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STORAGE STABILITY AND ANTIOXIDANT PROPERTIES OF CHICKEN BALL INCORPORATED WITH THREADFIN BREAM (*Nemipterus japonicas*) HYDROLYSATE

Normah Ismail[1*], Aishah Netylia Amat Ruba'iee[1] and Kyaw Zay Ya[2]

[*]*Department of Food Technology, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, 40450 Selangor Darul Ehsan, Malaysia*

[2]*Agri Asia Refinery Sdn. Bhd. Teluk Panglima Garang, Kuala Langat, Selangor Malaysia*

**Corresponding author: norismel@uitm.edu.my*

Abstract

This study was carried out to determine the antioxidant activity of threadfin bream (*Nemipterus japonicas)* hydrolysate (TFBH) in comparison with the commercial antioxidants; α-tocopherol and LYK Nanox 189 and to evaluate the oxidative stability of chicken balls added with 20% TFBH during the 15 days storage at 4°C. TFBH was prepared by hydrolysis with Alcalase at pH 8.5, 60°C, enzyme /substrate ratio of 2% for 2 hours. The results showed some antioxidant activities of the hydrolysate even though the activity was lower than the commercial antioxidants. This was based on the ferric reducing antioxidant power (FRAP) analysis and 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Based on the induction time measured by Rancimat, TFBH when added into the chicken balls was capable of delaying the oxidation process during the 15 days storage at 4°C. The observation was also supported by peroxide and TBARS values. Therefore, TFBH can be used in food to delay the oxidation process during storage.

Keywords: threadfin bream, *Nemipterus japonicas*, hydrolysate, antioxidant

Introduction

Fish protein hydrolysate is obtained from fish proteins in which peptides are broken down to various sizes by either chemical or enzymatic processes. In general, protein hydrolysate contains mixture of amino acids prepared by splitting a protein with acid, alkali or enzyme (Normah & Asmah, 2016). According to Benjakul *et al.* (2014), fish protein hydrolysates are suitable source of protein for human nutrition because of their positive effect on gastrointestinal absorption and balanced amino acid composition.

All types of foods that contain lipids even at a very low level (<1 %) are susceptible to oxidation and can cause oxidative rancidity (Sunantha *et al.,* 2016). Lipid oxidation is a major concern during processing and storage of fish because it contributes to quality deterioration and decreases in the quality of fish products (Secci & Parisi, 2016). Therefore, antioxidant is added to food to reduce oxidation. There are many sources of protein from plant and animal that showed significant antioxidant ability such as corn and eggs (Zhou *et al.,* 2012; Rao *et al.,* 2012). Bhat *et al.* (2015) also reported that marine organisms like fish exhibiting antioxidant active peptides and found that the antioxidant property of salmon protein hydrolysate functioning as protein hydrolysate and peptide fractions inhibited the oxidation of linoleic acid. Besides, Udenigwe and Aluko (2011) found that food protein hydrolysates such as from marine protein sources, including fish have bioactive peptides with antioxidant properties. The antioxidant properties of these peptides include scavenging of reactive oxygen species (ROS) or free radicals and inhibition of ROS-induced oxidation of biological macromolecules such as lipids and proteins. Protein hydrolysate are physically believed to be capable of adsorbing at the interface of lipid

droplets, therefore providing a physical protection of the lipid against reactive oxygen species due to the amphipathic nature of peptides (Damgaard *et al.*, 2015). Other factors that may affect the antioxidant activity of food protein hydrolysates include specificity of proteases used for hydrolysis and degree of hydrolysis itself.

Some protein hydrolysates have been reported to exhibit antioxidant activity. Jun *et al.* (2004) reported that yellowfin sole hydrolysate, prepared using pepsin at degree of hydrolysis of 22%, had a higher antioxidative activity than those produced using alcalase. Thiansilakul *et al.* (2007) found that hydrolysates produced from round scad muscle with flavourzyme exhibited a higher DPPH radical scavenging activity and reducing power, but a lower Fe^{2+} chelating ability than alcalase derived hydrolysates. Studies also showed that from bigeye tuna head, commercial alcalase enzyme can produce hydrolysate with higher antioxidant activities as tuna head protein hydrolysate was able to scavenge superoxide, hydroxyl and DPPH radicals (Yang *et al.*, 2011). Besides, Jeevitha *et al.* (2014) reported that protein hydrolysates from *Sardinella longiceps* produced with trypsin enzyme had shown higher hydroxyl radical scavenging activity and could be used as natural antioxidant in order to prevent oxidation. Several antioxidant activities studies from threadfin bream processing discards have also been reported. This include isolation and identification of antioxidative peptides from hydrolysate of threadfin bream surimi processing byproduct (Wiriyaphana et al., 2013). Protein hydrolysates from fish frame waste of threadfin breams (*N. japonicus*) have also been produced using papain and bromelain and the hydrolysates were evaluated for bioactive properties such as angiotensin-I-converting enzyme (ACE) inhibitory activity, antioxidant and functional properties (Gajanan et al., 2016).

The addition of TFBH may improve the oxidative stability of chicken balls. Besides, incorporation of hydrolysate in different food system may improve the nutritional values of the food especially in processed food such as chicken balls. The antioxidant derived from fish protein could also be used as an alternative to the commercial synthetic antioxidant. Thus, this study evaluated the potential antioxidant activities of TFBH and its ability to delay the oxidation of chicken balls during storage.

Materials and methods

Threadfin bream (*Nemipterus japonicas*) was purchased from a wet market in Selangor, Malaysia. Enzyme used was Alcalase 2.4 L with a declared activity of 2.4 AU/g and a density of 1.18 g/ml. The enzyme was obtained from Chemolab Supplies, Selangor, Malaysia. Commercial antioxidant used was *α*-tocopherol obtained from Sigma-Aldrich (M) Sdn. Bhd., Subang Jaya, Selangor and LYK Nanox 189 obtained from LYK Technologies Sdn. Bhd., Puchong. Chicken balls ingredients such as salt, seasoning and corn starch were purchased from local hypermarket.

Preparation of threadfin bream (*Nemipterus japonicas*) hydrolysate

TFBH was prepared according to Normah *et al.*, (2005). Alcalase was used at enzyme/substrate ratio of 2%. Other conditions set were temperature (60°C), pH 8.5 and hydrolysis duration of 2 hours. The supernatant collected was adjusted to pH 6 with 2 M HCl and dried in a freeze dryer (Alpha 1-4 Martin Christ, Germany). The powdered hydrolysate obtained after freeze drying was stored at -20°C until further analysis.

Determination of antioxidant activity of TFBH

Ferric reducing antioxidant power (FRAP) analysis

Ferric reducing antioxidant power (FRAP) analysis was conducted according to method described by Babu *et al*., (2013). The FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml 10 mM tripyridyl triazine (TPTZ) solution and 2.5 ml of 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$). FRAP reagent was heated at 37°C for 15 minutes prior to analysis. Next, 2.85 ml of freshly prepared FRAP reagent was mixed into 150 mg of hydrolysate. Then, the mixture was incubated at room temperature for 30 minutes. The absorbance was measured using UV-Vis Spectrophotometer at 595 nm. A standard curve was constructed with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at 200-1000 ppm. The FRAP values was expressed as milligram per ml sample. Commercial antioxidants; α-tocopherol and Nanox 189 were used as reference and the results were compared.

2, 2-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH radical-scavenging activity was measured for determination of antioxidant activity of TFBH according to the method of Huang and Mau (2006). Approximately 1 g of hydrolysate was mixed with 1 mL of 1 mM DPPH in methanol. The mixture was allowed to stand for 40 minutes in the dark and the absorbance was monitored at 517 nm. Distilled water was used as a blank. Scavenging DPPH activity was calculated according to the equation whereby percent (%) radical scavenging activity = $(A_0-A_1)/A_0 \times 100$ where A_1 is sample absorbance and A_0 is blank absorbance. Commercial antioxidants; α-tocopherol and Nanox 189 were used as reference and the results were compared.

Preparation of chicken balls

Chicken balls were prepared according to Monjurul *et al*. (2013). 700 g chicken meat was mixed with 15 g salt, 5 g monosodium glutamate and 10 g corn starch. Two sets of balls were prepared; one without TFBH (control) and the other with the addition of 20% TFBH based on weight of chicken balls mixture. Balls with a diameter of 2.5 cm were manually formed and then boiled until floated on the surface of water. After cooling, the balls were stored in refrigerator at 4°C. For peroxide values (PV), thiobarbituric acids (TBARS) and induction time analysis, the lipid was first extracted by soxhlet extraction according to AOAC (2005). The extracted lipid was kept and stored at 4°C prior to analysis.

Lipid oxidation induction time analysis

The induction time was measured using a Rancimat instrument (Metrohm CH series 743, MetrohmAG, Herisau, Switzerland). The oxidation process was analyzed on a 3 g extracted oil in Rancimat instrument set with air velocity of 20 L/h at 110°C according to Rižnar *et al.,* (2006). Samples induction time was automatically recorded by induction time corresponded to the break point of the plotted curves (the intersection point of the two extrapolated parts of the curve).

Peroxide value (PV) analysis

PV was determined according to AOAC method 965.33 (AOAC, 2005). 5 g extracted oil was weighed into a glass Erlenmeyer flask. 30 ml of chloroform and acetic acid mixture (2:3v/v) were added and dissolved by constant swirling. 0.5 ml of freshly prepared saturated potassium iodide solution was added and kept in dark in amber glass bottle for 1 minute. Then, 30 ml water was added. Next, 0.5 ml of 1% starch was added as an indicator and titrated against 0.01 N sodium thiosulfate. PV was calculated according to the following formula:

$$PV = \frac{(S-B)*N*1000}{\text{g of oil}}$$

where S= ml of sodium thiosulfate, N= normality of sodium thiosulfate, B is the volume of sodium thiosulfate in blank (ml) and 1000 is the conversion unit (g/kg).

Thiobarbituric acid reactive substances (TBARS) analysis

TBARS analysis was performed according to Xiong *et al.,* (2015). A 5 g extracted oil was mixed with 25 mL of 20% aqueous solution of tricholoroacetic acid (TCA) with addition of 20 mL water. Then, the mixture was left to stand at room temperature for 1 hour followed by centrifugation for 10 minutes at 2,000 rpm by using a centrifuge (Kubota Corporation, Japan). Two phases were formed. The lower layer was taken and made up to 50 mL with distilled water. Next, 5 mL of the solution was mixed with 5 mL of 0.02 M aqueous solution of thiobarbituric acid in the stoppered test tube. The test tube was kept at 95 °C in water bath for 20 minutes and followed by cooling. The absorbance was measured at 532 nm by using a spectrophotometer (Thermo Scientific, Australia). The concentration (mg MDA (malonaldehyde)) /g sample on the basis of wet weight) was calculated as follows:

TBARS value (mg MDA/g) = absorbance value at 532 nm x 7.8

Statistical analysis

The data obtained was analyzed using the Analysis of Variance (ANOVA) to determine significance at 5% level. Mean comparisons was carried out by using Duncan Multiple Range Test (DMRT) while statistical analysis was conducted using the Statistical Package for Social Science (SPSS for windows: SPSS Inc. Chicago, II, USA, 2006).

Results and discussion

Determination of antioxidant activity of TFBH

Ferric reducing antioxidant power (FRAP)

Figure 1 shows the antioxidant activity of TFBH in comparison with commercial antioxidants. All samples showed significant difference ($p<0.05$) in FRAP value with TFBH having the lowest value while Nanox 189 the highest (Figure 1a). Nanox 189 contains the combinations of BHA and BHT. BHA, BHT and α-tocopherol were known to have high value of ferric reducing ability. For example, Pereira *et al.* (2017) reported that α-tocopherol showed high antioxidant activity when evaluated in chicken burger which is 1328.80 µmol /g. BHA, BHT and α-tocopherol are commonly used in food products such as margarines, oils, and roasted nuts in order to prolong their shelf life (Race, 2009). Antioxidant activity relates to the ability to donate the electron to the free radicals. Hydrolysates that have this ability slows down the lipid oxidation process (Rajendran, 2012). Although FRAP value was lowest for TFBH, there is some antioxidant activity exhibited by the hydrolysate. Theodore *et al.* (2008) reported that catfish protein hydrolysate showed a high ferric reducing antioxidant activity as reducing properties are associated with the presence of compounds which exert their action by breaking free radical chain through donating hydrogen atom. Usually, the antioxidant activities in fish protein hydrolysates is attributed to its constituent peptides and / or amino acids (Margetts & Buttriss, 2003; Anusha *et al.,* 2008). Aromatic amino acids act effectively as radical scavengers due to their ability to donate proton easily to electron-deficient radicals (Rajapakse *et al.,* 2005). Amino acids such as histidine and tryptophan have been shown to exhibit higher antioxidative activity than methionine, cysteine, glycine and alanine (Rissom *et al.,* 1980). According to Je *et al.,* (2005), histidine residues in the peptide contain imidazole ring which enable it to chelate and trap free radicals whereas tyrosine may act as a potent hydrogen donor.

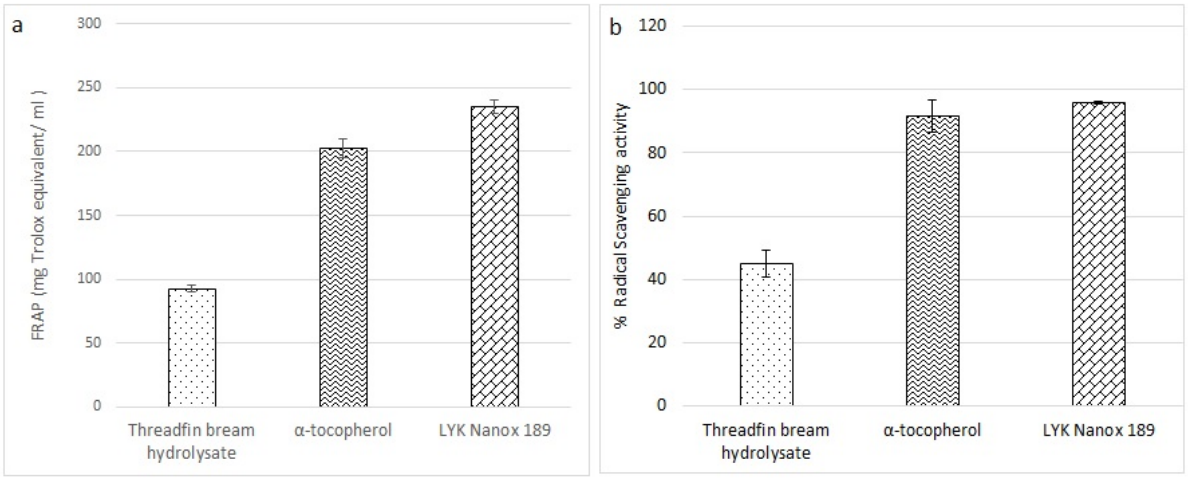

Figure 1 FRAP values (a) and radical scavenging activity (b) of threadfin bream hydrolysate in comparison with α-tocopherol and LYK Nanox 189

2, 2-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

Figure 1b shows the radical scavenging activity of TFBH in comparison with commercial antioxidants; α-tocopherol and Nanox 189. In general, free radical is reduced by antioxidant. This is because many antioxidants that react with radicals might react slowly or may be inert with DPPH (Prior *et al.,* 2005).

TFBH showed significant difference ($p<0.05$) in radical scavenging activity exhibiting the lowest value compared to commercial antioxidants. From the result, it indicates that TFBH has the ability to transfer electron and can be effectively used to terminate the free radical-induced chain reaction. Antioxidant activity of protein hydrolysates mainly depends on peptides that are present in the hydrolysate (Wiriyaphan *et al.,* 2012). Peptides that contribute to antioxidant activities contain hydrophobic amino acids such as tryptophan, phenylalanine and tyrosine (Liu *et al.,* 2010). Radical scavenging activity of TFBH (45.05 %) which is close to the previous report (52.2 %) for threadfin bream head and frame hydrolysates obtained from alcalase hydrolysis (Piyadhammaviboon *et al.,* 2012).

The effect of threadfin bream (*Nemipterus japonicas*) hydrolysate on the oxidative stability of chicken ball during storage

Lipid oxidation induction time analysis

Rancimat test is an accelerated aging test. At constant elevated temperature, air is going through the sample in the reaction vessel. From the result obtained, oil from chicken balls added with hydrolysate had longer induction time (19.785 hour) than oil in control chicken balls (1.885 hour) (Table 1). In general, the longer the induction time, the more stable is the lipid, therefore adding TFBH delayed the oxidation process of the chicken balls. Control chicken balls contained oil from chicken meat. However, in chicken balls containing 20% hydrolysate, the oil derived from both the meat and TFBH. Threadfin bream fish is categorized as low fat fish which have only 2-4 % fat (Nurnadia *et al.,* 2012). The fish also contains 1211.2 mg/ 100 g saturated fatty acid based on wet weight while polyunsaturated fatty acid content is 796.5 mg/ 100 g. Unsaturated fatty acid content in chicken ball was higher than in threadfin bream fish which is 1730.0 mg/ 100 g (Food Composition Japan, 2015). Oil containing high amount of unsaturated fatty acid is more prone to rancidity and oxidation. Hydrolysate that acts as antioxidant can retard the oxidation process.

Table 1 Induction time of chicken balls and chicken balls added with threadfin bream hydrolysate (TFBH)

Sample	Induction time (hr)
Chicken balls (control)	1.885 ± 0.134^{b}
Chicken balls added with 20% TFBH	19.785 ± 0.955^{a}

Peroxide value (PV) analysis

The purpose of using antioxidant is to delay the primary oxidation products and improving oxidative stability. Figure 2 shows the changes in PV of chicken balls added with TFBH during the 15 days of storage at 4°C. From the result, PV of both the chicken balls and chicken balls added with 20% hydrolysate increased during storage. However, PV of chicken balls added with 20% hydrolysate was lower than the control. The control chicken balls which reached a maximum PV of 26.417 meq kg^{-1} was oxidized at higher extent compared to chicken ball added with 20% hydrolysate (16.620 meq kg^{-1}). A significant difference ($p<0.05$) was found between control chicken balls and chicken balls added with 20% hydrolysate. Yang *et al.* (2011) reported that soybean oil containing tuna head protein hydrolysate delayed the rate of peroxide formation by exhibiting lower PV than control along the 5 days storage at 60°C. Debbarma *et al.* (2016) also reported that fried fish nuggets containing squilla (*Squilla Mantis*) protein hydrolysate showed relatively low PV during the 10 days storage at refrigerated temperature (4°C) compared to control. In general, fresh unsaturated oils have a PV of less than 10 meq/kg while rancid taste often begins to be noticeable when the PV is between 30-40 meq/kg (Amina *et al.,* 2017).

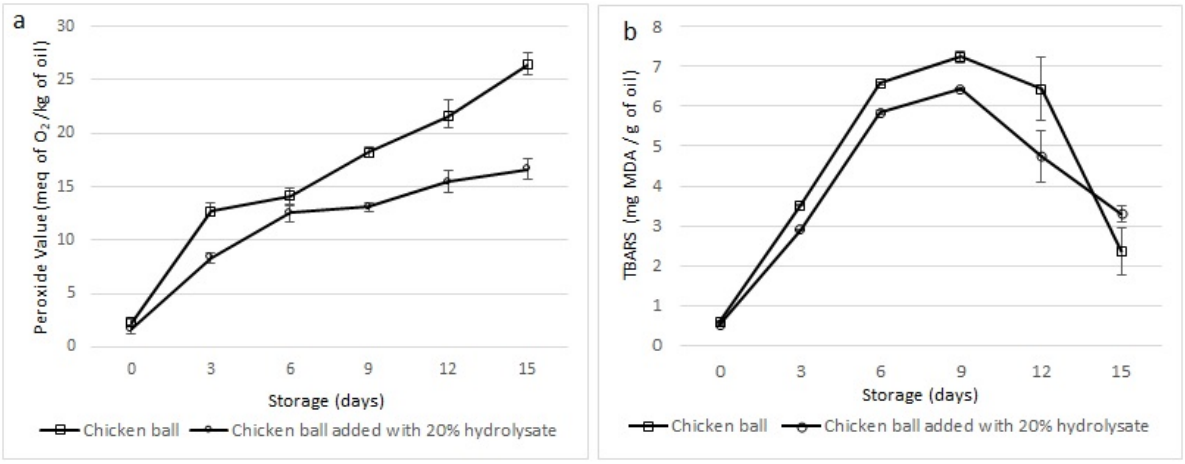

Figure 2 Peroxide values (a) and TBARS (b) of chicken balls added with threadfin bream hydrolysate during 15 days of storage at 4°C

Thiobarbituric acid reactive substances (TBARS) analysis

Figure 2b shows the changes in TBARS value of chicken balls added with TFBH during storage. According to Decker *et al.* (2008), antioxidant not only inhibit primary lipid oxidation but may also increase the secondary oxidation products, so the ability of TFBH to inhibit lipid secondary oxidation in chicken balls was observed as indicated by TBARS. TBARS were expressed as mg malondialdehyde/g of sample. From the result, TBARS value of both the control chicken balls and chicken balls added with 20 % hydrolysate increased from day 0 until day 9. A decrease in TBARS value was observed after 9 days of storage. The decrease in TBARS value might be due to loss of oxidation products in the form of malondialdehyde and other short-chain products of lipid oxidation which are not stable for a long period of storage (Khantaphant *et al.*, 2011). The TBARS value of chicken balls added with 20% hydrolysate were significantly lower ($p<0.05$) than the control. This showed that TFBH inhibited the formation of secondary oxidation products and delayed the oxidation as can be seen by the decreased in TBARS value. Besides, the presence of hydrolysate appeared to diminish lipid oxidation when compared to control where no hydrolysate was added (Oliveira *et al.*, 2014). Previous studies by Nasri *et al.* (2013) showed that goby protein hydrolysate analyzed in turkey meat sausage inhibit lipid peroxidation by reducing TBARS formations during 27 days of storage at 4°C. Besides, Kittiphattanabawon *et al.* (2012) reported that cooked pork meat added with gelatin hydrolysate from blacktip sharp skin had lower TBARS value during storage period of 14 days at 4°C.

Conclusion

Although the antioxidant activity of TFBH was lower than the commercial antioxidants; α-tocopherol and Nanox 189, TFBH does show some antioxidant activities based on its ability to slightly improve the oxidative stability of the chicken balls during the 15 days of storage at 4°C. These are supported by lower PV, TBARS value and longer induction time indicating the oxidation process of lipid from chicken balls containing the hydrolysate required longer time to occur compared to control. Thus, TFBH can be a source of a natural antioxidant from marine fish species.

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