.45 ± 0.46[b]
Threonine	4.45 ± 0.13[a]	3.11 ± 0.12[b]
Tyrosine	4.37 ± 0.25[a]	2.85 ± 0.10[b]
Valine	4.22 ± 0.04[a]	2.44 ± 0.06[b]

Values are mean ± SD of triplicates (n = 3). Means with different lowercase superscripts within a column are significantly different at $p \leq 0.05$.

amino acids were detected, including eight essential amino acids except for tryptophan, which was present in negligible amount or even non-existing in sea cucumbers due to the collagen nature of their tissue. Crude stone fish protein depicted a more prominent amino acid content (individual and total) as compared to the proteolysate. The most abundant amino acid was glycine with 13.95 ± 0.35 g/100 g for crude protein, and 10.40 ± 0.33 g/100 g for proteolysate, and followed by glutamic acid; while cysteine recorded the lowest amount with 0.22 ± 0.15 g/100 g for crude protein, and 0.10 ± 0.05 g/100 g for proteolysate. Similar amino acid profiles have been previously reported in other sea cucumber species such as *Isostichopus badionotus* (Pérez-Vega *et al.,* 2013) and *Parastichopus californicus* (Bechtel *et al.,* 2013).

The presence of acidic and hydrophobic amino acids demonstrated strong contribution towards the antioxidant activity of food protein hydrolysates. A positive relationship between these amino acids with DPPH$^{\bullet}$ scavenging activity and ferric ion reducing ability was postulated by Udenigwe and Aluko (2011). The amount of these amino acids in stone fish proteolysate obtained in the present work was 22.9 and 50.5%, respectively, partially explaining its antioxidative activity. In particular, stone fish proteolysate contained a substantial amounts of acidic residues, with glutamic acid and aspartic acid recording values of 8.02 ± 0.10 and 4.73 ± 0.15 g/100 g dry weight, respectively. These amino acids were responsible for ferric ion chelation as they can utilise the carbonyl and amino groups in the side chain to promote metal ion chelation (Suetsuna *et al.,* 2000). The presence of aromatic residues including histidine, phenylalanine, and tyrosine (consisted of 8.5% of total amino acid) also contributed towards the radical scavenging activity, as a result from their ability to donate proton to the electron-deficient radical, thus stabilising the highly reactive molecule (Rajapakse *et al.,* 2005).

Despite the fact that glycine and glutamic acid are non-essential amino acids, their high levels in stone fish protein and proteolysate could be an additional nutritional value, particularly in individuals with impaired amino acid synthesis

ability. Glycine is found to reduce total cholesterol level in serum (Wen and Hu, 2010), and glutamic acid is reported to play a key role in immune-based activities such as lymphocyte proliferation, macrophage phagocytic activity, and neutrophil bacterial killing (Cruzat *et al.,* 2018). The ratio of lysine:arginine was 0.77 for stone fish crude protein, and 0.61 for the proteolysate. This low ratio has been reported to reduce serum cholesterol significantly (Inhamuns *et al.,* 2009).

Membrane ultrafiltration fractionation

Stone fish proteolysate was fractionated based on the M_W of peptides after hydrolysis into four fractions with M_W cut-offs > 10, 5 - 10, 2 - 5, and < 2 kDa. The yield (%) of each fraction was 26.23 ± 0.07, 17.09 ± 0.38, 18.72 ± 0.30, and 38.00 ± 0.58, respectively. The < 2 kDa fraction was the most abundant peptide amount after membrane ultrafiltration, thus indicating the efficiency of papain to digest stone fish crude protein into low M_W peptides. This finding is in line with previous works reported by Yu *et al.* (2012) and Zhou *et al.* (2012), whereby the highest yield were obtained from the lowest M_W fraction for tilapia fish frame proteolysate (< 1 kDa) and abalone proteolysate (< 3 kDa) upon ultrafiltration separation.

All ultrafiltrated fractions were analysed in terms of DPPH• radical scavenging activity. Statistically, both 2 - 5 kDa (74.91%) and < 2 kDa (75.22%) fractions showed significantly higher DPPH• radical scavenging activity as compared to > 10 kDa (59.45%) and 5 - 10 kDa (69.61%) fractions. This result showed an inverse relation between the peptides' M_W and their antioxidant activities, whereby peptides with lower M_W demonstrated higher DPPH• radical scavenging activity. This is in agreement with previous studies reported by Qin *et al.* (2011), Yu *et al.* (2012), and Zhou *et al.* (2012), among others, who demonstrated that short-chain and low M_W peptide fractions would produce higher DPPH• scavenging activity in different marine protein samples. For instance, Qin *et al.* (2011) and Yu *et al.* (2012) reported that the proteolysate with M_w < 1 kDa obtained from sea urchin and tilapia fish frame, respectively, showed the strongest DPPH radical scavenging activity, while Zhou *et al.* (2012) reported that scallop proteolysate with M_w 1 - 3 kDa showed the strongest antioxidant activity.

Molecular weight distribution

The M_w distribution profiles of papain-digested stone fish proteolysate, both unfractionated and fractionated, were elucidated using SDS gel electrophoresis (Figure 2). Standard marker with different M_w was applied to approximate the M_W of peptides generated during hydrolysis. Based on Figure 2, all observed bands were below 45 kDa, including unfractionated and whole proteolysate. This indicated that stone fish proteolysate consisted mainly of peptides with low M_w, unlike fish muscle proteolysate which demonstrated two major bands at much higher M_w of 212 and 166 kDa (Shamloo *et al.,* 2012). Low M_w of stone fish proteolysate could be due to the extensive digestion of flesh proteins and long-chain polypeptides into small-chain peptides. The unfractionated proteolysate and > 10 kDa fractions showed two thick bands at M_w of 14.4 and 21.5 kDa, whereas for other fractions < 10 kDa, their peptide profile appeared as smear below 6.5 kDa.

Profiling based on hydrophobicity using RP-HPLC

Bioactive peptides with short chain length and smaller molecular size are considered more potent than large-sized peptides. Ultra-filtrated fractions with M_w of 2 - 5 and < 2 kDa both showed similarly high DPPH• scavenging activity (74.91 and 75.22%, respectively). However, fraction 2 - 5 kDa contained polypeptides that were bulky and not as favourable as short-chained oligopeptides in < 2 kDa fraction, in terms of antioxidative potency. Thus, fraction with the lowest M_w (< 2 kDa) was subjected to RP-HPLC to separate the peptides based on hydrophobicity. This result is in agreement with the previous findings from Zou *et al.* (2016), Li *et al.* (2008), Sarmadi and Ismail (2010), as well as Mishra and Nagarajan

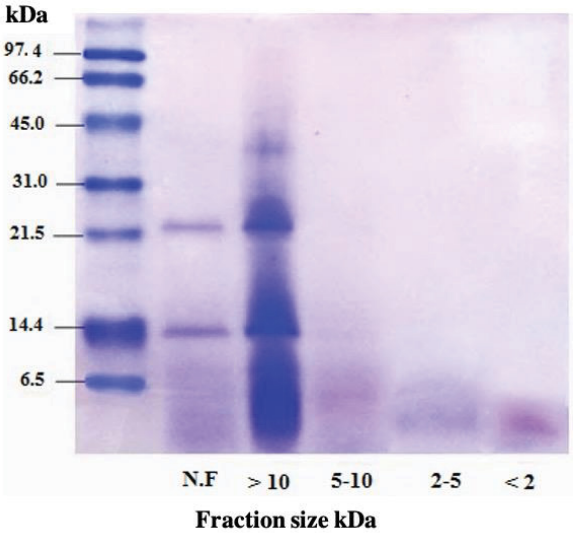

Figure 2. Molecular weight distribution profile for non-fractionated (NF) and fractionated papain-digested stone fish proteolysate, depicted as SDS-PAGE band patterns.

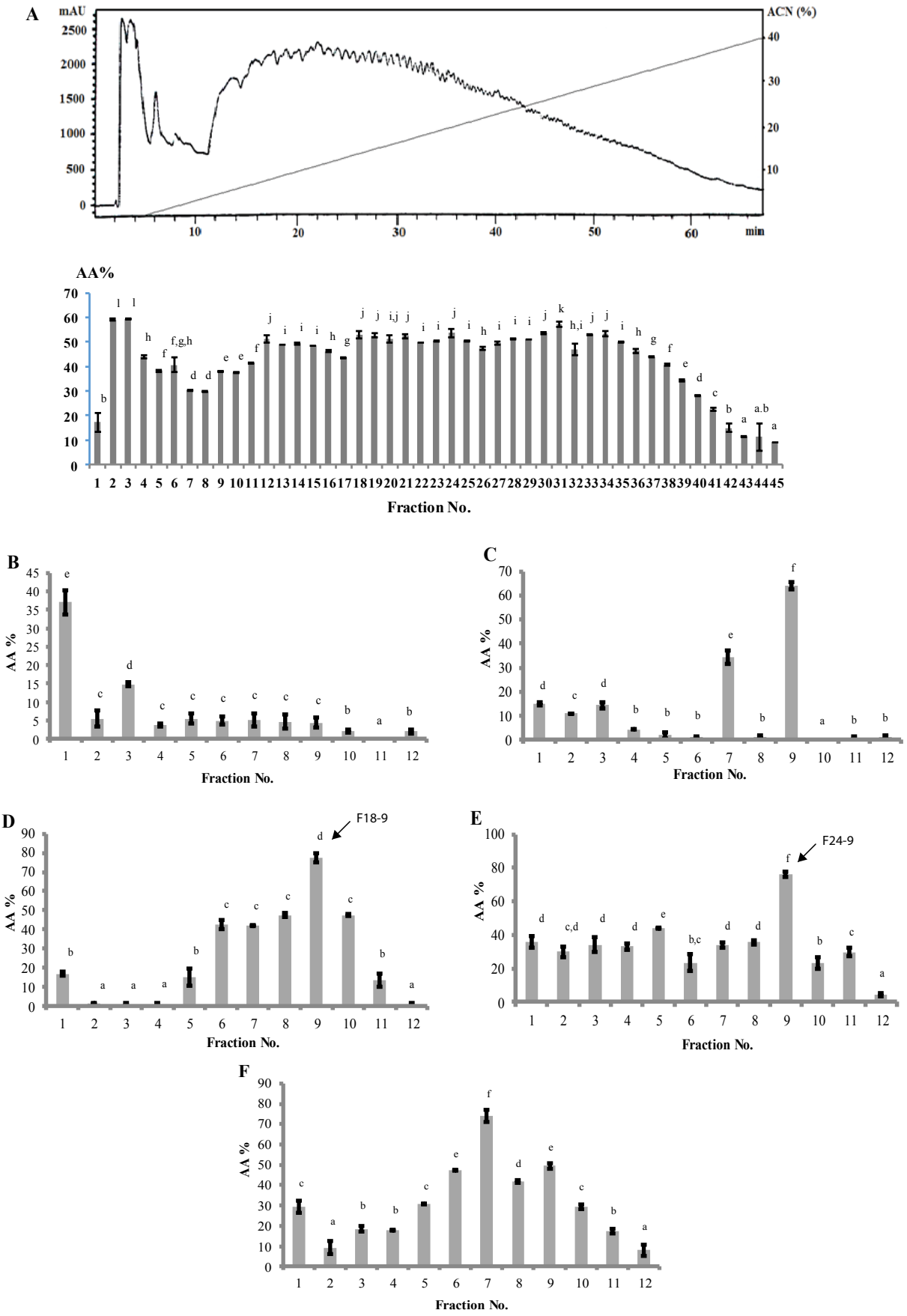

Figure 3. (A) Separation of stone fish proteolysate on acetonitrile gradient elution (0 - 40%) and the corresponding DPPH· activity from the separated HPLC fractions; DPPH· activities for five HPLC fractions, namely (B) F2, (C) F3, (D) F18, (E) F24, and (F) F31. Bars represent mean ± SD from triplicates (n = 3), and lowercase letters indicate significant differences at $p \leq 0.05$. AA% = Antioxidant activity (%).

(2020) who revealed that short-chain peptides were positively correlated with high antioxidant activity. In general, peptides with higher degree of hydrophobicity have longer retention time residing in the column as compared to peptides with lower hydrophobicity, thus requiring higher acetonitrile concentration, *i.e.,* higher hydrophobicity of mobile phase to be eluted out.

The correlation between peptides' hydrophobicity (characterised as acetonitrile gradient) and antioxidant activity is displayed in Figure 3A. Fractions 2 and 3 (1% hydrophobic), 18 (7% hydrophobic), 24 (12% hydrophobic), and 31 (17% hydrophobic) showed the most effective DPPH$^{\bullet}$ radical scavenging activity (59.16, 59.4, 53.01, 53.61, and 57.1%, respectively) despite the fact that they were diluted up to 100 times as compared to the initial proteolysate concentration. The optimum range of hydrophobicity for antioxidative peptides has been generally reported between 14 - 18% from different sources. The strongest DPPH$^{\bullet}$ scavenging activity recorded in the present work fell within this range, thus indicating that radical scavenging capacity is more prominent in polar and hydrophilic peptides instead of hydrophobic ones. This is supported by Hu *et al.* (2019) and Hou *et al.* (2019) who reported that the most potent antioxidative fractions were collected at early elution of RP-HPLC (*i.e.,* high hydrophilicity) for marine microalgae and sheep plasma protein, respectively. Findings from the present work also revealed that the antioxidant property of stone fish papain-digested proteolysates was not only related to the M_W and degree of hydrolysis, but also to the hydrophilicity of the generated peptides.

Profiling based on pI using OFFGEL fractionation

Five HPLC fractions with the highest antioxidant activity (Fraction 2, 3, 18, 24, and 31) were further fractionated based on their pI. A total of 60 sub-fractions were obtained, and each sub-fraction was tested for their antioxidant activity (Figure 3B - 3F). The highest DPPH$^{\bullet}$ radical scavenging activities were recorded for F18-9 (HPLC fraction 18, OFFGEL subfraction 9, 77.4 ± 4.4%) and F24-9 (76.2 ± 2.7%) with pI = 9, followed by F31-7 with an antioxidant activity of 74.1 ± 3.4% at pI = 6.7. Similarly, Zarei *et al.* (2012) reported that fractions with pI = 10 showed the highest antioxidant activity (71.7%), followed by fractions with pI = 5 - 7 in palm kernel cake proteolysate. The results obtained in the present work revealed that the bioactive peptides derived from stone fish flesh proteolysate could serve as an important source of natural antioxidants, which should be further identified and characterised by MS/MS spectroscopy in the future.

Conclusion

Stone fish was enzymatically hydrolysed using papain, and the resulting proteolysate was evaluated for its antioxidant capacity. Following 8-h hydrolysis, the proteolysate showed IC_{50} of 0.49 mg/mL for DPPH$^{\bullet}$ radical scavenging, IC_{50} of 0.36 mg/mL for ABTS$^{\bullet}$ radical scavenging, and 0.26 mM $FeSO_4$ equivalent for FRAP value. Stone fish protolysate was profiled via three approaches namely ultrafiltration, RP-HPLC, and isoelectric focusing fractionation to profile the peptides based on molecular weight, hydrophobicity, and isoelectric point, respectively. Results revealed that papain-digested stone fish protein produced appreciable amount of antioxidative peptides with M_W of less than 2 kDa, low hydrophobicity (≤ 20%), and basic isoelectric point (pI = 9). The present work concluded that papain-generated stone fish proteolysate and peptides demonstrated strong antioxidative potential that could be further explored in terms of sequence identification and peptide characterisation to foster its use as a natural functional ingredient in food products.

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References

Auwal, S. M., Zainal Abidin, N., Zarei, M., Tan, C. P. and Saari, N. 2019. Identification, structure-activity relationship and *in silico* molecular docking analyses of five novel angiotensin I-converting enzyme (ACE)-inhibitory peptides from stone fish (*Actinopyga lecanora*) hydrolysates. PLoS One 14(5): article ID e0197644.

Auwal, S. M., Zarei, M., Abdul Hamid, A. and Saari, N. 2017. Response surface optimisation for the production of antioxidant hydrolysates from stone fish protein using bromelain. Evidence-Based Complementary and Alternative Medicine 2017: article ID 4765463.

Auwal, S. M., Zarei, M., Tan, C. P., Basri, M. and Saari, N. 2018. Enhanced physicochemical stability and efficacy of angiotensin I-converting

enzyme (ACE)-inhibitory biopeptides by chitosan nanoparticles optimized using Box-Behnken design. Scientific Reports 8(1): article ID 10411.

Bar-Or, D., Bar-Or, R., Rael, L. T. and Brody, E. N. 2015. Oxidative stress in severe acute illness. Redox Biology 4: 340-345.

Bechtel, P. J., Oliveira, A. C., Demir, N. and Smiley, S. 2013. Chemical composition of the giant red sea cucumber, *Parastichopus californicus*, commercially harvested in Alaska. Food Science and Nutrition 1(1): 63-73.

Bersuder, P., Hole, M. and Smith, G. 1998. Antioxidants from a heated histidine-glucose model system. I: Investigation of the antioxidant role of histidine and isolation of antioxidants by high-performance liquid chromatography. Journal of the American Oil Chemists' Society 75(2): 181-187.

Bordbar, S., Anwar, F. and Saari, N. 2011. High-value components and bioactives from sea cucumbers for functional foods—a review. Marine Drugs 9(10): 1761-1805.

Bordbar, S., Ebrahimpour, A., Abdul Hamid, A., Manap, A., Yazid, M., Anwar, F. and Saari, N. 2013. The improvement of the endogenous antioxidant property of stone fish (*Actinopyga lecanora*) tissue using enzymatic proteolysis. BioMed Research International 2013: article ID 849529.

Bougatef, A., Nedjar Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D. and Nasri, M. 2010. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. Food Chemistry 118(3): 559-565.

Chasanah, E., Susilowati, R., Yuwono, P., Zilda, D. and Fawzya, Y. 2019. Amino acid profile of biologically processed fish protein hydrolysate (FPH) using local enzyme to combat stunting. IOP Conference Series - Earth and Environmental Science 278: article ID 012013.

Church, F., Swaisgood, H., Porter, D. and Catignani, G. 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. Journal of Dairy Science 66(6): 1219-1227.

Cruzat, V., Macedo Rogero, M., Noel Keane, K., Curi, R. and Newsholme, P. 2018. Glutamine: metabolism and immune function, supplementation and clinical translation. Nutrients 10(11): article no. 1564.

Elavarasan, K., Naveen Kumar, V. and Shamasundar, B. 2014. Antioxidant and functional properties of fish protein hydrolysates from fresh water carp (*Catla catla*) as influenced by the nature of enzyme. Journal of Food Processing and Preservation 38(3): 1207-1214.

Ghanbari, R. and Ebrahimpour, A. 2018. Separation and identification of bromelain-generated antibacterial peptides from *Actinopyga lecanora*. Food Science and Biotechnology 27(2): 591-598.

Ghanbari, R., Ebrahimpour, A., Zarei, M., Ismail, A., Abdul Hamid, A. and Saari, N. 2016. Purification and characterization of nitric oxide inhibitory peptides from *Actinopyga lecanora* through enzymatic hydrolysis. Food Biotechnology 30(4): 263-277.

Guo, C., Yang, J., Wei, J., Li, Y., Xu, J. and Jiang, Y. 2003. Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. Nutrition Research 23(12): 1719-1726.

Hou, C., Wu, L., Wang, Z., Saguer, E. and Zhang, D. 2019. Purification and identification of antioxidant alcalase-derived peptides from sheep plasma proteins. Antioxidants 8(12): article no. 592.

Hu, X., Yang, X., Wu, Q., Li, L., Wu, Y., Chen, S., … and Ren, J. 2019. Purification and identification of antioxidant peptides from *Schizochytrium limacinum* hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. Molecules 24(16): article no. 3004.

Hudson, B. 1992. Biochemistry of food proteins. 1st ed. United States: Springer.

Inhamuns, A. J., Franco, M. R. B. and Batista, W. S. 2009. Seasonal variations in total fatty acid composition of muscles and eye sockets of tucunaré (*Cichla* sp.) from the Brazilian Amazon area. Food Chemistry 117(2): 272-275.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Li, X. X., Han, L. J. and Chen, L. J. 2008. *In vitro* antioxidant activity of protein hydrolysates prepared from corn gluten meal. Journal of the Science of Food and Agriculture 88(9): 1660-1666.

Mishra, M. and Nagarajan, K. 2020. An insight for potent *in vitro* antioxidant status of short-chain peptides. International Journal of Peptide Research and Therapeutics 26(3): 1437-1449.

Nazeer, R. A. and Kulandai, K. A. 2012. Evaluation of antioxidant activity of muscle and skin protein

hydrolysates from giant kingfish, *Caranx ignobilis* (Forsskål, 1775). International Journal of Food Science and Technology 47(2): 274-281.

Neha, K., Haider, M. R., Pathak, A. and Yar, M. S. 2019. Medicinal prospects of antioxidants: a review. European Journal of Medicinal Chemistry 178: 687-704.

Pęksa, A. and Miedzianka, J. 2014. Amino acid composition of enzymatically hydrolysed potato protein preparations. Czech Journal of Food Sciences 32(3): 265-272.

Pérez-Vega, J. A., Olivera-Castillo, L., Gómez-Ruiz, J. Á. and Hernández-Ledesma, B. 2013. Release of multifunctional peptides by gastrointestinal digestion of sea cucumber (*Isostichopus badionotus*). Journal of Functional Foods 5(2): 869-877.

Qin, L., Zhu, B. W., Zhou, D. Y., Wu, H. T., Tan, H., Yang, J. F., … and Murata, Y. 2011. Preparation and antioxidant activity of enzymatic hydrolysates from purple sea urchin (*Strongylocentrotus nudus*) gonad. LWT - Food Science and Technology 44(4): 1113-1118.

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y. and Kim, S. K. 2005. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Research International 38(2): 175-182.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26(9-10): 1231-1237.

Rozan, P., Kuo, Y. H. and Lambein, F. 2000. Free amino acids present in commercially available seedlings sold for human consumption. A potential hazard for consumers. Journal of Agricultural and Food Chemistry 48(3): 716-723.

Sarmadi, B. H. and Ismail, A. 2010. Antioxidative peptides from food proteins: a review. Peptides 31(10): 1949-1956.

Shamloo, M., Bakar, J., Mat Hashim, D. and Khatib, A. 2012. Biochemical properties of red tilapia (*Oreochromis niloticus*) protein hydrolysates. International Food Research Journal 19(1): 183-188.

Suetsuna, K., Ukeda, H. and Ochi, H. 2000. Isolation and characterization of free radical scavenging activities peptides derived from casein. The Journal of Nutritional Biochemistry 11(3): 128-131.

Udenigwe, C. C. and Aluko, R. E. 2011. Chemometric analysis of the amino acid requirements of ``dant food protein hydrolysates. International Journal of Molecular Sciences 12(5): 3148-3161.

Wen, J. and Hu, C. 2010. Elemental composition of commercial sea cucumbers (holothurians). Food Additives and Contaminants 3(4): 246-252.

Wiriyaphan, C., Chitsomboon, B. and Yongsawadigul, J. 2012. Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts. Food Chemistry 132(1): 104-111.

Yu, L. N., Yang, Q. L., Sun, J., Bi, J., Zhang, C. S. and Liu, S. F. 2012. Study on the antioxidation activity of peanut antioxidant peptide. Applied Mechanics and Materials 140: 446-450.

Zaheer, K. and Akhtar, M. H. 2017. An updated review of dietary isoflavones: nutrition, processing, bioavailability and impacts on human health. Critical Reviews in Food Science and Nutrition 57(6): 1280-1293.

Zarei, M., Ebrahimpour, A., Abdul-Hamid, A., Anwar, F. and Saari, N. 2012. Production of defatted palm kernel cake protein hydrolysate as a valuable source of natural antioxidants. International Journal of Molecular Sciences 13(7): 8097-8111.

Zhang, J., Liu, S., Zhang, Y., Lu, Y., Wang, M., Wang, G. and Liu, X. 2017. Purification and antioxidant ability of peptide from egg in sea cucumber *Apostichopus japonicus*. International Journal of Food Properties 20(2): 306-317.

Zhong, Y., Khan, M. A. and Shahidi, F. 2007. Compositional characteristics and antioxidant properties of fresh and processed sea cucumber (*Cucumaria frondosa*). Journal of Agricultural and Food Chemistry 55(4): 1188-1192.

Zhou, D. Y., Tang, Y., Zhu, B. W., Qin, L., Li, D. M., Yang, J. F., … and Murata, Y. 2012. Antioxidant activity of hydrolysates obtained from scallop (*Patinopecten yessoensis*) and abalone (*Haliotis discus hannai* Ino) muscle. Food Chemistry 132(2): 815-822.

Zou, T. B., He, T. P., Li, H. B., Tang, H. W. and Xia, E. Q. 2016. The structure-activity relationship of the antioxidant peptides from natural proteins. Molecules 21(1): article no. 72.