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# Antioxidative properties and hydrolysis profile of Skipjack tuna dark muscle and skin

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**Abstract**

Alcalase and Flavourzyme hydrolysates from Skipjack tuna *(Katsuwonus pelamis)*, dark muscle and skin, were studied for their antioxidative properties. Time-dependent changes in degree of hydrolysis (DH) and nitrogen recovery (NR) were found to be linearly increase with enzyme concentration ($R^2$>0.98). Ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity were found to increase with increasing enzyme (Alcalase and Flavourzyme) hydrolysis; hydrolysates of a same DH were different in antioxidant properties. DPPH scavenging capacity showed strongest dependence on DH for dark muscle-Alcalase hydrolysates (DAL) reaching up to 70% scavenging capacity. Skin-Alcalase hydrolysates (SAL) were characteristically in stronger scavenging activities in an intermediate DH depending on enzyme concentration. $IC_{50}$ values (DPPH) for dark muscle hydrolysates were 2 times lower than $IC_{50}$ for skin hydrolysates. Reducing power (FRAP) of hydrolysate peptides increased with hydrolysis time and then slightly decrease. Dark muscle tuna and skin hydrolysates potentially could be further developed into functional food ingredients with biological activities which remain to be further investigated.

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## Introduction

Utilization of fish by-products for value-added purposes is vital for business of tuna industry. Thailand is known as the world's largest exporter of canned tuna with economic values approximately two billion US dollars (Foreign Trade Statistics of Thailand, 2013). Among tuna species, Skipjack *(Katsuwonus pelamis)* is a commercially important species used (Nalinanon *et al*., 2010). In a canning process, a large amount of by-products consisted of skin, bone, and viscera are generated in the amount as much as 70% solid waste of the original fish materials (Arason, 2003). To find more use, these by-products could be converted into valuable fish hydrolysate using protease hydrolysis (Chalamaiah *et al*., 2012). Several commercial proteases are used, such as papain, pepsin, trypsin, α-chymotrypsin, Alcalase, Neutase, Protamax, Flavourzyme (Je *et al*., 2009; Alemán *et al*., 2011; Nazeer and Kulandai, 2012). Alcalase and Flavourzyme have emerged to have shown excellent potential for to make highly functional fish protein hydrolysates with relatively low bitter taste (Wasswa *et al*., 2007; Pacheco-Aguilar *et al*., 2008). By controlling the degree of hydrolysis, various but unique peptides and amino acid combination can be created resulting in a wide range of functional properties including antioxidant and other potential health-related functions.

Degree of hydrolysis (DH) is defined as the proportion of peptides bond cleaved during proteolysis to the total peptide bond available in the protein structure. It is an empirical indicator that is most commonly used to monitor the progress of a hydrolytic process. However, industrial control of hydrolysis using temperature, time and enzymatic concentration, often needs further characterization and an ability to determine other parameters such as nitrogen recovery (yield) and DH. These can be further useful to understand and to control a process in order to achieve certain functionality.

Functional properties that can be of economic value is antioxidant properties. Several fish hydrolysate studies have demonstrated that fish by-products are good candidates for making value-added antioxidants. Some examples are protein hydrolysates from Yellowfin sole (Jun *et al*., 2004), cobia skin (Yang *et al*., 2008), tuna liver (Je *et al*., 2009), sardinelle by-products (Bougatef *et al*., 2010), marine skin gelatins (Alemán *et al*., 2011) and muscle and skin of giant kingfish (Nazeer and Kulandai, 2012). Oxidation is a natural process occurring in living tissues coupling with metabolism which always creates free radicals that in living systems

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need to be stabilized in order to prevent triggering an imbalance resulting in various aging diseases such as cardiovascular disease, diabetes mellitus, cancer and Alzheimer's disease (Braca *et al.*, 2002; Maxwell, 1995). Such is known in generic term as oxidative stress which can also be created from various inherent (e.g., genetics) and environmental factors (e.g., pollution, UV exposure of skin, and contamination of heavy metals in water and foods) or unhealthy behaviors, such as smoking and over eating. Production of free radicals generated from an oxidation process is believed to be counteracted by antioxidants and used in health products.

Antioxidant can deactivate free radicals by two mechanisms, hydrogen atom transfer reaction and electron transfer reaction (Prior *et al*., 2005). Anti-oxidative mechanism of marine-derived peptides has been postulated to be based on their metal ion chelation activity, free radical scavenging capacity, and lipid peroxidation inhibiting activity (Harnedy and FitzGerald, 2012). Peptides with anti-oxidative properties often contain hydrophobic amino acid residues of Val or Leu at the N-terminus of the peptides, and Pro, His, Tyr, Trp, Met, and Cys in their sequences (Samaranayaka and Li-Chan, 2011). In addition, Some amino acids such as Leu, Lys, Met, Tyr, Ile, His, Val, Hyp, Pro, Ala and Cys and Trp, have been reported for the relatively strong reducing power and other antioxidative properties of hydrolysates (Samaranyaka and Li-Chan, 2011; Najafian and Babji, 2012; Dong *et al.*, 2008; Ren *et al*., 2008; Wang *et al.*, 2007; Cumby *et al*., 2008; Jun et al., 2004; Ranathaunga *et al*., 2006). Also important are the position that amino acids occupy in the sequence (Bernardini *et al.*, 2011). For instance, tri-peptides with Trp and Tyr at their C-terminus exhibited strong radical-scavenging activity, and different combinations of amino acids in tri-peptide chains also exhibited different antioxidant activity (Cumby *et al*., 2008). James and Kumar (2013) has reported the major amino acids in red meat of Skipjack tuna are glutamate (12.841%), aspartate (10,257%), leucine (9.947%), alanine (8.179%), histidine (7.940%), glycine (7.430%) and serine (6.927%).

In this work, protein hydrolysates from dark muscle and skin of Skipjack tuna will be made with Alcalase and Flavourzyme in order to investigate hydrolysis predictive and controlling factors of the degree of hydrolysis and development of peptides with anti-oxidative properties.

## Materials and Methods

*Chemicals and reagents*

Alcalase® 2.4L (declared activity of 2.4 AU/kg, density of 1.18 g/ml), an endoproteinase from Bacillus licheniformis, and Flavourzyme (declared activity of 1000 LAPU/kg, density of 1.27 g/ml) an exoproteinase from Aspergillus oryzae were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), Leucine, α-tocopherol, Trolox, L-Ascorbic acid, 2,4,6-trinitrobenzenesulfonic acid (TNBS), and 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH), were purchased from Sigma-Aldrich, Inc. (St. Louis, MO., USA). All other chemicals used in the experiments were of analytical grade.

*Samples*

Dark muscle and skin by-products of Skipjack tuna from tuna canning process was kindly donated by Songkla Canning Public Co., Ltd. (Songkhla, Thailand). Whole tuna was cooked by steaming at 100°C for 30 min after which skin was scraped off by hand. Dark muscle was then separated from the steamed meat. These two fractions were packed in polyethylene bags and then transferred on ice to university laboratory within 1 h. Upon arrival, foreign materials such as bone, scale, etc. were removed manually and then vacuum-sealed and stored at -20°C until use. Sample collection was done during March –June of 2013.

*Preparation protein hydrolysates*

To prepare hydrolysates, frozen tuna by-products were thawed overnight at 4°C. The thawed samples were washed twice with deionized water (55°C). The quantity of water used for washing was 1:3 (sample: water). The slurry was agitated for 5 min. Water was decanted through muslin cloth and samples were allowed to drain for 10 min. Washed samples were minced (particle size ≤ 0.5 cm) using a grinder (Moulinex Charlotte HV3, France). One hundred of the resulting ground tuna dark muscle (TDM) and tuna skin (TS) paste were continually mixed with deionized water at a ratio of 1:2 (W/V) and homogenized at a speed of 13,000 rpm for 1 min using IKA Labortechnik homogenizer (Selangor, Malaysia). The pH of homogenates was adjusted to 8.5 for Alcalase and 7.0 for Flavourzyme reaction using 6 N NaOH. The mixtures were then incubated at 55°C (Alcalase) or 50°C (Flavouzyme) for 20 min prior to enzymatic hydrolysis. The enzymatic hydrolysis was started when different amounts of enzymes at 0.5, 1, 2 and 4% w/w (protein basis) were added. Hydrolysis was carried out for 0, 30, 60, 90,

120, 180 and 240 min when the reaction was stopped by heating at 95°C for 15 min in a water bath with occasional agitation.

All samples were cooled immediately in ice and the pH's of the sample were subsequently adjusted to 7.0 using 1 M HCl (if needed). These were then filtered through muslin cloth 3 times and supernatants were collected. Total volume of collected supernatant was recorded before subjecting to nitrogen recovery analysis (see details below). The rest of supernatant were freeze-dried using Christ Delta 2-24 LSC Freeze dryer (Christ, Osterode, Germany) at 0.055 mbar for 12 hr. The freeze-dried hydrolysates were packed in amber bottles with $N_2$ gas flush and kept at -20°C for further study.

*Proximate chemical composition*

Moisture content was determined following AOAC (2000) by placing approximately 2 g of sample into a pre-weighted aluminum dish. Samples were dried in an oven at 105°C until constant weight was obtained. The total crude protein (N x 6.25) in the samples was determined using the Kjeldahl method (AOAC, 2000). Total lipid in samples was determined by Soxhlet apparatus (AOAC, 2000). Ash content was determined by charring a pre-dried sample in a crucible at 550°C until constant weight of white ash was obtained (AOAC, 2000). Total iron was also determined following method number 990.08 of AOAC (2000) with some slight modification by wet ash digestion sample with nitric acid and using ICP-OES in evaluation value of total iron.

*Degree of hydrolysis*

The degree of hydrolysis (DH) was estimated by determination of free amino acid groups by reaction with Trinitobenzene sulfonic acid (TNBS) (Adler-Nissen, 1979). Hydrolysate samples with the appropriate dilution (125 µL) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite before cooling at room temperature for 15 min. The absorbance was read at 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and α-amino group was expressed in terms of L-leucine. DH was calculated as follows:

$$DH = [(L-L_0)/(L_{max}-L_0)]\times 100$$

where $L$ is the amount of α-amino groups of hydrolysate samples. $L_0$ is the amount of α-amino groups of the unhydrolyzed samples. $L_{max}$ is the total α-amino groups of the unhydrolyzed samples obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

*Nitrogen Recovery*

Nitrogen recovery (NR), an index of recovered soluble nitrogen, was used to describe the hydrolysis yield. Total nitrogen in the soluble fraction and total nitrogen in the substrate (non-hydrolyzes sample) was determined using Kjeldahl method (AOAC, 2000). NR were calculated as follows:

$$NR(\%) = \frac{(\text{nitrogen in hydrolysate x total volume of supernatant})\times 100}{\text{Total nitrogen in substrate}}$$

*DPPH radical scavenging capacity*

The scavenging effect of hydrolysates against DPPH radicals was determined according to method described by Brand-Williams *et al.* (1995) with slight modification in order to perform it, in a microplate reader. DPPH solution (0.1 ml, 0.2 mM in 95% ethanol) was mixed with 0.1 ml of hydrolysate samples (at concentration 0.25, 0.5, 1, 2, 4 and 8 mg/ml). The reaction mixture was shaken well and incubated for 30 min at room temperature before absorbance measurement at 517 nm using a microplate reader (Synergy 2 multi-mode microplate reader, Biotek, Germamay). The test was carried out in triplicate using Trolox, ascorbic acid and α-tocopherol as positive control. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging capacity (\%)} = [1- A_{sample}/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of control reaction (containing of all reagent except the hydrolysate), and $A_{sample}$ is the absorbance of the reaction mixture with the mixture. The $IC_{50}$ value, denoting the concentration of the sample required to scavenge 50% of the DPPH free radicals, was calculated from scavenging capacity (%) versus hydrolysate concentration curves (Hira *et al*., 2013).

*Ferric reducing antioxidant power (FRAP)*

FRAP assay was according to Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \bullet 6H_2O$ solution. A working solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \bullet 6H_2O$ solution. The mixed solution was incubated at 37°C

Table 1. Proximate chemical composition of dark muscle/skin and dark muscle/skin hydrolysates hydrolysed by Alcalase and Flavourzyme at 0-240 mins at optimum conditions (see methods)

| Chemical composition | Dark muscle and Dark muscle hydrolysates | | | | | |
|---|---|---|---|---|---|---|
| | *Alcalase (2%)* | | | *Flavourzyme (2%)* | | |
| | 0 min | 60 min | 240 min | 0 min | 60 min | 240 min |
| Protein (%) | 83.30±1.91$^{a}$ | 88.96±0.36$^{b}$ | 91.62±1.44$^{b}$ | 83.30±1.91$^{a}$ | 89.14±1.47$^{b}$ | 91.96±1.09$^{b}$ |
| Lipid (%) | 8.25±0.43$^{b}$ | 2.62±0.80$^{a}$ | 2.84±1.30$^{a}$ | 8.25±0.43$^{b}$ | 3.50±1.09$^{a}$ | 3.70±0.96$^{a}$ |
| Ash (%) | 1.81±0.31$^{a}$ | 2.72±0.51$^{b}$ | 2.82±0.42$^{b}$ | 1.81±0.31$^{a}$ | 2.55±0.69$^{a}$ | 2.65±0.70$^{a}$ |
| Total Fe (mg/kg) | 1221.88.16±7.29$^{c}$ | 167.48±0.95$^{a}$ | 246.85±0.99$^{b}$ | 1221.88.16±7.29$^{c}$ | 144.45±2.66$^{a}$ | 255.18±0.45$^{b}$ |
| **Chemical composition** | **Skin and Skin hydrolysates** | | | | | |
| | *Alcalase (2%)* | | | *Flavourzyme (2%)* | | |
| | 0 min | 60 min | 240 min | 0 min | 60 min | 240 min |
| Protein (%) | 79.57±1.27$^{a}$ | 83.91±0.83$^{b}$ | 85.91±1.03$^{b}$ | 79.57±1.27$^{a}$ | 83.16±1.32$^{b}$ | 86.42±1.15$^{c}$ |
| Lipid (%) | 9.81±0.54$^{b}$ | 4.45±0.96$^{a}$ | 4.46±1.24$^{a}$ | 9.81±0.54$^{b}$ | 5.16±0.87$^{a}$ | 4.96±0.72$^{a}$ |
| Ash (%) | 8.22±0.18$^{a}$ | 8.53±1.22$^{a}$ | 8.66±1.16$^{a}$ | 8.22±0.18$^{a}$ | 8.25±0.54$^{a}$ | 8.25±0.54$^{a}$ |
| Total Fe (mg/kg) | 310.07±1.39$^{c}$ | 34.70±0.20$^{a}$ | 42.50±0.42$^{b}$ | 310.07±1.39$^{b}$ | 52.36±0.39$^{a}$ | 53.89±0.43$^{a}$ |

All data are given as mean values± S.D. in dry basis (N=3).
Different superscripts in small letters in a same row depict significantly difference among hydrolysis time in the same enzyme used (P<0.05)

for 30 min and was referred to as FRAP solution. Sample (1 mg/ml; 150 µL) was mixed with 2850 µL of FRAP solution and kept for 30 min in the dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm and compared against the standard curve which was absorbance of Trolox at 50 to 600 µM. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

*Statistical analysis*

Data were subjected to Analysis of Variance (ANOVA) and by Duncan's Multiple Range Test (Steel and Torrie, 1980). Paired comparisons were done using t-test ($p < 0.05$). The SPSS statistics program (Version 16.0) was used for data analysis.

## Results and Discussion

*Proximate chemical composition*

Hydrolysates from tuna dark muscle and skin (by Alcalase and Flavourzyme at 2% concentration) are shown in Table 1. The protein content increased with hydrolysis time in all cases with dark muscle protein being slightly higher than skin. This was due to protein solubilization during hydrolysis and the partial removal of insoluble undigested protein. Lipid contents after hydrolysis decreased ($p < 0.05$) as the lipids were separated out with insoluble fractions. The ash content in skin was higher than dark muscle but iron content was considerably higher in dark muscle due to the presence of myoglobin in dark muscle. After hydrolysis of dark muscle, the iron content in the hydrolysates from 60 min hydrolysis decreased from the control ($p < 0.05$) indicating that most of the iron was in the insoluble fractions but after 240 min hydrolysis the iron content in the soluble fraction of the hydrolysate increased in the dark muscle samples suggestion a release of free iron as the myoglobin porphyrin rings were hydrolyzed. Similar was found in the skin samples but the amount of iron was much lower than dark muscle hydrolysates.

*Prediction of DH and NR at various enzyme concentrations*

During hydrolysis, DH increased curvilinearly with incubation time (data not shown). As more soluble protein is released in the soluble fraction, NR also increased. This was found to be in similar fashion for both Alcalase and Flavourzyme hydrolyses. Tuna dark muscle and skin hydrolysis also showed enzyme concentration dependence. During the enzymatic hydrolysis, two distinctive stages could be recognized. The first stage involved a rapid rise in DH and the second stage when DH levelling off. The same also was observed for NR.

DH was also found to be linearly correlated with log enzyme concentration. The correlation coefficients of are shown in Table 2 with correlation coefficients ($r^2$) around 0.98 for dark muscle-Alcalase hydrolysate (DAL), dark muscle-Flavourzyme hydrolysate (DF), skin-Alcalase hydrolysate (SAL)

Table 2 Regression analysis between hydrolysis parameters (DH and NR) and enzyme concentration (at 60 min)

| y vs x | equation | $R^2$ |
|---|---|---|
| ***NR (%) vs log enzyme concentration (%) at 60 min*** | | |
| Dark muscle hydrolysis using Flavouzyme | y = 14.484x+67.672 | 0.989 |
| Dark muscle hydrolysis using Alcalase | y = 15.252x+56.458 | 0.998 |
| Skin using Flavouzyme | y = 23.031+60.652 | 0.984 |
| Skin using Alcalase | y = 16.359x+54.671 | 0.993 |
| ***DH (%) vs log enzyme concentration (%) at 60 min*** | | |
| Dark muscle hydrolysis using Flavouzyme | y = 20.312x+40.968 | 0.9836 |
| Dark muscle hydrolysis using Alcalase | y = 19.967x+35.36 | 0.9815 |
| Skin using Flavouzyme | y = 21.953x+24.416 | 0.985 |
| Skin using Alcalase | y =18.678x+20.069 | 0.976 |

X = enzyme concentration; y = NR or DH

and skin-Flavourzyme hydrolysate (SF). The results are in agreement with earlier reports (Guérard *et al*., 2001; Klompong *et al.*, 2007). From this relationship, the exact concentration of enzyme required to hydrolyze dark muscle and skin from Skipjack tuna to a required DH, at a given hydrolysis time (or vice versa) could be calculated. Similarly, NR linear correlation with log enzyme concentration gave $r^2 > 0.98$ (Table 2).

*DPPH radical scavenging capacity*

DPPH radical scavenging capacity of DAL, DF, SAL and SF was found to increase with hydrolysis time (data not shown) but it did not so clearly correlate with hydrolysis time as for the case of DH and NR. However, the trends showed DPPH radical scavenging capacity of each enzymatic hydrolysate to have interesting relationships with DH as can be seen in Figure 1. For the case of dark muscle, Alcalase hydrolysis resulted in a clear increase in DPPH scavenging capacity as DH increased for each enzyme concentration ($r^2$ 0.80-0.90, Figure 1a). However, Flavourzyme hydrolysis to the same extent of DH as Alcalase, showed very slight increase in DPPH scavenging activity with DH ($r^2$ 0.13-0.46, Figure 1b). For the case of skin hydrolysis, hydrolysates obtained from Alcalase hydrolysis increased in DPPH scavenging capacity with DH to a maximum at different levels (25-40% activity) depending on enzyme concentration (Figure 1c). Therefore, antioxidative peptides in this case apparently are intermediate hydrolytic products of endopeptidase. When hydrolyzed with Flavourzyme, a small increase in antioxidative scavenging property was observed ($r^2$ 0.19-0.59 Figure 1d) from 15% to 30% Flavourzyme concentration.

The highest amount of DPPH radical scavenging capacity was observed in Alcalase hydrolyzed dark muscle hydrolysate (DAL). The differences in of DPPH radical scavenging capacity observed partly due to the greater inherent scavenging property of dark muscle meat and also difference peptides developed. Hydrolysates (both from dark muscle and skin) prepared using Alcalase showed significantly higher DPPH radical scavenging capacity than did Flavourzyme hydrolysates ($p< 0.05$).

DPPH scavenging capacity was used to calculate $IC_{50}$ for all hydrolysates. In all cases, hydrolysates exhibited greater antioxidant property according to DPPH. $IC_{50}$ for hydrolysates was lower than the unhydrolyzed control and decreased with enzyme concentration and at least in some cases with hydrolysis time (Figure 2). $IC_{50}$ of all samples was found to be higher (lower in scavenging capacity) than measured $IC_{50}$ of Trolox (11.86 µg/ml), ascorbic acid (26.19 µg/ml) and α-tocopherol (14.78 µg/ml). DAL showed a significantly greater radical scavenging capacity than other hydrolysates of a same enzyme concentration and hydrolysis time. Also, dark muscle meat was a better substrate to render a higher DPPH antioxidant property than skin.

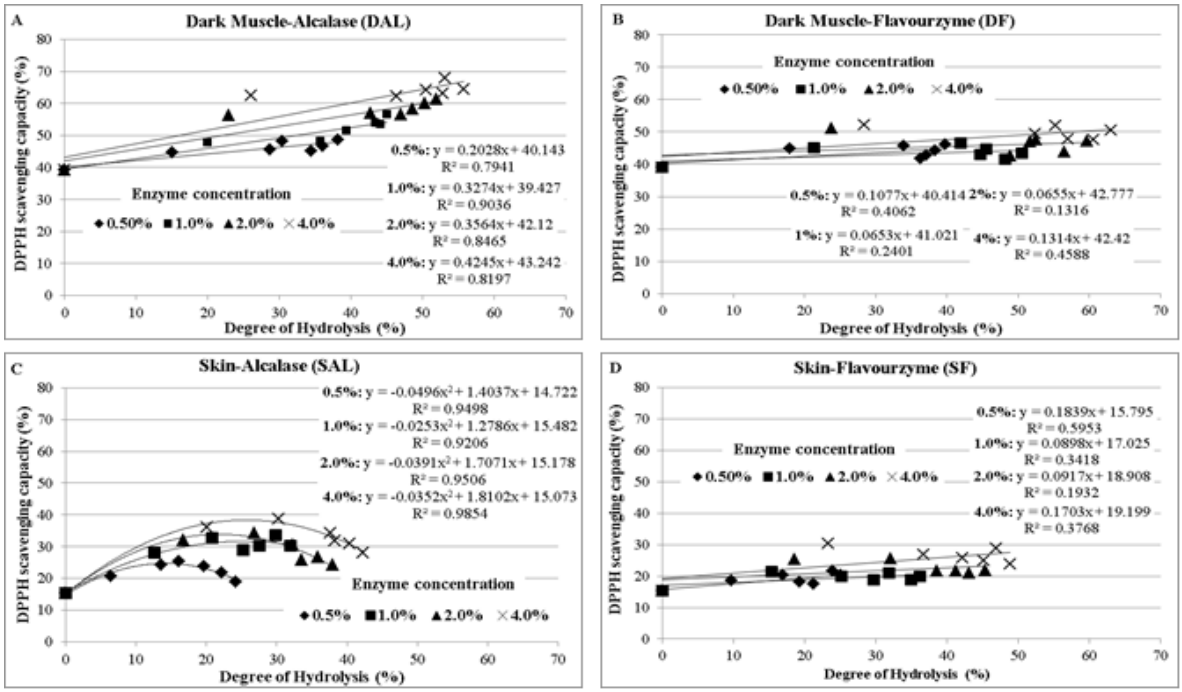


Figure 1 Relationship between DPPH scavenging capacity and degree of hydrolysis for dark muscle and skin hydrolysates (A) Dark muscle-Alcalase (DAL), (B) Dark muscle-Flavourzyme (DF), (C) Skin-Alcalase (SAL), (D) Skin-Flavourzyme (SF) at various enzyme concentrations.

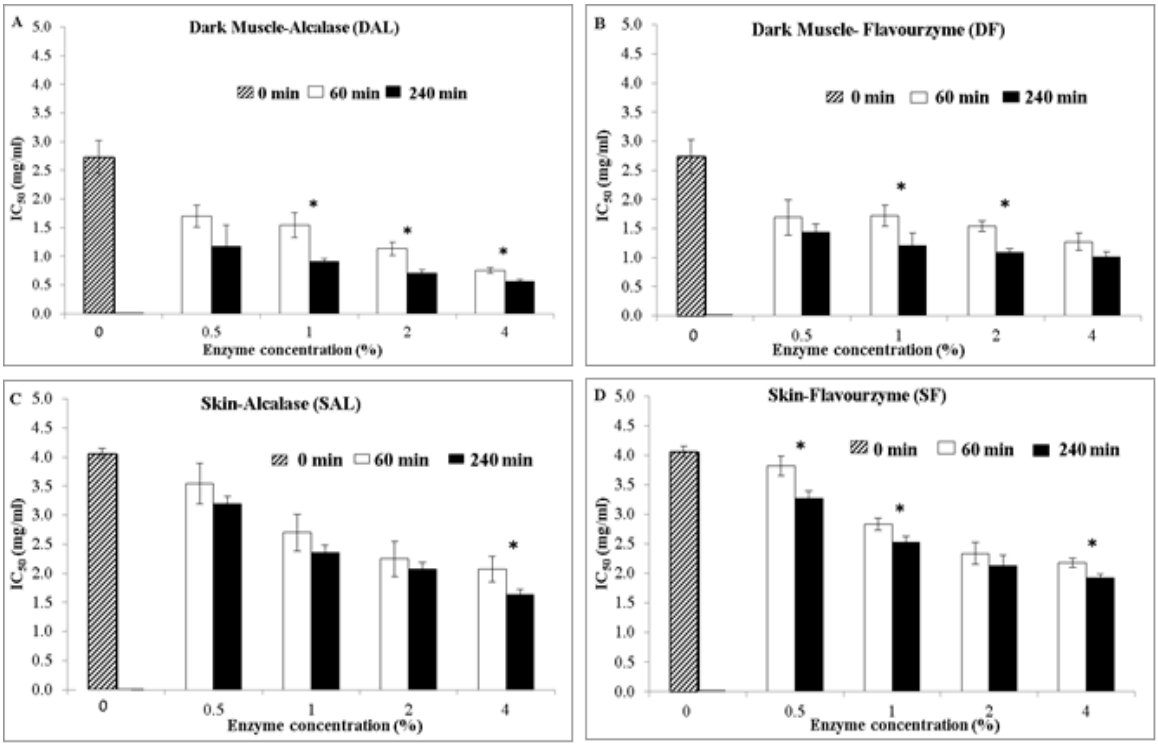


Figure 2 $IC_{50}$ (DPPH scavenging capacity) of Skipjack tuna dark muscle and skin hydrolysates (A) Dark muscle-Alcalase (DAL), (B) Dark muscle-Flavourzyme (DF), (C) Skin-Alcalase (SAL), (D) Skin-Flavourzyme (SF) at various enzyme concentrations. * depict significantly difference between hydrolysis time in the same enzyme used ($P<0.05$)

*Ferric reducing antioxidant power (FRAP)*

The reducing power by FRAP measures the ability of an antioxidant to donate an electron to Fe(III) ion (FeIII TPTZ) to Fe(II) (which has an intense 593 nm blue color at low pH). Ferric reducing power of DAL, DF, SAL and SF are shown in Figure 3. Reducing power of all hydrolysate samples increased with hydrolysis time to a maximum at 60 min for DAL and SF and at 90 - 120 min for DF and SAL. After the maxima, the reducing power decreased was observed in all hydrolysate samples. DAL showed a significantly higher ($p< 0.05$) reducing power than

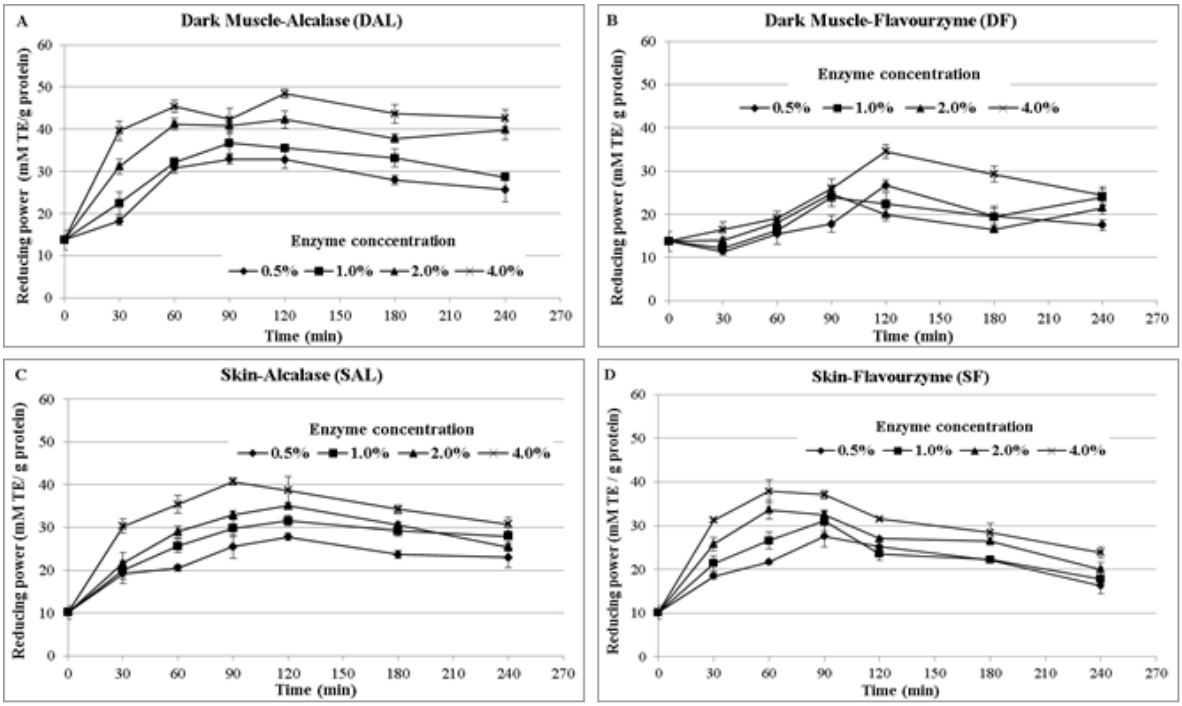


Figure 3 Ferric reducing power(FRAP) of dark muscle and skin hydrolysates (A) Dark muscle-Alcalase (DAL), (B) Dark muscle-Flavourzyme (DF), (C) Skin-Alcalase (SAL), (D) Skin-Flavourzyme (SF) at various enzyme concentrations and hydrolysis time.

other samples (Figure 3). The lowest reducing power was observed in DF. The increase and then decrease in reducing power during hydrolysis in some systems (such as Figure 3c and 3d) indicated that the reducing peptides were intermediate fractions.

The increase and then decline DPPH and FRAP data suggest some specific peptide property that are transient. Decker *et al*. (1996) discussed the possible antioxidative mechanisms for amino acids and peptides (such as carnosine) that various amino acids and peptides might exhibit antioxidant properties that varied among them in the ability to interact free radicals, lipid oxidation products, prooxidants, and lipid membranes. Although carnosine may not be present in a significant quantity in tuna, similar peptide properties could follow the same principles. A decrease in antioxidant properties after rising to a peak is not known but it could involve increased free radicals and prooxidants in cooked systems some of which might come from free iron released from myoglobin. In addition, Maillard reactions may inhibit antioxidative peptides or heat-induced disruption of lipids structure that may decrease molecular interaction between the antioxidants and the lipids (Decker *et al*., 1996).

For the case of dark muscle proteins, measuring FRAP could be prone to oxidative effect of released heme iron from myoglobin. In a FRAP assay, in order for metmoglobin-released iron to affect the reducing power, one would need to have iron (heme or non-heme) in a reduced form in order for it to affect FRAP results (i.e., Fe(II)). So Fe(II) would itself be oxidized by contributing the electron for use in the FRAP assay. It is hypothesized that the released heme iron in ferrous state would influence FRAP results. The literature has noted that Fe(II) is more pro-oxidative toward lipid than Fe(III) and, thus, a situation whereby ferrous iron in the dark muscle tissue matrix would be able to contribute to lipid oxidation or be measured by the FRAP assay – there would be a competition for the reducing equivalents by both processes.

A significant release of heme iron can be observed in the soluble portion of the hydrolysates (Table 1). However, it was also observed from PV and and TBARS analyses (data not shown) that at least the lipid phase there was an advanced lipid oxidation (PV >>50), which could be taken to mean that the heme iron was likely in the ferric Fe(III) state from oxidized metmyoglobin. Per earlier discussion, Fe(III) cannot participate and influence FRAP assay as it cannot be further oxidized by contributing the electrons. The decline in reducing power beyond a maximum (Figure 3) could possibly due oxidative effects from factors that could spontaneously promote further lipid oxidation. This could be postulated for the case of dark muscle tuna that this could involve a number of possibilities, including 1) polyunsaturated fatty acids oxidation, 2) generation of micelle or liposomes upon hydrolysis, 3) concurrent oxidation of myoglobin and 4) release of free pro-oxidants.

## Conclusion

Skipjack tuna, dark muscle and skin hydrolysis studied at various time-enzyme concentration combination produced series of positive correlation among hydrolysis parameters including degree of hydrolysis and nitrogen recovery with the log enzyme concentration. Alcalase and Flavourzyme hydrolysates yielded similar extent of hydrolysis but different patterns of the development of antioxidant properties. DPPH scavenging capacity showed strongest correlations against DH for dark muscle-Alcalase hydrolysates. Skin-Alcalase hydrolysates exhibited stronger scavenging activities in an intermediate DH. Flavourzyme resulted in lowest development of antioxidant (DPPH) ability with hydrolysis. $IC_{50}$ values (DPPH data) significantly decreased with the extent of hydrolysis (i.e., enzyme concentration and hydrolysis time) at a greater extent for the case of DAL. Dark muscle-Alcalase hydrolysates showed the greatest DPPH scavenging and reducing power properties. Similar results were also found in reducing power although heme iron released when hydrolyzing dark muscle could have affected the final reducing power. This study is presented here as a preliminary results from hydrolysis study of tuna proteins by-products. Antioxidative peptides from tuna dark muscle and skin warrant further investigation with respect to characterization and some bioactivities.

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