In vitro study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species

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Abstract

Fish by-products from different fish species may be utilized to produce compounds possessing physiological and physical functional properties. A simple extraction process involving use of endogenous proteases and/or addition of exogenous enzyme preparation resulted in proteinaceous hydrolysates. The extracts from Salmon, Flathead, Silver warehou and Barramundi by-products were evaluated for selected physiological and physical properties. Fish by-products were subjected to four different treatments, with or without the addition of acid fungal protease (AFP). The peptides produced in fish protein hydrolysate (FPH) were examined for bioactive properties based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and angiotensin I-converting enzyme (ACE) inhibitory activities. Furthermore, dried FPHs were also examined for properties such as colour, solubility, heat stability, emulsion activity, and rheological properties. Time and addition of AFP to FPHs, influenced the degree of hydrolysis (DH), DPPH scavenging and ACE inhibition activities. Salmon FPH treated with AFP showed the highest DH (43.86 %) and ACE inhibitory activity (95.50 %). High antioxidant activity was observed for Flathead and Barramundi FPHs with DPPH scavenging activity of 45.86 % and 43.03%, respectively. The solubility of dried FPHs ranged from 93 to 100% and increased with decreased DH. Emulsifying capacity of FPH for Barramundi was the highest, whereas FPH for Silver warehou showed the highest emulsion stability. Heat induced the gelation of Barramundi and Silver warehou FPHs but not for Salmon and Flathead FPHs. These results have implications for the use of protein hydrolysate from fish by-products in food formulation technology and serve as important sources of bioactive compounds.

Introduction

Fish is an important source of nutritional compounds for the world's population. In 2010, fish accounted for 16.7% of the global intake of animal protein. From 137 million tons of fish marketed for edible purposes, only 46 percent were sold live, fresh or in a chilled form (FAO Fisheries and Aquaculture Department, 2014). Handling and processing raw fish into consumable products generate by-products, as it requires removal of bones, skin, head, shell and viscera. Fish by-products represent 50-75% of total weight of the catch (Rustad *et al.*, 2011), thus good fish waste management is inevitable. Fish by-products, as well as underutilized fish, are commonly recognized as low-value resources with almost insignificant market value. They are normally used for the production of fish meal, fish silage and animal feed. Fortunately, research has directed the conversion of fish by-products into cheap nutritious protein sources for human consumption since 1960. This involved implementation of hydrolysis processes to improve the palatability and functional properties of fish protein hydrolysate (FPH) (Kristinsson and Rasco, 2000a) or simple extraction processes which separate major nutrients – oils and proteins (Nurdiani *et al.*, 2015).

The common industrial practice for the production of FPH involves the use of highly concentrated hydrogen chloride or sodium hydroxide. It assures high recovery yield, yet reduces nutritional qualities and results in poor functionalities (Kristinsson and Rasco, 2000a). In order to minimize the use of chemicals and control the quality of FPH produced, hydrolysis using

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commercial microbial and/or digestive enzymes may be carried out (Diniz and Martin, 1997) or by utilizing endogenous enzymes from fish (Herpandi *et al.*, 2011). Enzymatic hydrolysis requires a relatively small amount of exogenous enzyme to cleave specific peptide bonds and create desired properties of FPH. Pepsin, papain, chymotrypsin and trypsin are among commercial proteases that have been widely used for the production of FPH with potential physical properties (Hoyle and Merrit, 1994; Balti *et al,* 2010a; Cheung and Li-Chan, 2010). FPH produced with endogenous enzymes obtained from fish viscera (Aspmo *et al*., 2005), intestines (Barkia *et al*., 2010) and hepatopancreas (Balti *et al.*, 2010a) have also shown desirable physicochemical properties.

When enzymes of microbial origin were used, several advantages including a wide variety of available catalytic activities, greater pH and temperature stabilities were observed (Diniz and Martin, 1997). Many researchers have utilized enzymes produced from Bacillus and Aspergillus strains for the production of FPH with potential bioactivities (Benjakul and Morrissey 1997; Qian *et al.*, 2007; Thiansilakul *et al.*, 2007a; Ovissipour *et al.*, 2012;). *Aspergillus niger* var. are known to produce acid and semi-alkaline proteases (Pourrat *et al.*, 1988; Barthomeuf *et al.*, 1988; Jarai and Buxton 1994; O'Donnell *et al.*, 2001) nevertheless, reports on the use of protease from *A. niger* for FPH production have been absent.

Our previous work has shown that application of low pH optimized the extraction of oil and protein from fish by-products incubated overnight (Nurdiani *et al*., 2015). During incubation, endogenous proteases including calpains and cathepsins may cause limited breakdown of fish proteins to release a range of polypeptides or oligopeptides. Further degradation of polypeptides to small peptides with potential physiological activities can be achieved by addition of exogenous proteases or the use of human digestive enzymes (Ahmed *et al*., 2015; Manikkam *et al*., 2015). One of the objectives of this study, therefore, was to examine whether the addition of Acid Fungal Protease (AFP, a protease from *A. niger* with optimum pH range of 2.5 to 3.5) would enhance the production of bioactive peptides from fish by-products. The physiological and functional properties of FPH, nevertheless, are not only determined by the selection of appropriate protease but also the nature of fish species used as a substrate (Medenieks and Vasiljevic, 2008). In addition, the extent of hydrolysis is an essential factor to determine the chain length of peptide which may affect the functional properties of FPH produced (Kristinsson and Rasco, 2000a). For

this reason, the study further observed the effect of different incubation periods of fish by-products of four Australian fish species on selected physiological activities (bioactivities). The physical functionality of dried FPH was also evaluated.

Materials and Methods

Materials

Aquacultured Atlantic Salmon *(Salmo salar)*, and wild caught Barramundi *(Lates calcarifer)*, Flathead *(Platycephalus fuscus)*, and Silver warehou *(Seriolella punctata)* were analysed in this study. By-products (heads, backbones, and frames) from these four Australian fish species were generously provided by Barwon Foods (Geelong, VIC, Australia). The samples were collected immediately after processing and kept on ice during transport to the laboratory and processed immediately as described below.

Food grade Acid Fungal Protease (AFP, activity 2000 SAPU/g), obtained by controlled fermentation of *Aspergillus niger* var, *(A. niger)* was donated by Enzyme Solutions Pty. Ltd (Croydon South, VIC, Australia). Hippuryl–histidyl–leucine (HHL), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, U.S.A). Acetonitrile was purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Sample preparation

Heads, backbones and frames of each fish species were minced without water using a laboratory mincer (MG-22SS, Handy Imports, Smithfield, NSW, Australia). Subsequently, the mince was mixed with an appropriate volume of 1% sulphuric acid to adjust pH to 2.5 and obtain fish:water ratio of 1:1 (Nurdiani et al., 2015). The mixture was then divided into four equal parts and processed as described below (Figure 1): a) incubation at room temperature for 1 hour without enzyme addition, b) hydrolysis with AFP for 1 hour at room temperature, c) overnight incubation at room temperature without enzyme addition; and d) overnight incubation at room temperature followed by addition of AFP and hydrolysed for 1 hour. The enzyme activity of the commercially prepared protease added was 25 SAPU/g of sample. After each treatment, the samples were centrifuged at 4,000 rpm for 10 minutes at 20°C using a Sorvall centrifuge (RT-H750 swing bucket rotor, RT model, DuPont Company, Newtown, CT, USA).

Upon centrifugation, five distinct layers were formed in the tube (Nurdiani *et al.*, 2015). The
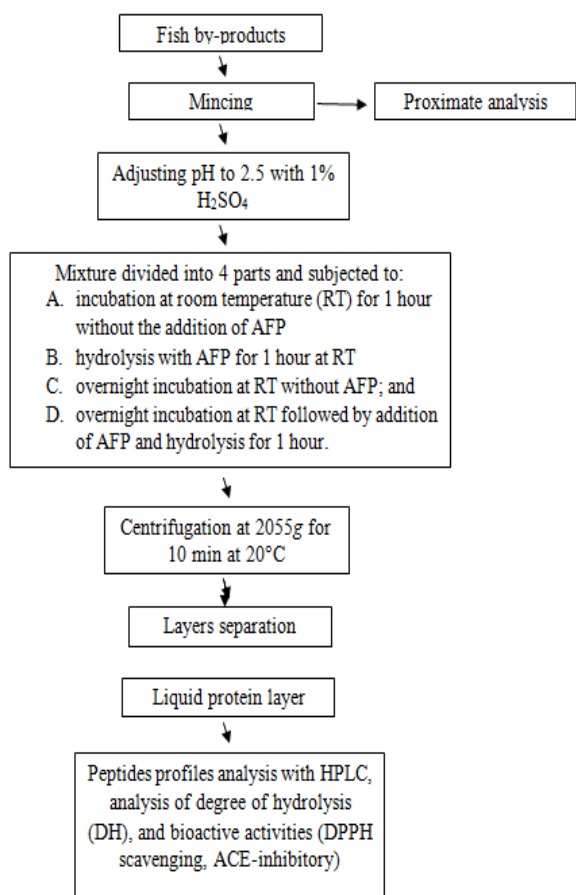
Figure 1. Schematic diagram representing method used for the isolation of bioactive compounds from fish byproducts

soluble protein/peptide extract layers were then collected to be used for analysis of degree of hydrolysis (DH), peptide profile (using reversed phase HPLC), and corresponding radical scavenging (DPPH assay) and ACE inhibitory activities. Additionally, selected functional properties (colour, solubility, heat stability, and emulsifying properties) of FPH powders were also assessed. For this determination, liquid proteins were collected after overnight incubation (treatment c), freeze rapidly with liquid nitrogen to inactivate endogenous enzymes and lyophilized to obtain FPH powders.

Evaluation of degree of hydrolysis

The degree of hydrolysis of hydrolysed soluble protein/peptide extract was assessed according to Hoyle and Merritt (1994). An aliquot of 2 mL of soluble extract was mixed with an equal volume of 20% TCA followed by centrifugation at 10,000 rpm for 20 min at room temperature (Eppendorf Centrifuge 5415C, Crown Scientific Pty Ltd, Moorebank, NSW, Australia). The supernatant was collected and extent of hydrolysis was established by determining the nitrogen content using Kjeldahl method (AOAC, 2005) and expressed as:

$$\%DH = \left(\frac{10\% \text{ TCA soluble nitrogen in the sample}}{\text{Total nitrogen in the sample}}\right) \times 100\%$$

Peptide profiling by reversed-phase high performance liquid chromatography (RP-HPLC)

The peptides in various fish soluble extracts were profiled using RP-HPLC according to Elfahri *et al.* (2014) with minor modifications. All samples were centrifuged at 14,000 rpm for 30 min (Eppendorf Centrifuge) and filtered through 0.45µm membrane filter (Schleicher and Schuell GmbH, Germany) into HPLC sample vials. Peptides were separated on a Varian HPLC system (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with Vydac Everest C18 column (250mm x 4.6 mm, particle size 5 µm, pore size 300Å; Grace Davison Discovery Sciences, Rowville, VIC, Australia). The peptides were eluted by a linear gradient of 0% to 100% of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in deionized water) over 90 min. The flow rate was maintained at 0.75 mL/min and eluted peptides were detected at 214 nm using a Varian 9050 variable wavelength UV/vis detector. All solvents were filtered through a 0.45 µm membrane filter.

DPPH radical scavenging activity

The scavenging effect of liberated peptides on DPPH free radical was measured according to Donkor *et al.* (2012) with some modifications. An aliquot of 50 µL soluble extract was added to 1950 µL of 0.075 mM DPPH in 95% ethanol. The mixture was allowed to stand in the dark for 30 min and absorbance of the resulting solution was recorded at 517 nm using a Biochrom Libra S12 UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, Cambridgeshire, United Kingdom). A lower absorbance represented a higher DPPH scavenging activity. Water was used as a blank. The scavenging effect was expressed as shown in the following equation:

$$\text{Scavenging activity (\%)} = \left|\frac{(\text{Blank absorbance} - \text{Sample absorbance})}{\text{Blank absorbance}}\right| \times 100$$

Determination of ACE inhibitory activity

Approximately 0.5 g of hydrolysed liquid fish protein was diluted appropriately with Milli-Q water and centrifuged at 14,000 rpm for 30 min at 4°C (Eppendorf Centrifuge). The supernatant was filtered through 0.45 µm filter and the resulting solution was characterized for its inhibitory activity toward rabbit lung ACE using method as described in Medenieks and Vasiljevic (2008). The ACE inhibitory activity was calculated as follows:

$$\text{ACE inhibitor activity (\%)} = \left[1 - \frac{C - D}{A - B}\right] \times 100$$

where A is the absorbance of the ACE solution with

the substrate hippuryl–histidyl–leucine (HHL), B is the absorbance of the HHL without the ACE solution, C is the absorbance of the solutions of ACE, HHL and the ACE-inhibitory component (fish protein hydrolysate samples, FPH), and D is the absorbance without ACE but with solutions of HHL and FPH. All determinations were carried out in triplicate.

Physical functionality of fish protein powders

The colour of each fish protein powder was assessed with a Minolta Chromameter (CR-300, Minolta Corporation, Ramsey, NJ, USA) using CIE 1976 (L^* - Lightness; Positive a^* is red and negative a^* is green; Positive b^* is yellow and negative b^* is blue) colour system (Dissanayake *et al.*, 2010). Fish powder was placed on a plastic petri dish with a black paper as dark background and a white tile was used to correct for the white balance.

Selected functional properties including solubility, heat stability and emulsifying capacity of FPHs were examined. FPH powder required to prepare 5% (w/w) protein dispersion was mixed with Milli-Q water, stirred for 2 hours and further homogenized with a Polytron homogenizer (Model PT 2000, Kinematica AG, Luzern, Switzerland) at room temperature for 1 min. The dispersions were kept overnight at 4°C for full hydration. The pH of the dispersions was then adjusted to 7 at room temperature using 1M NaOH and the weights were corrected before being used for functional property analyses.

Solubility

Protein solubility of FPH sample was estimated following the method of Dissanayake and Vasiljevic (2009) with several adjustments. Approximately 10 mL portions of 5% (w/w) protein dispersion were centrifuged at 3,000 rpm for 20 min at 20°C (Sorvall RT). Protein contents in the supernatant and in the original 5% protein dispersion were determined by the Kjeldahl method (AOAC, 2005) and solubility was calculated using the following equation:

$$\text{Heat stability} = \frac{\text{Protein content of supernatant after heating, mg/mL}}{\text{Total protein content of original dispersion, mg/mL}} \times 100$$

Heat stability

Two different methods were used to assess the heat stability of FPHs at 100 °C: a) heat coagulation time (HCT); and b) solubility after brief exposure to heat. HCT is defined as the time required to form visible aggregates during exposure to excessive heating (Dissanayake and Vasiljevic, 2009).

Approximately 3.0 mL of 5% (w/w) FPH dispersion was placed in 10 mL glass tubes, sealed, immersed in a shaking oil bath (Ratek Shaking oil bath, Australian Scientific Pty. Ltd., Kotara, NSW, Australia) maintained at 100°C. A time point at which the first visible aggregates appeared was recorded as HCT.

For the second method, a 3 mL sample of FPH dispersion was exposed to the same conditions. After 30 seconds, tubes were quickly removed from the oil bath, cooled instantly in an ice bath and centrifuged at 3,000 rpm at 20°C for 20 min (Sorvall RT). The supernatant was then filtered through 0.45 μm filter. The protein content of the original 5% protein sample and supernatant of heat treated sample were estimated using the Kjeldahl method. Heat stability was expressed using the following equation:

$$\text{Solubility} = \frac{\text{Protein content of supernatant, mg/mL}}{\text{Total protein content of dispersion, mg/mL}} \times 100$$

Emulsifying properties

Emulsifying characteristics of FPHs were monitored using a turbidimetric method previously described by Pearce and Kinsella (1978). Emulsifying activity index (EAI), emulsion stability and adsorbed protein of 5% (w/w) protein dispersions were measured following a protocol fully described previously (Dissanayake and Vasiljevic, 2009).

Rheological properties

The rheological properties of FPHs were measured using a controlled-stress rheometer (Physica MCR 301, Anton Paar GmbH, 73760 Ostfildern-Scharnhausen, Germany) equipped with a cone and plate measuring system (CP50-1- SN9151, Anton Parr) as described previously (Dissanayake *et al*., 2010). An appropriate volume of 5% (w/w) FPH dispersion was pre-sheared for 30 s at a shear rate of 500 s^{-1} held for 30 s at 20°C to reach equilibrium. The heat-induced gelation was carried out using a dynamic small amplitude oscillatory measurement (SAOM) at 0.5% strain and a frequency of 1 Hz. Measurements were acquired by heating the mixtures from 20 to 80°C at a heating rate of 5°C min^{-1}, held at 80°C for 1 min, and cooled to 20°C at 5°C min^{-1} then held at 20°C for 10 min. All rheological measurement data were analysed with the Rheoplus/32 v 2.81 software (Anton Paar, Germany).

Statistical analysis

Statistical analyses were performed using Statistical Analysis System (SAS) software (SAS Institute Inc., 2009). All experiments were repeated at least in triplicate and the mean values were reported. Data were analysed as a randomized block design using fish species and replications as the main factors. The means were also compared using the
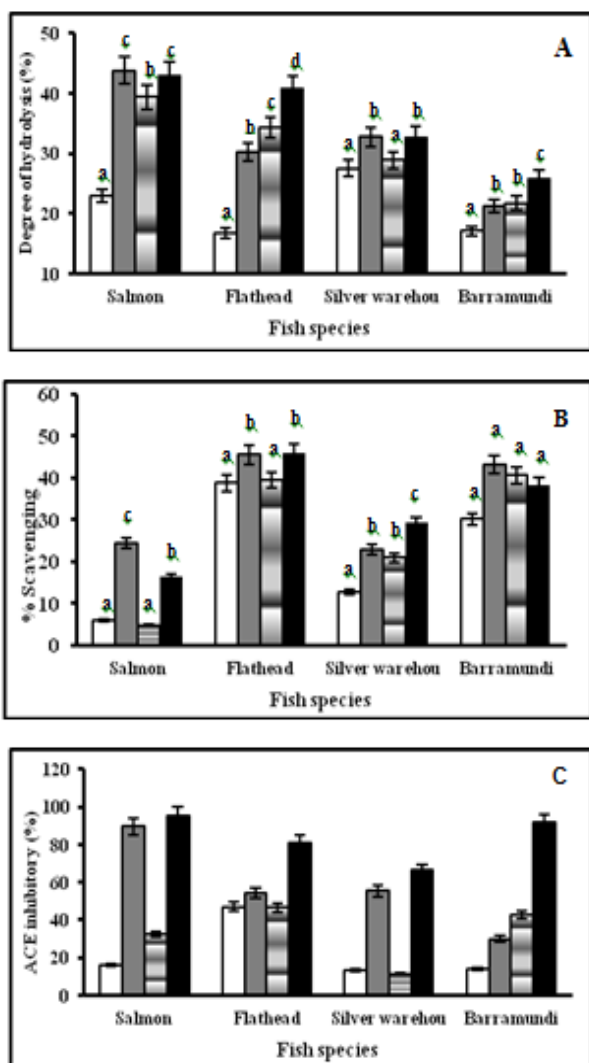
Figure 2. Degree of Hydrolysis (A); DPPH Scavenging activity (B); ACE-inhibitory activity (C) of Salmon, Flathead, Silver warehou, and Barramundi hydrolysed at pH 2.5. Results represent means ± standard deviation. Values with different superscripts indicate significant difference within the same fish species (P < 0.05). ☐ = 1 hour; ■ = 1 hour AFP; ▨ = overnight; ■ = overnight with AFP.

Tukey test with the pre-set level of significance at p < 0.05.

Results and Discussion

Degree of hydrolysis and peptide profiles

Degree of hydrolysis describes the extent of peptide bonds cleaved in a substrate. The DH of Salmon, Flathead, Silver warehou, and Barramundi by-products hydrolysed at pH 2.5 is presented in Figure 2A. As depicted, four different treatments were applied to solubilize fish proteins via hydrolysis to enhance the extraction of valuable proteinaceous components. All fish species contained endogenous proteases with varying degree of activity (Ahmed *et al*., 2013), and incubation time played important

role in governing the extent of hydrolysis (Šližytė *et al.*, 2009). For this reason, the extent of hydrolytic cleavage was significantly (p<0.05) lower after an hour of hydrolysis in comparison to overnight incubation. The rate of hydrolysis was however substantially improved upon addition of exogenous protease preparation, doubling DH during the first hour of hydrolysis (Figure 2A). Two main endogenous proteolytic systems involved in hydrolysis of fish proteins are the cytoplasmic calpains and lysosomal cathepsins (Chéret *et al.*, 2007). Calpains are optimally active at neutral pH, whereas cathepsins have their optimum activity in acidic environment (Ahmed *et al*., 2015). Sovik and Rustad (2004) however found that the highest proteolytic activity in fish viscera occurred at low pH.

Acid fungal protease (AFP) is a food grade enzyme optimized to hydrolyse proteins under acidic conditions. The optimum pH range for AFP is 2.5 to 3.5 (Enzyme Solutions, 2010). Addition of AFP to fish proteins at pH 2.5 almost doubled (p < 0.05) DH in Salmon and Flathead (Figure 2A). This result is in agreement with Wu *et al.* (2003) who observed higher levels of free amino acids, anserine, and other peptides from mackerel hydrolysed with protease compared to autolysis by endogenous proteolytic enzymes. Overall, highest DH was achieved in Salmon by-product than in other fish by-product/species, with Barramundi showing the lowest DH. The chemical composition and the type of fish by-products used as substrate played a role in determining the rate of DH. Interestingly, DH achieved for Salmon by-product in the present study was substantially higher than that obtained by Kristinsson and Rasco (2000b) who used various alkaline proteases, an indication that AFP may be more suitable in these applications.

In order to confirm hydrolysis of fish protein, peptides in the soluble protein/peptide extract fractions were profiled using RP-HPLC (Figure 3). For all fish species, the chromatograms of the fish proteins incubated for 1 hour with no added enzyme showed lower degrees of liberated peptides, compared to other treatments. However, the addition of AFP and longer hydrolysis time resulted in improve DH and higher concentrations of smaller peptides. Despite the fact that most of the peaks appeared during the first 20 min of elution time in the hydrophilic region, distribution and concentration of peptides varied among species. Salmon and Flathead contained more hydrophobic peptides (20 to 30 min region) than the other two species. Overall, the water soluble extract fish hydrolysates, contained more hydrophilic than hydrophobic peptides. This result is in agreement with a previously reported study on
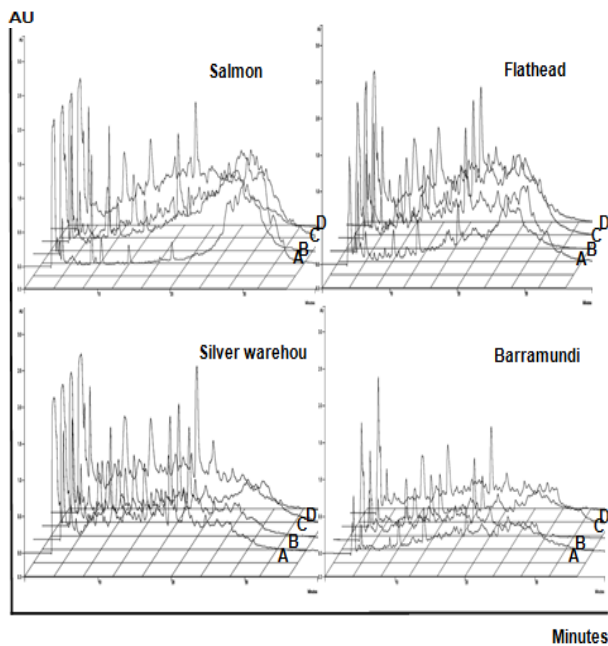
Figure 3. Reversed-phase HPLC profile of the soluble protein fraction of fish by-products hydrolysed at pH 2.5 for: (A) 1 hour without enzyme, (B) 1 hour with enzyme, (C) overnight without enzyme, (D) overnight and 1 hour with enzyme. The chromatographic conditions were detailed in materials and methods section.

less hydrophobic peptides in hydrolysed proteins of Cuttlefish by-product (Balti *et al.*, 2010b). Wilding *et al.* (1984) mentioned that hydrophilic/hydrophobic peptide ratio is an important factor influencing functional properties such as foaming, whippability and emulsifying capacity.

DPPH radical scavenging activity of peptides

The antioxidant activity of hydrolysates from Salmon, Flathead, Silver warehou and Barramundi, determined using DPPH radical scavenging assay has been depicted in Figure 2B. DPPH assay is widely used to test the free radical-scavenging ability of various natural antioxidants. As illustrated in Figure 2B, the DPPH scavenging activities of Flathead and Barramundi soluble extracts were significantly ($p<0.05$) higher than those of Salmon and Silver warehou. For all species on the other hand, antioxidant activity increased with increasing hydrolysis as a result of the addition of enzyme.

The antioxidant activity of fish hydrolysates, however does not correlate with DH or vice versa. Salmon hydrolysate, for example, with high DH showed low antioxidant activity than Barramundi hydrolysate. Similarly, Klompong *et al.* (2007) observed that DPPH radical scavenging activity of Yellow trevally *(Selaroides leptolepis)* hydrolysates obtained using Alcalase, decreased with increasing DH. Furthermore, Jun *et al.* (2004) reported low DH
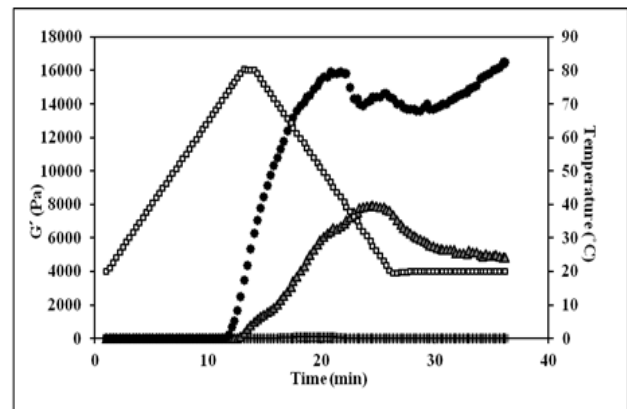

Figure 4. Changes in storage modulus (G') of 5% (w/w) fish protein dispersions. □ = Temperature; ◆ = Salmon; ⊕ = Flathead; ▲ = Silver warehou; ● = Barramundi.

of Yellowfin sole *(Limanda aspera)* which showed high antioxidant acitivity. Antioxidative activity of hydrolysates depends on the hydrolysis condition, type of enzyme used (Peña-Ramos and Xiong, 2003; Jun *et al.*, 2004) and liberated peptides containing specific amino acid sequence motifs. Enzymes cleave protein selectively at certain peptide bonds to produce specific amino acid sequence. In order to possess high antioxidant activity, peptides should contain certain types of amino acids such as Tyrosine and Histidine which have shown strong antioxidant activities (Mendis *et al.*, 2005; Je *et al.*, 2005; Fan *et al.*, 2012).

ACE inhibitory activity of fish protein fraction

The angiotensin converting enzyme (ACE; EC. 3.4.15.1) have been reported to play important physiological role in the regulation of blood pressure and cardiovascular function (Li *et al.*, 2004). The enzyme converts angiotensin I, to angiotensin II, and also inactivates bradykinin, a vasodilator (Ondetti *et al.*, 1977), resulting in the inhibition of ACE activity which appears to be a good target for hypertension. The ACE inhibitory activity of fish by-products protein hydrolysates from Salmon, Flathead, Silver warehou, Barramundi is presented in Figure 2C., and ranged from 11.58 to 95.50%. Fish protein hydrolysate treated with AFP showed substantially high levels of ACE inhibitory activity, although there was no statistical difference ($p > 0.05$) between treatments. The highest inhibition (95.50%) was observed for peptide in Salmon hydrolysate prepared by overnight incubation and further 1 hour with enzyme. This result is in agreement with a study by Ono *et al.* (2003) who similarly reported high ACE-inhibitory activity (85.5%) from Chum salmon hydrolysate. Low molecular weight peptides from Salmon skin hydrolysates (Gu *et al.*, 2011) and Chum salmon head hydrolysate (Ohta *et al.*, 1997) also showed high

Table 1. Functional properties and physical characteristics of fish waste protein

Fish sample	Solubility, %	EAI, m²g⁻¹	ESI, h	HCT, s	Heat stability, %	Colour of protein powder		
						L^*	a*	b*
Salmon	96.71±0.39ᵃ	4303.20±18.91ᶜ	59.36±1.87ᵃ	56.0±2.83ᶜ	96.69±1.92ᵇ	95.38±0.10ᶜ	-1.30±0.01ᵃ	14.54±0.02ᵇ
Flathead	99.35±0.63ᵇ	1203.47±46.75ᵃ	203.31±56.73ᵇ	36.0±1.41ᵃ	98.24±0.78ᵇ	96.13±0.09ᵈ	-0.91±0.02ᵃ	11.37±0.04ᵃ
Silver warehou	93.40±2.78ᵃ	2383.94±1.64ᵇ	205.88±8.32ᵇ	41.5±2.12ᵇ	80.93±0.45ᵃ	76.10±0.08ᵇ	1.87±0.01ᵇ	17.27±0.01ᶜ
Barra-mundi	100.02±0.02ᶜ	4945.98±26.57ᵈ	148.94±25.26ᵈ	31.0±2.83ᵃ	85.92±4.89ᵃ	72.95±0.18ᵃ	1.86±0.01ᵇ	11.42±0.02ᵃ

Values with different superscripts (a–d) within the same column indicate significant difference ($P < 0.05$).
EAI -Emulsifying activity index; ESI – Emulsifying stability index; HCT – Heat coagulation time.

ACE inhibitor activity. The ACE inhibitory activity of Silver warehou hydrolysate was the lowest among four species which ranged from 11.58 to 66.24% in our study. Medenieks and Vasiljevic (2008), reported slightly higher ACE inhibitory activity with FPH extracted from Silver warehou fillet using simulated human digestion. Flathead and Barramundi by-products hydrolysates incubated overnight and with further 1 hour AFP hydrolysis, yielded hydrolysates possessing high ACE inhibitory activity. No previous ACE inhibition studies have been conducted for these two fish species. Therefore, the current study has shown that Flathead and Barramundi by-products are promising sources of ACE inhibitory peptides. Using various proteolytic enzymes, many studies have assessed the release of these bioactive peptides from other fish species (Seki *et al.*, 1995; Lin *et al.*, 2012; Nasri *et al.*, 2013; Akagündüz *et al.*, 2014). Several specific amino acid sequences were observed from potent ACE inhibitory peptides. Matsumura *et al.* (1993) found four potential ACE inhibitor peptides from bonito bowel contained carboxyl terminal proline residues. Whereas Gu *et al.* (2011) reported two dipeptides (Ala-Pro and Val-Arg) from Atlantic salmon skin as showing high ACE inhibitory activities. Meanwhile, Shiozaki *et al.* (2010), isolated peptides from oyster and suggested that Asp-Tyr was the main effectors of peptides exhibiting hypotensive activity *in vivo*.

Physical functionality of fish protein powders

In addition to their physiological functionalities, FPH can be used as valuable ingredients in formulated foods. For this purpose, establishing its physical functionality is important as reported previously in selected fish species (Nurdiani *et al.*, 2015).

Colour

Colour is one of the most important aspects in terms of presentation, value of fish powders and consumer acceptance, as it may contribute to quality and appeal of product to the consumer (Bueno-Solan*o et al.*, 2009). Results of the colours of freeze-dried protein hydrolysates investigated using colorimeter are shown in Table 1. There was a significant difference ($p < 0.05$) in L^* values of Salmon, Flathead, Silver warehou and Barramundi fish powders. Visually, Flathead and Salmon powders showed light yellowish colour, whereas Silver warehou and Barramundi powders were brownish in colour. Darker colour of fish protein hydrolysate may be less attractive for consumers thus limit its application as food ingredient (Kristinsson and Rasco, 2000a). Darker colour may be the results of genetics or pigments possessed by different fish species (Chaijan *et al.*, 2005). The L^* values of Silver warehou and Barramundi were however higher than blue whiting protein isolate (Geirsdottir *et al.*, 2011) and round scad (Thiansilakul *et al.*, 2007b); therefore, may be more promising as an additive in certain food products.

Solubility

Solubility is important functional property of protein hydrolysates, as other properties such as emulsification and foaming are directly affected by it (Kristinsson and Rasco, 2000a). High solubility is a desirable attribute of fish protein hydrolysates as low solubility may cause a sandy mouth feel and unattractive final product (Petersen, 1981). The solubility values of fish hydrolysate are provided in Table 1. Salmon, Silver warehou, Flathead and Barramundi showed very high solubility between 93 to 100%.

High solubility may have resulted from the

unfolding protein molecules, both non polar and polar amino acid groups, during hydrolysis. These exposed amino acids likely interacted with water molecules via hydrogen bonds resulting in increased solubility (Wu *et al.*, 1998). Increased solubility correlated with increased DH (Kristinsson and Rasco 2000b; Dong *et al.*, 2008). Souissi *et al.* (2007) similarly reported increasing DH of sardinella protein increased the solubility of hydrolysate. Excellent solubility at high DH was also observed for yellow stripe trevally meat protein (Klompong *et al.*, 2007) and Salmon by-product (Gbogouri *et al.*, 2004). Interestingly, our results showed no correlation between DH and solubility. Barramundi sample with the highest solubility (100%) showed the lowest DH (Fig. 2 A). Factors other than DH may likely influence solubility, balance between hydrophilic and hydrophobic forces of fish hydrolysates and solution pH have been reported to influence solubility (Gbogouri *et al.*, 2004). FPH containing smaller peptides are expected to have proportionally more polar residues than intact proteins with increased ability to form hydrogen bonds with water and hence increase solubility (Kristinsson and Rasco 2000b; Gbogouri *et al.*, 2004).

Emulsifying properties

Table 1 shows the emulsifying capacity and stability for fish protein hydrolysates. Barramundi exhibited the highest emulsifying capacity while Silver warehou hydrolysate yielded the most stable emulsion. Many factors may account for the ability of hydrolysates to form stabilized emulsions. Low emulsifying stability of Salmon hydrolysate and low emulsifying activity of Flathead hydrolysate may be as a result of their relatively small peptides. Both FPHs showed relatively high DH (Figure 2A). Our previous work showed that Salmon and Flathead FPHs did not have any distinguishable proteins in SDS gels. It was suggested that myosin and heavy myosin chain in the samples had been hydrolysed and yielded low molecular weight (less than 10 kDa) proteins or peptides (Nurdiani *et al.*, 2015). A peptide should have a minimum length of >20 residue to have good emulsifying properties (Lee *et al.*, 1987). Barramundi and Silver warehou hydrolysates, on the other hand, retained some larger peptides (Nurdiani *et al.*, 2015) that were likely responsible for the high emulsifying capacity observed for the hydrolysates. Decrease in emulsifying capacity as the DH increases was also observed by Spinelli *et al.*, (1972); Gbogouri *et al.*, (2004) and Klompong *et al.*, (2007). Relatively poor emulsification ability of FPH was documented for acylated rockfish protein (Miller and Groninger,

1976), Brazilian lobster (Vieira *et al.*, 1995), and capelin (Shahidi *et al.*, 1995) hydrolysates.

Heat stability

Heat has a major influence on fish proteins including myofibrillar proteins (myosin and actin), connective tissue proteins and sarcoplasmic proteins (Kong *et al.*, 2008). Application of heat on proteins may cause denaturation, dissociation of myofibrillar proteins, transversal and longitudinal shrinkage of muscle fibre (Murphy and Marks 2000; Kong *et al.*, 2007). As shown in Table 1, there were significant (p<0.05) differences in the heat coagulation time among the species. Salmon and Barramundi hydrolysates showed the longest and shortest HCT, respectively. Long HCT indicates good ability of fish protein to withstand heat which may be an advantage for food formulation. Slightly different experiment was conducted by Taguchi *et al.*, (1981), where fish proteins from several species were exposed to various temperatures (40, 50, 60, 70, 80 and 100°C) and HCT was determined when equilibrium was established. It was concluded that heat coagulation time was within 15 min and established that protein coagulation started immediately after heating to 100°C.

The heat stabilities of four fish species differed significantly (Table 1) with Flathead and Silver warehou showing the highest and the lowest, respectively. FPH with high heat stability is suitable as additive for foods that undergo high temperature processing. Relatively low heat stability of Silver warehou and Barramundi was due to coagulation and precipitation of protein molecules at the treatment temperature (100°C) (Dissanayake and Vasiljevic, 2009). This result agreed with results of the SDS-PAGE (Nurdiani *et al.*, 2015) where Barramundi and Silver warehou samples contained high amounts of myosin heavy chains which are more unstable and easily denatured (Poulter *et al.*, 1985; Thorarinsdottir *et al.*, 2002). Heat stability of fish protein is also species-dependent (Taguchi *et al.*, 1981; Hastings *et al.*, 1985). Fish that live in the waters of higher ambient temperatures show more stable proteins (Poulter *et al.*, 1985).

Rheological properties

Gelling of FPH upon heating provides structural and textural benefits in many food applications. During heat-induced gelation, proteins dissociate and reactive sites (such as thiols) and hydrophobic groups are exposed (Totosaus *et al.*, 2002; Dissanayake et al., 2010). Under adequate environmental conditions, association and aggregation reactions result in a gel formation (Totosaus *et al.*, 2002). Figure 4 shows

the changes in storage modulus (G') of 5% (w/w) fish protein dispersions. Only Barramundi and Silver warehou formed heat-induced gels. The failure of both Salmon and Flathead hydrolysates to yield heat-induced gels may be due to the unavailability of required sites for cross-linking as well as molecular size of fish proteins. Barramundi and Silver warehou possess higher molecular weight protein bands than Salmon and Flathead (Nurdiani *et al.*, 2015) indicating that molecular size might have played a role in governing rheological properties of these proteins (Damodaran, 1989).

The ability of fish protein to form gels is important in food formulation and product development. It is influenced by several factors including myosin and actin content, type of muscles, protein concentration, pH, ionic strength, processing (heating rate, temperature and pressure), fat content, the presence of gelatine, addition of transglutaminase, protein additives and non-protein additives (Visessanguan and An, 2000; Sun and Holley, 2011.).

Conclusion

Fish by-products contained valuable compounds that have potential applications in food formulations and in pharmaceuticals. Results showed that fish by-products are good natural sources of antioxidants and ACE-inhibitory peptides. High antioxidant and ACE inhibitor activities observed from Flathead and Baramundi by-products revealed new promising sources of bioactive peptides. Hydrolysis duration and addition of enzyme increased DH and bioactivity of hydrolysates. The DH however was not entirely related to functional properties of fish proteins. Other factors such as fish species and balance between hydrophilic and hydrophobic forces of fish proteins appeared to be involved. The FPH obtained from Salmon showed relatively high emulsification capacity, HCT and heat stability which makes it suitable as additive for foods that undergo high temperature processing. Silver warehou and Baramundi FPHs formed heat-induced gels, important for food structuring. In order to maximize the utilisation of fish by-products, further detailed studies on isolation and purification of useful compounds from fish by-products are needed.

Acknowledgments

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