Characterization and Profiling of Protein Hydrolysates from White and Red Meat of Tuna (Euthynnus affinis)

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Proper utilization of these protein rich fish processing discards could be achieved by enzymatic conversion of these sources into protein hydrolysates which has immense application scope in food and pharmaceutical areas (Chalamaiah et al., 2012; He et al., 2013).

Protein hydrolysates are bioactive peptides obtained by the breakdown of proteins by hydrolysis either chemically or enzymatically. Previous studies have indicated variations with respect to the properties in red and white meat of tuna hydrolysate which may be on account of species, seasonal variations etc (Sanchez-Zapata et al., 2011; Parvathy et al., 2018). The present study was focused on comparative evaluation of the properties of hydrolysates derived from white and red meat of tuna (*Euthynnus affinis*), a high demand species in the domestic market. Protein hydrolysate was prepared using 1% (w/w) papain for 60 min under optimal hydrolytic conditions to obtain spray dried tuna white meat (TWPH) and tuna red meat protein hydrolysates (TRPH) which were further evaluated for nutritional, functional and antioxidant properties.

Materials and Methods

Fresh Tuna (*Euthynnus affinis*) was purchased from local fish market at Vashi, Navi Mumbai and brought to the laboratory in iced condition. Tuna white and red meat was separated and was used as raw material for the preparation of protein hydrolysates. Enzyme papain (Hi Media) from Papaya latex was used for hydrolysis. All chemicals used for the study were of analytical grade.

Tuna white and red meat was comminuted separately and thoroughly using an electric grinder and mixed with water (1:2 (w/v)). The resultant slurry was cooked for 30 min at 80-90°C to completely inactivate the endogenous enzymes in the raw material. Hydrolysis was initiated by adding enzyme papain at physiological pH and temperature of 60°C. The enzyme:substrate (E/S) concentration and hydrolysis time were kept as 1% (raw material weight basis (w/w)) and 60 min, respectively. On completion of hydrolysis, the solution was immediately heated to 80-90°C for 15-20 min to arrest the hydrolytic process. The resultant solution was course filtered and centrifuged (K-24A, Remi Instruments, Mumbai) at 8000 g for 20 min at 10°C and the supernatant obtained thereof was spray dried (Hemaraj Enterprises, Mumbai) to get

hydrolysate powder which was transferred to air tight plastic containers for further analysis.

Proximate composition of tuna white meat and red meat as well as their respective hydrolysates were estimated as per AOAC (2012). Degree of Hydrolysis (DH) was determined by evaluating the α -amino nitrogen in the protein hydrolysates, by formol titration method (Taylor, 1957) and the total nitrogen content in the wet meat by Kjeldahl method (AOAC, 2012) and expressed as percentage of α-amino nitrogen to total nitrogen.The protein recovery in hydrolysate was defined as the percentage of protein obtained during the extraction process in hydrolysate to the total amount of protein in raw material. Yield of fish protein hydrolysate was calculated as the percentage of the amount of hydrolysate powder gained after spray drying to the amount of raw material used for hydrolysis.

Proteolytic activity was measured by determining the tyrosine content of the hydrolysate inorder to assess the extent of hydrolysis under given conditions. A known quantity of diluted liquid hydrolysate solution was measured for the absorbance at 280 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). A standard curve of L-tyrosine in 0.2 M HCl was generated and the tyrosine content of sample was expressed in μ mole of tyrosine liberated mg^{-1} of protein. Higher tyrosine content indicates higher proteolytic activity.

Colour of the sample (1% hydrolysate solution) was evaluated using Hunter Lab colorimeter (Colorflex EZ 45/0, Hunter Associates Lab inc., Reston, Virginia, USA) to produce numeric results indicative of the colour of the sample *viz.,* L* (the degree of lightness), a* (redness(+)/greenness (-)) and b* (yellowness (+) or blueness (-)).

Ultraviolet absorption spectra of the samples (2 mg ml^{-1}) were monitored by employing UV-VIS spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). The wavelength in the range of 200 to 330 nm was taken and a scan speed of 120 nm per minute was set. UV absorption spectra of the samples were obtained by plotting absorbance against the wavelength (Elavarasan & Shamasundar, 2016). Samples were dissolved in double distilled water to a concentration of 80 mg ml^{-1}, filtered and the absorbance of filtrate was spectrophotometrically (Lambda 25 UV/ Vis, Perkin Elmer Life and Analytical Sciences,

Singapore) measured at 420 nm to determine the browning intensity of the samples (Elavarasan & Shamasundar, 2016).

Protein sample (10 mg ml^{-1}) in distilled water at neutral pH was vortexed for 30 min at room temperature and centrifuged at 7500 g for 15 min. Protein contents in the supernatant were determined by Kjeldahl method and protein solubility was calculated as follows (Morr et al., 1985).

Protein Solubility (%) =

$$\frac{\text{Total Protein content in Supernatant}}{\text{Total Protein content in Sample}} \times 100$$

For determining the oil absorption capacity, about 0.5 g hydrolysate sample was thoroughly mixed with 10 ml sunflower oil in a pre-weighed centrifuge tube, vortexed (Expo Hitech, India) for 30 sec and centrifuged (K-24A, Remi Instruments, Mumbai) at 2800 g for 25 min (Shahidi et al., 1995). The supernatant was drained off at 45° immediately and the centrifuge tube weighed again and calculated as weight of oil held per gram of the sample (g oil/g sample).

Determination of foaming capacity and stability of protein hydrolysates were done as per the methodology of Sathe & Salunkhe (1981). A known volume of protein solution (0.5%) was homogenized (230 VAC T-25 digital Ultra-turrax, IKA, India) @ 16,000 rpm for 2 min under ambient conditions, to entrap air. The total volume of the whipped sample immediately and after 3 min was noted and the foaming properties were calculated according to the following equation:

Foaming capacity/stability % = $(V_t - V_0)/ V_0 \times 100$

where V_t is the volume after whipping for foaming capacity and volume after standing (foam stability), V_0 is the initial volume prior to whipping.

For evaluating emulsifying properties, 10 ml vegetable oil and 30 ml of 1% protein hydrolysate solution were thoroughly homogenized (230 VAC T-25 digital Ultra-turrax, IKA, India) for 1 min @ 20,000 rpm and an aliquot (50 µl) of the emulsion taken from the container bottom immediately (0 min) and after 10 min were mixed with sodium dodecyl sulphate (0.1%, 5 ml). The absorbance of the solution was measured at 500 nm (Lambda 25 UV/ Vis, Perkin Elmer Life and Analytical Sciences, Singapore) immediately (A_0) and 10 min (A_{10}) and

emulsifying properties were determined (Pearce & Kinsella, 1978).

EAI (Emulsifying Activity Index) (m^2/g) =

$$\frac{2 \times 2.303 \times A_0}{0.25 \times \text{wt of protein}}$$

ESI (Emulsion Stability Index) (min) = $\dfrac{A_{10} \times \Delta t}{\Delta A}$

The acceptability level of the hydrolysate was measured using nine point hedonic scale as per the methodology by Normah et al. (2013). For 100 g of plain cooked rawa porridge which was used as the carrier, 20 ml of 1% (w/v) hydrolysate solutions were mixed and panellists evaluated the color, odour, taste and overall acceptability of the same. The degree of bitterness of hydrolysate was evaluated by 10 point scale (01: no bitterness to 10 indicating extreme bitterness), adopting the modified methodology of Nilsang et al. (2005). Standard solution prepared from caffeine was used as the reference for anchoring the scale of bitterness.

Protein hydrolysate solution (2.0 mg ml^{-1}) was mixed with equal volume of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was then vortexed for 10 min and kept in the dark for 30 min. The absorbance of the resultant solution was recorded at 517 nm (Lambda 25 UV/V is, Perkin Elmer Life and Analytical Sciences, Singapore). Sample solution and ethanol (1:1) (v/v) was used as sample blank and DPPH together with distilled water (1:1) (v/v) was used as control. The DPPH scavenging activity was calculated using the following equation (Shimada et al., 1992):

DPPH (%) =

$$\frac{(\text{Abs control} - (\text{Abs sample} - \text{Abs sample blank}) \times 100}{\text{Abs control}}$$

Reducing power of the samples were determined following the methodology of Oyaiza (1986). About 0.5 ml of 1% protein solution mixed with 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide (2.5 ml each) was incubated at 50°C for 20 min. To this mixture, an aliquot (2.5 ml) of 10% TCA was added and centrifuged @ 3000 rpm (K-24A, Remi Instruments, Mumbai) for 10 min. An aliquot of the resultant solution (2.5 ml) was mixed with equal volume of distilled water and 0.1% ferric chloride and absorbance read at 700 nm (Lambda

25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore).

The method of Decker & Welch (1990) was followed for determining the metal chelating activity where one ml of protein solution (80 mg ml^{-1}) was dispersed in 3.7 ml of distilled water to which 2 mM FeCl$_2$ (0.1 ml) and 5 mM 3-(2-pyridyl)-5, 6- bis (4-phenyl-sulfonic acid)-1, 2, 4-triazine (ferrozine) (0.2 ml) was added and incubated for 20 min under ambient conditions. The absorbance was read at 562 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore).

One gram of sample was aseptically taken and transferred to 9 ml of Butterfields phosphate buffer (BPBS) and properly mixed by vortexing. This mixture was serially diluted and one ml from each dilution was transferred into sterile empty petriplates and 25 ml of molten plate count agar was poured over the plate and firmly rotated for uniform distribution of sample. Further the plates were incubated at 37°C for 48 h for aerobic plate count enumeration (USFDA, 2001).

The data obtained was analysed in triplicate and subjected to analysis of variance (ANOVA). The difference in means was evaluated by Duncan's multiple range test. SPSS statistic programme (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL,) was used for statistical interpretation.

Results and Discussion

Determination of proximate composition of tuna meats and their respective protein hydrolysates (TPH) revealed an increase in protein from 26.34±0.79% to 78.01±1.09% for tuna white meat and its hydrolysate and 28.34±1.63 to 75.17±1.55% for tuna red meat and its hydrolysate (Table 1). This is in agreement to the results observed by several authors who reported a protein content in similar range in fish protein hydrolysates (Ovissipour et al., 2009; Motamedzadegan et al., 2010). Hydrolysis facilitates solubilisation of proteins as well as removal of insoluble undigested non-protein substances yielding high protein content in hydrolysates (Thiansilakul et al., 2007). As the hydrolysates were spray dried they indicated moisture in the range of 12-13% which facilitates better storage stability to the samples. The fat content in the hydrolysates were about 4% whereas ash content was higher for TRPH compared to TWPH which might be due to the presence of higher minerals in red meat than white meat (Table 1). Reports suggest high ash content to be a drawback of protein hydrolysate, from the food application point of view which can be reduced by pre-treatment of raw material prior to hydrolysis (Slizyte et al., 2005; Picot et al., 2006).

DH, defined as the percentage of peptide bonds cleaved, is commonly used as a monitoring parameter for proteolysis and it facilitates the comparative evaluation among different protein hydrolysates with regard to the properties they exhibit (Guérard et al., 2002; Slizyte et al., 2005). Under the same hydrolysis condition a significantly different ($p < 0.05$) DH of 22.08 ± 0.54 % and 29.87± 0.59% was observed for TRPH and TWPH, respectively after 60 min hydrolysis time. This difference in DH may be on account of the variations in raw material composition. However, properties exhibited by the hydrolysate can't be explained with respect to the extent of hydrolysis as the source of raw material itself used were different which also have influence on the resultant hydrolysate properties.

Protein recovery is one of the fundamental parameter deciding the efficiency of the hydrolysis process. In the present investigation, a protein recovery of 42.14 and 36.87% was observed for TWPH and TRPH, respectively from their parent

Table 1. Proximate composition of white meat and red meat of *Euthynnus affinis* and their respective hydrolysates

	White Meat	Red Meat	TWPH	TRPH
Moisture	74.54 ± 0.46[a]	72.94 ± 0.23[b]	11.96 ± 0.12[a]	12.95 ± 0.14[b]
Protein	26.34 ± 0.79[a]	28.34 ± 1.63[a]	78.01 ± 1.09[a]	75.17 ± 1.55[a]
Fat	1.10 ± 0.07[a]	1.11 ± 0.04[a]	4.28 ± 0.49[a]	4.05 ± 0.48[a]
Ash	1.12 ± 0.02[a]	1.34 ± 0.10[b]	6.31 ± 0.80[a]	7.32 ± 0.61[a]

Values are expressed as Mean ± SD; n = 3. Different superscripts for groups in the same row indicates significant difference ($p < 0.05$)

protein sources. Recovery of protein from substrate to the final product is influenced by the degree of hydrolysis with more peptide bonds being cleaved resulting in low molecular weight protein hydrolysates which are more water soluble thereby increasing the protein recovery in hydrolysate solution (He et al., 2013). In the present study also, TWPH exhibited more DH which coincided with the extraction of more protein from raw material to the hydrolysate solution. Pacheco-Aguilar et al. (2008) reported an increase in protein recovery from 48.6% at 10% DH to 67.8% at 20% DH in Pacific whiting hydrolysates.
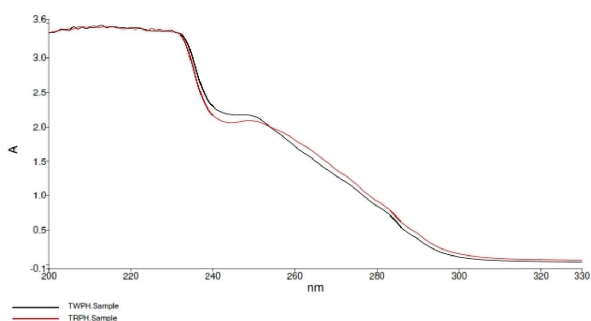
Fig. 1. Ultra Violet Absorption spectra of TWPH and TRPH

Product yield is a major criterion which determines the economic viability of a process. In the present study, the yield obtained for spray dried TWPH and TRPH were 2.18 and 3.74%, respectively. Similar observations were reported by Parvathy et al. (2016) in protein hydrolysates from yellowfin tuna waste with yield of about 3.9%. The lower yields obtained in the study may be because only the soluble fraction was spray–dried. Further losses also occurred as a result of small batch drying, particularly in the high capacity spray drier. However, when the hydrolysate solution was oven dried, a higher yield of 8.89% for TRPH and 8.93% for TWPH was observed in comparison to spray drying as losses were negligible during oven drying.

Proteolytic activity, measured as the amount of tyrosine liberated is indicative of the extent of hydrolysis. It was found to be in direct proportion with the DH values with a significantly higher (p<0.05) proteolytic activity for TWPH (0.215± 0.015) than TRPH (0.179±0.008). Similar observations of increased proteolyic activity with the progress of hydrolysis were reported by Gajanan (2014) in threadfin bream frame waste hydrolysate.

Fig. 2. Functional properties a. Protein solubility, b. Oil Absorption Capacity, c. Foaming properties, d. Emulsifying properties and e. Sensory properties of TWPH and TRPH

Factors *viz.,* composition of the raw material, the hydrolysis conditions and the drying method adopted influences the colour of the derived product. In the present study, colour analysis revealed darker colour for TRPH than TWPH. TWPH had an L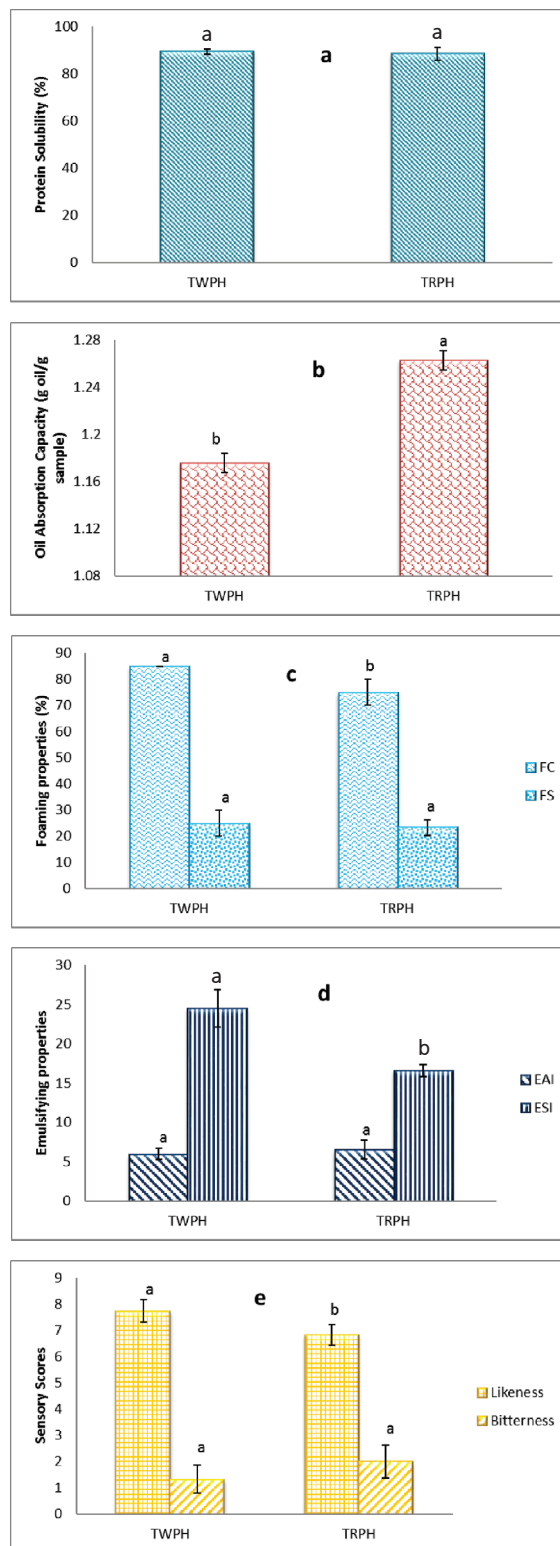, a*, b* value of 92.56±0.10, -1.52±0.14 and 15.34±0.08, respectively while it was 83.14±0.11, 2.88±0.10, and 29.86±0.24, respectively for TRPH. a* values indicated TRPH to have redness and whereas greenness was prominent in TWPH. b* values revealed more yellowness for TRPH compared to TWPH. These colour variations in hydrolysate was due to the compositional variation of the raw material used as reported by Parvathy et al. (2016).

UV spectrophotometry is a qualitative analysis of protein wherein enzymatic hydrolysis of protein results to have a prominent effect on hydrolysate spectra. The UV absorption spectra of TWPH and TRPH samples are depicted in Fig. 1. Both the hydrolysates revealed UV spectral pattern identical in nature with absorbance maxima at 200- 230 nm. A rapid decrease in absorbance from 230-240 nm was observed while the decrease was gradual from 240–300 nm. Reports by Elavarasan & Shamasundar (2016) mention that in peptide bonds, light absorption in the wavelength ranging between 180–230 nm is noticed, while in the case of aromatic side chains of Phenyl alanine, Tyrosine and Trytophan it ranges from 270–280 nm. The UV absorption spectra of hydrolysate samples indicate the absence of aromatic side chains as well as point out that both red meat as well as white meat compositional variations have not altered the spectral properties of the resultant hydrolysate.

Hydrolysates derived from tuna red meat had significantly higher (p<0.05) browning intensity than their counterpart. The TRPH indicated an index of 0.200±0.002 while that of TWPH was 0.045±0.001. This must be on account of the higher pigments present in the red meat used for deriving their respective hydrolysate. This was substantiated with the higher redness and yellowness observed in the colour values of TRPH solution compared to TWPH.

Solubility is the amount of protein that goes into the solution under specified conditions and it is regarded as the most important functional property as many of the other functional properties like emulsifying and foaming properties are influenced by this parameter (Halim et al., 2016). Intact fish myofibrillar proteins lack the solubility in water over a wide range of pH whereas enzymatic hydrolysis facilitates enhancement of this property. The present study revealed a fairly high protein solubility of 89.45±1.05% and 88.48±2.61%, respectively for TWPH and TRPH (Fig. 2a). Studies by Kristinsson & Rasco (2000) have suggested a higher extent of hydrolysis resulting in protein solutions of smaller molecular weights and higher solubility which is hypothesized to be due to an increased exposure of hydrophilic polar groups on hydrolysis.

The capacity of hydrolysate to absorb fat / oil is an important attribute that influences the taste of the product and thereby its food applicability. OAC is related to surface hydrophobicity and protein hydrolysates develop this hydrophobicity on account of the hydrolysis cleaving the protein chain thereby exposing more internal hydrophobic groups (Kristinsson & Rasco, 2000). In the present study, OAC was observed to be 1.176±0.008 g/g and 1.263±0.008 g/g, respectively for TWPH and TRPH (p<0.05) (Fig. 2b). Sanchez-Zapata et al. (2011) reported a high OAC exhibited by the dark meat of yellowfin tuna attributed to the high bulk density of protein as well as suggested that the presence of high phospholipids in meat to be linked to higher fat absorption. Reports indicate OAC of protein hydrolysates from fish ranging between 1.0 to 10.8 ml g^{-1} based on the hydrolysis conditions (He et al., 2013; Halim et al., 2016).

Foaming properties *viz.,* foaming capacity and foam stability are the physicochemical characteristics of proteins to form and stabilise foams. Compared to parent protein, on hydrolysis, the resultant molecule exposes more of hydrophobic residues thus improving the foaming properties. Foaming capacity of TWPH and TRPH were 85±0% and 75±5%, respectively indicating significant difference (p<0.05) whereas foam stability after 3 min was observed to be 25±5% and 23.33±2.89%, respectively (Fig. 2c). The variations with respect to the foaming properties in the present study must be due to the variations exhibited in the raw material composition, especially the type of protein (Sanchez-Zapata et al., 2011) used for hydrolysate preparation. Galla et al. (2012) reported a foaming capacity of 88% and foam stability of 64% in roe hydrolysate from *Channa striatus*. A higher activity with 130% and 90% foaming capacity and stability, respectively was reported by Elavarasan et al. (2014) in Catla hydrolysate.

Emulsifiers are able to form a protective coating around the oil droplets leading to prevention of coalescence phenomenon (Kasapis et al., 2009). The emulsifying properties of FPH are directly connected to their surface properties. The superior amphiphilic nature of protein solution assists in effective adsorption by proper orientation at the oil–water interface. Emulsifying properties *viz.,* emulsifying activity index and emulsion stability index were observed to be 5.94 ±0.73 m^2/g and 24.51 ±9.39 min, respectively for TWPH and 6.52 ±1.21 m^2/g and 16.57± 4.75 min, respectively for TRPH (Fig. 2d). From the results though EAI were in similar range, ESI between the samples indicated significant difference (p<0.05) which indicated that even though TRPH and TWPH assembled in similar efficiency at the interface, stabilization of the oil droplet to a higher extent was observed for TWPH than TRPH. This must be the influence of amount and type of protein and amino acid in substrate on the property of the derived hydrolysate (Hulting and Kelleher, 2000; Sanchez-Zapata et al., 2011). However Jemil et al. (2014) reported a higher EAI and ESI of 47.58 m^2/g and 47.75 min in hydrolysate from Sardinella by fermentation.

For effective application of a product in food system, enhanced functional properties together with sensory acceptability is of prime importance. Reports suggest that enzymatic hydrolysis of protein develops desirable functional properties but also has the limitation of bitterness generation thus becoming a major obstruction regarding utilization and commercialization of bioactive protein hydrolysates (Kim & Wijesekara, 2010). Though a number of reasons are suggested for the bitterness generation, it is generally established that hydrophobic amino acids of peptides are a major cause for this. The degree of bitterness varies with the raw material used, type of enzymes, hydrolytic conditions etc. Present study indicated no bitterness (1.33±0.52) in TWPH and very slight bitterness (2±0.63) in TRPH when incorporated in porridge at 0.2%, which was not significant (Fig. 2e). Similarly the acceptability studies were influenced by the source of hydrolysate as indicated by a score of 7.75 ± 0.42 and 6.83±0.41, respectively in TWPH and TRPH (p<0.05).

The chemical composition as well as physical properties of peptides assists their role to be potential antioxidants. DPPH radical-scavenging activity is generally used to determine hydrogen-donating ability of protein hydrolysates which

facilitates termination of the radical chain reaction (Nalinanon et al., 2011; Yarnpakdee et al., 2015). DPPH of 0.2% protein hydrolysate solutions differed significantly (p<0.05) with a value of 81.5 ±0.63 % and 66.24 ±2.42 %, respectively for TRPH and TWPH (Fig. 3a). Observations by Gajanan et al. (2016) on DPPH radical scavenging activity of hydrolysates derived from frame meat of threadfin bream indicated an increase in the activity with DH from 5 to 15%. In studies associated with muscle protein hydrolysate derived from giant squid, Rajapakse et al. (2005) mentioned high DPPH or other radical scavenging activities to be usually associated with hydrophobicity of aminoacids. The results from the present study suggest the applicability of hydrolysates as electron donors capable of reacting with free radicals to convert them to more
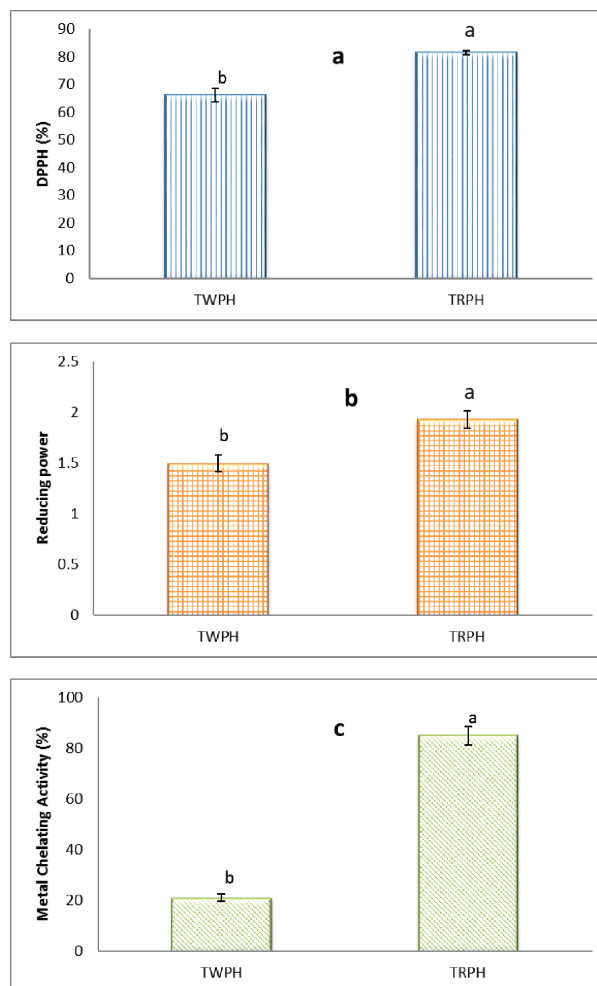

Fig. 3. Antioxidative properties a. DPPH radical scavenging activity, b. Reducing power and c. Metal chelating activity of TWPH and TRPH

stable products and terminate the radical chain reaction. Further the results revealed more antioxidant potential for TRPH compared to TWPH, though it can't be related to the extent of hydrolysis undergone by the sample due to raw material compositional variations.

Reducing power is a measure of the iron-reducing capacity and samples with higher reducing power have better abilities to donate electron and free radicals to form stable substances, thereby interrupting the free radical chain reactions (Daud et al., 2015). The reducing power of 1% protein solution (Fig. 3b) was observed as 1.929±0.086 and 1.497±0.086, respectively for TRPH and TWPH with significant difference ($p<0.05$). Liu et al. (2016) reported that enzymatic hydrolysis results in a mixture of peptides which could be responsible for the different range of antioxidant capacity.

Peptides have the ability to reduce lipid oxidation in foods on account of their property to chelate prooxidants. In foods, transition metals like Fe, Cu, Co influences the autoxidation rate as well as the breakdown of hydroperoxide to volatile compounds. Chelation of these metal ions by antioxidative peptide helps in retarding the oxidation process (Klompong et al., 2007). Metal chelating activity of the hydrolysates revealed significantly higher activity ($p<0.05$) for TRPH than TWPH with 85±3.6% and 21.04±1.4%, respectively (Fig. 3c).

The microbiological stability of tuna meat as well as their respective hydrolysates revealed a log total plate count of 5.56 and 5.3 for tuna white and red meat, respectively. However the log TPC count of TWPH was 3.81 and that of TRPH were 4.45. Similar to the findings in the present study, reports indicated a lower bacterial count for the hydrolysed protein powder compared to the raw material which is suggested to be on account of the drying adopted by the application of heat which might have destroyed some bacteria which are susceptible to high temperature (Utomo et al., 2014).

Effective utilization of fishery waste generated enormously from fish processing industry by recovering in the form of hydrolysates by enzyme application can satisfy numerous food and pharmaceutical applications. The present study indicated the nutritional composition of tuna red meat comparable to that of white meat with abundance in recoverable proteins. Assessment of the properties also indicated the potential of tuna red meat

hydrolysate for its application in food and pharmaceutical sector. Further detailed studies are required on the extent of variations in the properties exhibited with respect to white and red meat of tuna as it is influenced by intrinsic as well as extrinsic factors.

Acknowledgements

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