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Influence of Enzyme type and hydrolysis time on Antioxidant activity of hydrolyzed Protein from Longtail Tuna (*Thunnus tonggol*) dark muscle

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ABSTRACT

Aims: In this study, the antioxidant properties of hydrolyzed protein from longtail tuna dark muscle with commercial enzymes (i.e., Alcalase, Alkaline Protease, and Evatase) were investigated.

Materials & Methods: Protein hydrolysates from tuna dark muscle were prepared by different enzymes. The degree of hydrolysis (DH) was performed by the TCA technique. The five aliquots at 60, 180, 240, 300, and 360 min were gathered during hydrolysis. The antioxidant activity of aliquots was monitored by in vitro assays (DPPH inhibition ability and Ferric (Fe^{3+}) reducing power).

Findings: The antioxidant activities of protein hydrolysate from tuna dark muscle (TDM) increase with increasing time and DH. Alcalase hydrolyzed protein (AHP) generally showed higher antioxidative activity than evatase hydrolyzed protein (EHP) and alkaline protease hydrolyzed protein (APHP). Among the samples (concentration 3 $mg.ml^{-1}$), AHP at 360 min significantly exhibited the highest ability to scavenge DPPH radical (72.6 %). Furthermore, AHP and APHP significantly showed a minimum IC50 value of 1.1 $mg.ml^{-1}$ at 240 and 360 min hydrolysis. APHP significantly exhibited the highest ferric reducing power of 0.83 at 300 min and 0.76 at 240 min. AHP and APHP significantly showed the highest ferric reducing power of 0.74 at 360 min ($p < 0.05$).

Conclusion: This study confirmed that protein hydrolysate from TDM could be a good source of antioxidant peptides. In addition, the antioxidant activity of hydrolyzed protein relies on protease type and hydrolysis condition.

Keywords: Antioxidant; Enzyme; Dark muscle; Protein hydrolysate; Tuna fish.

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Introduction

The amount of tuna catch is about 30% of the total fishery production in Iran and 45% of the fishery in the southern waters. The annual commercial landings of tuna is 174,234 tons, which around 46% (80883 tons) of them include longtail tuna. Hormozgan province, with an average catch of 19,801 tons, accounted for about 64% of longtail tuna catch [1–3]. Longtail tuna is a commercial tuna species in the Persian Gulf and it is commonly used in canning and fish processing. Tuna fishing and processing leaves more than 60% of by-products, of which dark muscle is 10-13%[4]. Since dark muscle has unpleasant smell and high oxidation, it is limitedly used in the seafood industry. Dark muscle is mainly turned into low-grade products, such as fishmeal and fertilizer [5]. However, bioactive peptides could be recovered from seafood protein in different methods, including enzymatic, fermentation and organic solvents [6–8]. Enzymatic hydrolysis has been used as an appropriate technique to produce protein hydrolysate with short-chain peptides (2-50 amino acid residues) [9].

The functional properties of protein hydrolysate depend on the source of protein, hydrolysis time, degree of hydrolysis and enzyme types. Since the hydrolyzed protein obtained from fish by-products have health-promoting and nutritional functions, including antioxidative, antihypertensive, etc. hey have attracted the attention of pharmaceutical and food industries [10–13]. Recently, TDM has been used to obtain bioactive peptides by hydrolysis [14–16].

Oxidative damage is caused by a dis-balance between oxidants and antioxidants in cells and, ultimately, in tissue. These damages have a crucial role in developing inflammation, chronic diseases and cancer [17]. Research has demonstrated that using antioxidant peptides from natural sources could decrease oxidative stress risks. [18]. Recently, researchers have shown the antioxidant activity of protein hydrolysates obtained from different tuna species byproducts such as yellowfin dark muscle. [19], Spanish mackerel muscle [20], skipjack tuna head [21], yellowfin tuna waste [22] and dark muscle [23]. However, there are limited studies on the production of antioxidant hydrolysate from tuna byproducts in the Persian Gulf and Oman Sea.

Global fishmeal consumption is estimated to attain 183000000 tons in 2031, which shows a total growth of 24000000 tons (more than 15%) compared to the baseline period of 3 years (2019-2021) [24]. Therefore, a significant amount of nutrient-rich by-products is discarded annually and ends up in landfills and oceans. With this background, this study can suggest an enzymatic solution to convert fish by-products into new products with higher profit and a significant demand called protein hydrolysates. The proposed solution is an original recycling method using enzymatic hydrolysis that allows the production of the desired products. Considering the richness of proteins in dark muscle and the prevention of discarding in tuna fishery and canning industry, dark muscle hydrolysis using enzymes (alcalase, evatase, and alkaline protease) was performed. Then, influence of enzyme type and hydrolysis time on antioxidant activity of hydrolyzed protein was investigated.

Material & Methods

Sample

Longtail tuna were obtained from a fish market on Qeshm Island, located in the Persian Gulf. After cutting internal organs and head, dark muscle was separated from white muscle. Dark muscle was frozen at -20ºC and immediately transferred to the Biochemistry Laboratory of Tarbiat Modares University.

Preparation of dark muscle protein hydrolysate

TDM samples were hydrolyzed using alcalase (temperature 55ºC, enzyme/substrate ratio 1 %, pH 7.5) (Alcalase® EC 3.4.21.62, Novozymes, Denmark)[25], evatase (temperature 55ºC, enzyme/substrate ratio 1 %, pH 7.5) (EvaTase, liquid form, India), and alkaline protease (temperature 65ºC, enzyme/substrate ratio 1 %, pH 8) (powder form, India)[26]. Before hydrolysis, samples are finely crushed by the grinder. Afterward, 25 grams of sample were put at 85ºC temperature for deactivation of internal enzymes (serine proteases, metalloproteases and cathepsins). Then, the sample was mixed with 25 milliliters of deionized water and pH of blend was changed to the desired level (7.5 for alcalase and evatase and 8 for alkaline protease) with NaOH (1N). Enzymes were added to the samples and put in a shaking incubator (200 rpm). To stop the reaction, the samples were heated at 85ºC and obtained 60, 180, 240, 300, and 360 min after starting hydrolysis. Afterward, samples were chilled and centrifuged (6000 rpm).

Determination of DH and protein content of samples

The DH was obtained based on method performed by Hoyle and Merritt [27] with some changes. An amount of 5 milliliters of sample was totally mixed with 5 milliliters of 20% trichloroacetic acid, and the blend was centrifuged at 7000 rpm for 10 min. The DH was obtained according to Equation (1):

$$DH = \left(\frac{N_2}{N_1}\right) \times 100\ \%. \qquad \text{Eq.(1)}$$

N2 is 20% TCA soluble nitrogen, and N1 is sample soluble nitrogen.

The protein content of samples was obtained by a BCA kit, and bovine serum albumin was applied as standard protein (Figure 2).

Antioxidant assays

DPPH radical scavenging assay

The DPPH radical scavenging assay was determined based on the method of Yang et al. [28]. The sample (1 milliliter) with the concentration of 0.5, 1, 2, 3 and 5 mg.ml^{-1} was added to 1 milliliter of 0.16 mM DPPH in 96% ethanol. The blend was kept at room temperature in dark for 30 min, and the absorbance of the sample was read at 517 nm by ELISA Reader. Then, DPPH scavenging activity was obtained according to Equation (2):

$$DPPH\ inhibition\ (\%) = 1 - \left(\frac{A_{sample}}{A_{blank}}\right) \times 100. \qquad \text{Eq.(2)}$$

A_{sample} is the sample absorbance and A_{blank} is the sample absorbance without any antioxidant. The IC50 was calculated and expressed as mg sample.ml^{-1}.

Ferric reducing power (FRAP)

Reducing power was determined by the method described by Chalamaiah et al. [29]. Absorbance of the final solution was measured at 700 nm by Elisa Reader.

Statistical analysis

Statistic analyses were done with SPSS (version 16) by one-way analysis of variance. Data normality was analyzed by Kolmogorov-Smirnov test. The significant differences among the means were compared with Duncan's multiple range test. Differences were assumed significant at $p < 0.05$.

Findings

Degree of hydrolysis

As illustrated (Figure 1), DH increased linearly with increasing time up to 240 min, and then it became stable. The greatest DH at 240, 300 and 360 min was significantly obtained using alcalase (DH, 53 %) ($p < 0.05$). Hydrolyzed protein by alkaline protease significantly showed higher DH of 43.5 % at 240, 300 and 360 min ($p < 0.05$). Hydrolyzed protein by evatase significantly showed higher DH of 45 % at 300 and 360 min ($p < 0.05$).

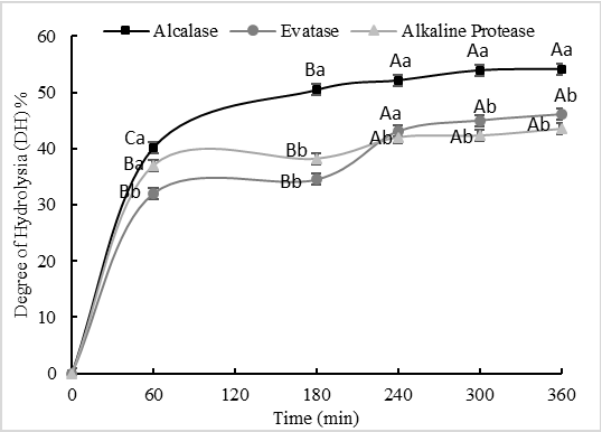

Figure 1) Samples DH treated with various enzymes at different times. Different lowercase letters represent significant differences in DH within same time. Different uppercase letters represent significant difference in DH within same enzyme ($p < 0.05$).

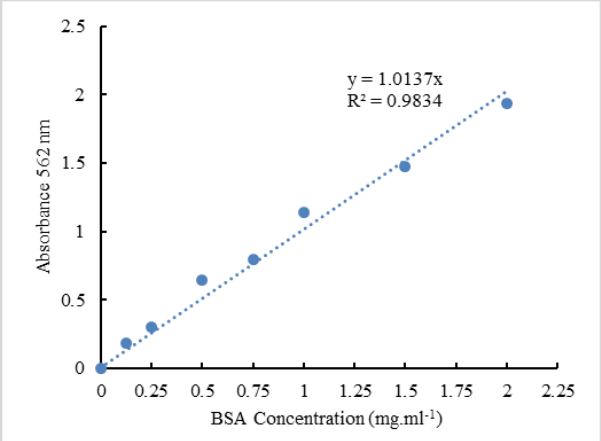

Figure 2) Bicinchoninic acid (BCA) protein calibration curve performed with BSA standards.

Antioxidant assays

DPPH scavenging activity

All studied samples showed DPPH scavenging activity. Result of DPPH assay for protein hydrolysates by three enzymes within the same hydrolysis time was compared (Figure 3). AHP at 360 min significantly showed highest DPPH inhibition among all samples. APHP significantly showed highest DPPH activity at 60 min ($p < 0.05$) (Figure 3). AHP significantly showed higher DPPH scavenging activity of 72.6 % at 360 min ($p < 0.05$). EHP significantly showed higher DPPH of 56.4 % at 360 min ($p < 0.05$).

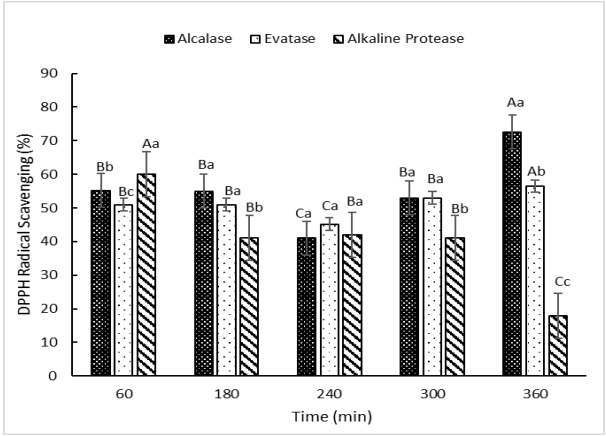

Figure 3) DPPH inhibition activities of sample at different times and same concentration. Different lowercase letters represent significant differences among different enzymes (same time). Different uppercase letters represent significant differences among different hydrolysis times (same enzyme) ($p < 0.05$).

Samples at different times were analyzed for IC50 determination. The protein hydrolysates produced with all three enzymes showed considerable DPPH scavenging activity varying over a range of IC50 from 1 - 3.4 mg.ml^{-1}. Among the samples, APH and APHP significantly exhibited a minimum IC50 of 1.1 mg.ml^{-1} at 240 min. Furthermore, APHP significantly showed a minimum IC50 value of 1.1 mg.ml^{-1} at 240, 300 and 360 min ($p < 0.05$) (Figure 4).

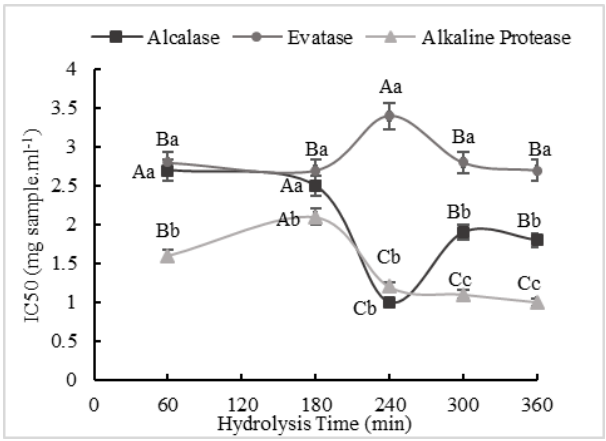

Figure 4) The IC50 values of DPPH scavenging activity of TDM hydrolysates by alcalase, evatase, and alkaline protease. Different lowercase letters within same hydrolysis time and different enzymes represent significant differences. Different uppercase letters represent differences among hydrolysis times (same enzyme) ($p < 0.05$).

Ferric reducing power

As shown (Figure 5), all samples showed good ferric reducing power. Among the samples within the same hydrolysis time, APHP significantly showed the highest ferric reducing power of 0.83 at 300 min and 0.76 at 240 min. AHP and APHP significantly showed the highest ferric reducing power of 0.74 at 360 min ($p < 0.05$).

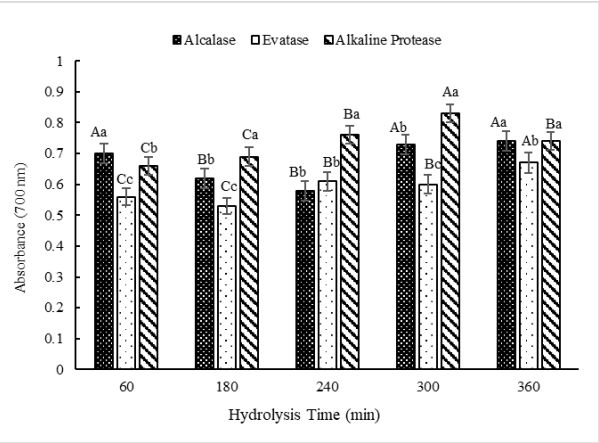

Figure 5) Ferric reducing power of TDM hydrolysates at different times. Different lowercase letters within the same hydrolysis time represent significant differences. Different uppercase letters represent significant differences among hydrolysis times (same enzyme) ($p < 0.05$).

Discussion

The global catch of commercial tuna fish in 2020 was about 4.9 million tons and its economic value at the wharf and without added value was about 11.7 billion dollars and with added value was more than 40.8 billion dollars, which is 9% of the value of the global marine catch [30]. Iran's percentage of tuna caught in the northwest Indian Ocean has improved from 5% in 1995 to more than 12% in 2003. Iran owns about 4% of commercial tuna catch in the world and about 15% catch in the Indian Ocean [2,3]. Tuna species are highly appreciated worldwide because of their high nutritive and health-promoting value. Tuna muscle consists of light and dark muscle, which dark muscle is discarded in the processing process. In the study of Abd Aziz et al.[31], the average amount of protein, lipid, ash and moisture was reported as 33.9%, 12.1%, 3.8% and 59.1%, respectively. Nevertheless, enzymatic hydrolysis can reduce the wastage of valuable dark muscle compounds and produce new marketable products for the food and pharmaceutical industries. Enzyme type has a significant effect on protein yield, DH, and features of protein hydrolysate.

DH is an important factor that is correlated to the output of the hydrolysis procedure. Based on the results, DH was high in the early hours, which indicates the greatest breakup of peptide bonds happened at 180 and 240 min of hydrolysis, then the reaction rate decreased, which indicated that the hydrolysis was in a stable phase. This steady trend after 180 and 240 minutes is likely attributed to reduce peptide bonds for reaction. Outcome is in accordance with the result of Noman et al. [32] which, demonstrated a stable trend after two hours of hydrolysis by alcalase. Guerard et al.[2] also found that DH increased with increment incubation time in yellowfin tuna by alcalase and umamizyme. Bougatef et al.[33] have observed that DH of hydrolysate using proteases is raised when the incubation time is increased. Alcalase significantly showed the highest efficiency compared to evatase and alkaline protease for the hydrolysis of TDM. This result is in agreement with the results formerly demonstrated for the enzymatic hydrolysis of tuna by-products by alcalase [34], and Silver carp by alcalase and flavourzyme [35]. However, Bougatef et al. [33] showed that DH of hydrolysate from tuna (*T. thynnus*) head with alkaline protease was higher than alcalase.

The antioxidant property of hydrolyzed proteins is affected by type of enzyme, nature of protein and process condition [36]. Due to their unique characteristics, various enzymes can produce different peptides and

free amino acids with various properties in hydrolysis [37]. DPPH assay is broadly utilized to estimate the antioxidative features of compounds. The antioxidant compound can donate a hydrogen atom to the DPPH free radical and turn the free radical solution into a reduced form with purple color. [38]α-diphenyl-β-picrylhydrazyl (DPPH. Protein hydrolysate from TDM showed DPPH scavenging activity and the results varied with different enzymes. Results revealed that DPPH inhibition ability of protein hydrolysate is affected by time and enzyme type at 3 $mg.ml^{-1}$ concentration. DPPH inhibition ability of AHP and EHP significantly decreased, from 55.2% to 41% and 51% to 45.2%, when the hydrolysis was prolonged from 60 to 240 min ($p < 0.05$) (Figure 3). The inhibitory activity improved by increasing the time up to 360 min. These fluctuations probably indicate that two simultaneous mechanisms might influence antioxidative activity. The first mechanism is production of antioxidant peptides, and the second is degradation of produced antioxidant peptides. Proteases cut polypeptide chains from different and unique cleavage positions. Therefore, protein breakdown by various proteases generates different protein hydrolysates that contain a blend of peptides and amino acids with different molecular weight. Nguyen et al. [39] reported the highest radical scavenging activity in three hours after hydrolysis and a decrease in scavenging activity after 4.5 hours, which indicated that the duration of hydrolysis affects the antioxidant activity. which indicated that the duration of hydrolysis affected the antioxidant activity. Yarnpakde et al. [40] showed that DPPH scavenging activity of hydrolyzed protein from Nile tilapia has a direct relationship with DH. In addition, Bougatef et al.[33] demonstrated that DPPH scavenging activity of hydrolyzed protein from tuna head increased as the DH increased. However, DPPH scavenging activity of APHP decreased when DH and time increased. In two other studies, the hydrolysis of scad and catfish was performed by proteases and reported that DPPH scavenging activity decreased with increasing hydrolysis time and DH [41, 42]. AHP obtained at 360 min significantly exhibited the highest DPPH inhibition activity (72.6 %) among different hydrolyzed proteins and times ($p < 0.05$*)*. Comparison of EHP at different times showed the most significant difference at 240 min (56.4%), while APHP at different times showed the most significant difference at 60 min (60%) ($p < 0.05$*)* (Figure 3). Esmaieli et al. [21] reported that DPPH inhibition ability of hydrolyzed protein from skipjack tuna was significantly increased at 240 min.

The IC50 values were determined and APHP significantly exhibited the lowest IC50 values of 1.1 $mg.ml^{-1}$ at 240, 300, and 360 minutes of hydrolysis. APH significantly exhibited a minimum IC50 value of 1 $mg.ml^{-1}$ at 240 min ($p < 0.05$). Bougatef et al.[33] found the IC50 of 0.7 $mg.ml^{-1}$ by alkaline protease and 2 $mg.ml^{-1}$ by alcalase. Mongkonkamthorn et al. [43] reported the IC50 of 0.29-1.11 $mg.ml^{-1}$ by different enzymes at 180 min of hydrolysis. Therefore, the overall decrease in IC50 value with increasing hydrolysis time indicates that hydrolysis is effectively enhances DPPH scavenging of hydrolyzed protein. The results showed that the protease type and DH are important factors in detremining the efficiency of peptides in hydrolyzed protein for hydrogen donation.

The Fe^{3+} reducing assay uses to estimate the capability of compounds for electron donation. APHP significantly showed the highest reducing power (0.83 at 300 min and 0.76 at 240 min), while the lowest value was obtained from EHP (0.53 at 180 min). Ferric reducing power results were higher than results reported for *T. obesus* head protein hydrolysate (0.4 at 340

min by alcalase) [44], *C. carpio* roe protein hydrolysate (0.63 at 180 min by alcalase) [29] and tilapia protein hydrolysate (0.629 by papain) [45]. Antioxidant peptides in protein hydrolysates could reduce Fe^{3+} to Fe^{2+} and it can be analyzed by a colorimetric reaction. Protein hydrolysate from TDM showed reducing power and the results varied with different enzymes used for hydrolysis. Various proteases produce peptides with different arrangements, designs, and sizes, relying on enzyme specificity.

The best results of DPPH and FRAP for different enzymes were different. These results could be because antioxidant assay methods have different reaction mechanisms. The DPPH method is based on electron and Hydrogen transfer, while FRAP method is based on electron transfer. Electron transfer methods are based on the measurement of antioxidant capacity to reduce oxidants [46]. Alcalase and alkaline protease hydrolyzed protein showed better antioxidant activities (scavenging and reducing activity) than evatase. Since alkaline protease is cheaper than alcalase, it can be used as a suitable enzyme for enzymatic hydrolysis of tuna by-products.

Conclusion

The results demonstrated that enzymatic hydrolysis is an effective method for extracting protein compounds with antioxidant effects from TDM. Alcalase hydrolyzed protein generally exhibited higher antioxidant activity than evatase and alkaline protease hydrolyzed proteins, as indicated by the higher DPPH radical scavenging activity. APHP significantly showed the highest reducing power than AHP and EHP. In general, AHP and APHP showed better antioxidant activities than EHP. It can be concluded that the antioxidant activity of hydrolyzed protein depends on protease type and hydrolysis time. Nevertheless, additional investigation is required to separate and identify peptide fractions from *T. tonggol* dark muscle.

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Authors Contribution: Aliyeh Daryanavard (first author), main researcher/ data analyzer; Saber Khodabandeh (second author) methodologist/ discussion author, Reza Hasan Sajedi (third author) methodologist, Mehrdad Behmanesh (forth author) methodologist.

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