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Antioxidant Activities of Threadfin Bream *(Nemipterus Japonicus)* Hydrolysate and Its Effect on Oxidative Stability of Frying Oil

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ABSTRACT

The antioxidant activities and oxidative stability of palm olein frying oil added with threadfin bream (TFB) hydrolysate and Nanox 189 were studied. The DPPH radical scavenging activities and chelating effects of ferrous ion were evaluated and compared with α-tocopherol. Oxidative stability after 30 frying cycles were analysed for the induction period, free fatty acids (FFA), peroxide values (PV), total polar compounds (TPC), and viscosity. Palm olein without any antioxidant was used as control. Nanox 189 exhibited a higher percentage on the DPPH scavenging effect and ferrous ion chelating effect than TFB hydrolysate. Frying oil added with Nanox 189 recorded the longest induction period, which was up to 11 hr. while TFB hydrolysate added oil showed an induction period of nine hours. Nanox 189 addition in frying oil yielded the lowest FFA and PV, followed by those added with TFB hydrolysate. The highest readings of TPC were recorded for TFB hydrolysate added oil, which is 13%. Throughout the 30 frying cycles, the addition of TFB hydrolysate recorded a lower percentage increase for viscosity than

Nanox 189 with 4.7% compared to the latter, which is 9.6%. Even though the antioxidant activities of TFB hydrolysate was lower than that of Nanox 189, the study suggested some antioxidant potential of TFB hydrolysate based on the DPPH radical scavenging activities and chelating effects of the ferrous ion as well as its ability to slightly improve the oxidative stability of palm olein during the 30 frying cycles.

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

INTRODUCTION

Frying of foods is one of the most common food preparation methods. The high temperature used during frying in the presence of oxygen and water-induced several chemical changes of the oil, reducing their shelf life and directly affecting the quality of the final fried food [1]. According to Karakaya and Şimşek[2], during the frying process via a series of complex physical and chemical reactions, oils are subjected to thermal oxidation, polymerisation, and hydrolysis. These reactions lead to a decrease in tocopherols, total phenols, an increase in the peroxide value (PV), and the formation of decomposition products with high molecular weights such as polar compounds and polymeric triacylglycerides. Furthermore, changes in the viscosity and density of the frying medium during the repeated frying cycle can be expected to affect buoyant bubble removal from the food surface, and thus the convective heat transfer from the oil to the food that undergoes frying [3].

Recently, a great deal of interest has been expressed regarding marine-derived bioactive peptides because of their numerous health benefits. Protein hydrolysates are the breakdown results of an enzymatic change of proteins into smaller peptides. For the most part, protein hydrolysates are little pieces of peptides that contain two to twenty amino acids, which were created by the enzymatic hydrolysis of proteins [4]. Bioactive peptides from enzymatic hydrolysis of various food proteins such as soy protein, casein, whey protein, and gelatin have been shown to possess antioxidant activities [5]. Thus, hydrolysates of different protein sources have received increased attention because they are sources of bioactive peptides [6]. The degradation of the

hydroperoxides, the primary oxidation products to secondary oxidation products with aldehydic and ketonic functions negatively affect oil flavour. The susceptibility of these oils towards oxidation has limited their utilisation and contributes to some significant economic losses as the shelf life of the oil becomes shorter. Therefore, the oils can only be used for a less frying cycle. Besides, the susceptibility of the oil towards oxidation causes several undesirable changes towards the quality of oil, such as an increase in viscosity, foaming, colour, and development of rancid odour [7].

Protein hydrolysate has been studied for the development of food functionalities and technological properties, nutritional and medical purposes, as well as for the improvement of food taste [8]. However, studies on the addition of threadfin bream (Nemipterusjaponicus) hydrolysate in frying oil are unavailable. Besides, research on the antioxidant properties of threadfin bream(Nemipterusjaponicus) as the substitute for the synthetic antioxidant is still scarce. In this study, the antioxidant activity of TFB hydrolysate and its effect on the oxidative stability of frying oil after several frying cycles were evaluated.

MATERIALS AND METHODS

Threadfin bream fish were obtained from a local supplier around Shah Alam, Selangor, stored in a box filled with ice and immediately transported to the laboratory. Potatoes (*Solanumtuberosum*) were obtained from the nearby local market around Shah Alam, Selangor. A fresh palm olein with Iodine Value (IV) of 56 was obtained from Agri Asia Refinery Sdn. Bhd. (Teluk Panglima Garang, Selangor, Malaysia). Flavourzyme 500 L with a declared activity of 500 Leucine Amino Peptidase Units per gram (LAPU/g) was purchased from Novozyme Sdn. Bhd. Malaysia. Artificial antioxidant, Nanox 189, was obtained from LYK Technologies Sdn. Bhd. (Puchong, Selangor, Malaysia). All chemical reagents which were used for analysis are of analytical grade and purchased from Sigma-Aldrich Sdn. Bhd. Malaysia (Subang Jaya, Selangor).

Preparation of Threadfin Bream (TFB) Hydrolysate

TFB hydrolysate was produced by hydrolysing the fish muscle in flavourzyme at an enzyme-substrate ratio of 2%, pH (8.5), temperature (65°C), for two hours, according to Normah *et al*.[9].

Analysis of the Antioxidant Activity of TFB Hydrolysate

Chelating effects on the ferrous ion

The chelating effects of TFB hydrolysate were determined according to the method described by Razali *et al*.[10]. Approximately 0.5 ml of 200 ppm of hydrolysate solution was mixed with 1.6 ml of distilled water and 0.05 ml of 2 mM FeCl2. 0.1 ml of ferrozine (5 mM) was added after 15 minutes, after which each mixture was vigorously shaken and left at room temperature for ten minutes. The absorbance of the Fe2+ ferrozine complex was measured at 562 nm using a UV-Visible spectrophotometer (ThermoScientific, USA). α-tocopherol and Nanox 189 were used as positive control and prepared in the same manner. Chelating antioxidant activity for Fe2+ was calculated as follows:

$$\text{Chelating effect (\%)} = \frac{A_{control} - A_{sample} \times 100}{A_{control}}$$

Where $A_{control}$ is the absorbance of the control, and Asample is the absorbance of TFB hydrolysate.

DPPH radical-scavenging activities

The ability of TFB hydrolysate to scavenge free radicals was analysed by using 1, 1- diphenyl-2-picrylhydrazyl (DPPH), according to the method described by Razali *et al*.[10]. Approximately 0.5 ml of 200 ppm TFB hydrolysate solution was mixed with 0.5 ml of 90% methanol and 0.125 ml 0.02% DPPH. The mixture was vigorously vortexed and then incubated in the dark for 60 min. Absorbance was measured at 517 nm using a UV-Visible spectrophotometer (ThermoScientific, USA). A DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activities (\%)} = \frac{A_{control} - A_{sample} \times 100}{A_{control}}$$

Where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of TFB hydrolysate.

Preparation of potato strips and frying oil

Potatoes were washed, hand-peeled, and cut into strips (10 x 10 x 70 mm) and then dried with tissues before frying. The palm olein was divided into three consisting of control, palm olein added with Nanox 189, and palm olein added with TFB hydrolysate at the concentration of 200ppm for each antioxidant.

Frying of sample

An amount of 100 g of potato strips was fried in a deep fryer containing 4 L oil at 130 °C for two minutes. The oil used was palm olein (control), palm olein containing Nanox 189, and palm olein containing TFB hydrolysate. Antioxidants in the palm olein were prepared at 200ppm. After frying, the remaining oil was allowed to cool at room temperature. After every fifth cycle of frying, the oil was analysed. This was carried on until the 30th frying cycle.

Analysis of the Oil

Induction Period

The induction period of the oil was measured using the 743 Rancimat (Metrohm, Herisau, Switzerland) at 120°C following the AOCS Official Method Cd 12b-92 [11]. About 0.3 g of palm olein sample was initially placed at the bottom of the reaction tube. The tubing from the air manifold was connected to the conductivity measurement tube. The aeration tube was adjusted within 5mm from the bottom of the reaction and conductivity tube before measuring the airflow at 2.5 ± 0.2 ml s^{-1}. The reaction vessel was closed with a reaction vessel cover assembled with an air inlet tube. The two tubings between Rancimat and reaction vessel and between the reaction vessel and measuring vessel were connected. Then the reaction

vessel was placed in the heating block, and the measurement was started immediately. At the end of the reaction, the induction period of the oil sample was recorded.

Free fatty acid (FFA)

FFA analysis was conducted according to the standard AOCS Official Method Ca 5a-40 [12]. The frying oil sample was weighed at 20 g and poured into a 250 ml Erlenmeyer flask. 50 ml of 0.002 N of ethanol at 40°C was added, followed by three drops of 1% phenolphthalein and 25 ml of diethyl ether. The mixture was titrated with 0.1 M aqueous sodium hydroxide until the appearance of the first pink colour which persisted for at least 30 seconds. The FFA value, expressed in percentage (%) was calculated using the formula:

$$\%FFA = \frac{\text{volume of titre (ml) x factor (0.404)}}{\text{weight of sample (g)}}$$

Peroxide value (PV)

PV was determined by AOCS Official Method Cd 8b-90 [13]. Approximately 5 g of oil sample was placed in a 100 ml Erlenmeyer flask. A solution of 50 ml of 3:2 acetic acid: chloroform was then added, followed by the addition of 0.5 ml of saturated potassium iodide solution. The mixture was swirled for exactly one minute. Then 30 ml of distilled water was added into the flask before the titration with 0.001 N Na2S2O3. A few drops of the starch solution were added as an indicator. The endpoint was observed as the solution turned colourless. The PV, expressed in miliequivalent of active oxygen per kilogram (meq/kg), was calculated using the formula:

$$PV\ (meq/kg) = \frac{\text{volume of titre (ml) x factor (9.81)}}{\text{weight of sample (g)}}$$

Total polar compounds (TPC)

Total polar compounds of the frying oil were analysed using Testo 270 Deep-frying Oil Tester (Testo Inc., Germany). The oil samples were analysed by inserting the sensor into the oil samples [14]. The oil was stirred gently for 20 seconds to allow even distribution. Testo270 typically showed a stable reading of total polar compounds (%) after one minute. The equipment was cleaned with warm water and detergent and dried well using a tissue between measurements of the oil samples.

Viscosity

Viscosity was measured by using a Brookfield RVDV-I+viscometer and spindle number 3 with a speed of 100 rpm. About 200 ml of frying oil sample was taken for viscosity measurements. The reading was recorded after one minute until a constant reading is observed. All the viscosity measurements were conducted at 25 ±2 °C.

Statistical analysis

All measurements were performed in triplicate. Data were analysed using the Analysis of Variance (ANOVA) to determine significance at 5% level. Duncan Multiple Range Test (DMRT) was used to identify differences between means. All the data analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

Antioxidant Activity of TFB Hydrolysate

Ferrous chelating effects and DPPH radical scavenging activities

Chelating effects and radical scavenging activities of TFB hydrolysate, Nanox 189, and α-tocopherol are shown in Figures 1a and b. From the results, α-tocopherol showed the highest percentage of chelation (88.29%), while Nanox 189 (80.99%) followed by TFB hydrolysate (42.82%). Similar

findings were obtained for DPPH radical scavenging effect with α-tocopherol (90.67%) showing the highest activity, followed by Nanox 189 (84.21%) and TFB hydrolysate (43.97%). α-tocopherol and TBHQ were known to have excellent chelation ability towards ferrous ion. Nanox 189 is the derivative of TBHQ. Generally, hydrolysates contained peptides, which were hydrogen donors, and could react with radicals to convert them to more stable products, thereby terminating the radical chain reaction [15]. Such peptides usually contain between 2 to 16 amino acid residues [16]. Antioxidant activities of hydrolysate also depend on the type of enzyme used during the hydrolysis, which then influences the resulting degree of hydrolysis [17,18]. It has been shown that a higher degree of hydrolysis produced higher chelating activity [18]. This observation suggested that TFB hydrolysate contains the antioxidant properties; nevertheless, α-tocopherol and Nanox 189 remained the better antioxidant.

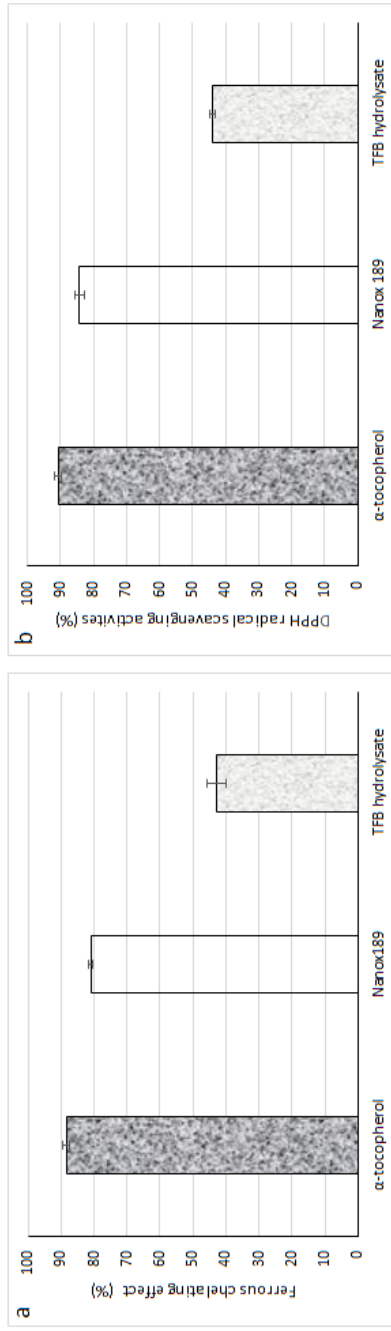

Figure 1: Ferrous Chelating Effect (a) and DPPH Radical Scavenging Activities (b) of α-Tocopherol, Nanox 189 and TFB Hydrolysate

Oxidative Stability of the Oil

Induction period

The duration of the induction period is a measure of oil and fat resistance to oxidation. The rancimat method is based on the fact that in oxidised oils or fats, volatiles is formed at the end of the induction period [19]. The difference between the induction periods of the three palm olein samples can be seen in Table 1. In this analysis, the addition of Nanox 189 into the palm olein increased the induction period of the oil to 11.08 hr. In comparison, the addition of TFB hydrolysate showed a slight increment towards the induction period of the palm olein with 9.43 hr. A previous study by Taghvaei *et al*. [20] stated that the addition of 200 ppm of fish protein hydrolysate from Crucian carp fish increased the induction period of soybean oil sample to approximately seven hours, compared to the control, which was six hours, which might arise due to strong oxidation prevention activity. Nanox 189, which consists of tert-butyl hydroquinone (TBHQ), showed the longest induction period among the three samples. This is correlated with the study by Liang *et al*.[21], which indicates that TBHQ showed more significant enhancement towards the induction period of palm biodiesel in a much lower concentration compared to α-tocopherol and butylated hydroxytoluene (BHT).

Table 1: Effect of TFB Hydrolysate and Nanox189 Addition on the Induction Period of Palm Olein

Sample	Induction period (hr)
Palm olein	8.71 ± 0.07c
Palm olein added with Nanox 189	11.08 ± 0.03a
Palm olein added with TFB hydrolysate	9.43 ± 0.04b

[a-c] Values with different letters are significantly different ($p<0.05$).

Analysis of Frying Oil

Free fatty acid (FFA)

Previous studies of frying oils such as palm oil have shown that the content of FFA increases during deep-frying [22]. In this study, the addition of TFB hydrolysate and Nanox 189 gives different results towards the analysis of FFA of the palm olein (Figure 2a). From the results, FFA composition in the palm olein increased with the number of frying cycles. Hydrolysis of triglycerides is among the major cause in the production of FFA, although it is also possible to produce FFA through oxidative reactions [23]. Nanox 189 possesses the lowest amount of FFA, with almost 0.1% at the 5th frying cycle, which increases to 0.14% in the 30th cycle. For the addition of TFB hydrolysate, the FFA value increased from about 0.12% at the 5th cycle to about 0.145% at the 30th frying cycle. This might be due to the inhibitory effect of antioxidants from TBHQ, which can be found in Nanox 189, as studied by Zhang *et al*. [24] on sunflower oil. For TFB hydrolysate, it does affect the FFAas shown by the slight increment of the FFA amount of the palm olein compared to the control. The hydrophobicity of the peptide also appears to be an important factor for its antioxidant activity due to increased accessibility to hydrophobic targets such as lipophilic fatty acids [25]. The final percentage of FFA for the three oil samples after the 30 frying cycle is approximately 0.14%, which is still low and does not exceed the FFA levels according to Malaysian Standard for palm oil (MS 814), which is 5.0% [26].

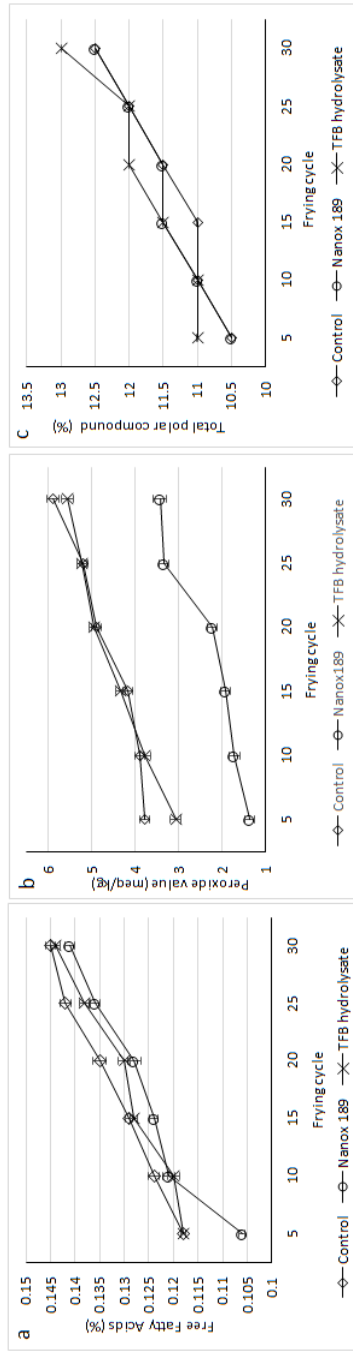

Figure 2: Free Fatty Acids (a), Peroxide Value (b) and Total Polar Compound (c) of Palm Olein (Control), Palm Olein Added with Nanox 189 and Palm Olein Added with TFB Hydrolysate After Frying for Five to 30 Frying Cycles

Peroxide value (PV)

Peroxide value (PV) is a measure of the concentration of peroxides and hydroperoxides formed in the early stages of lipid oxidation. Peroxide value is one of the most widely used tests for the measurement of oxidative rancidity in fats and oil. In this study, the oxidation degree of palm olein samples was determined by measuring PV in the presence of antioxidants from Nanox 189 and TFB hydrolysate through 30 frying cycles. The results obtained are shown in Figure 2b. Peroxide value increased with an increasing frying cycle. Palm olein with the addition of Nanox 189 showed the lowest peroxide value with 1.5 meq/kg in the 5th cycle, which then increased to about 3 meq/kg at the 30th cycle. This was studied by Zhang *et al*. [24] in which TBHQ remained the most effective and gave the lowest PV in oxidative stability of sunflower oil compared to other antioxidants such as BHT and BHA. However, for TFB hydrolysate, there is only a slight inhibition effect on the peroxide value compared to the control. The addition of TFB hydrolysate recorded the peroxide value of the oil at 3 meq/kg at the 5th frying cycle, which then increased to 5.5 meq/kg at the 30th cycle. Kim *et al*. [27] stated that the potent lipid peroxidation inhibition activity is thought to be associated with the presence of hydrophobic amino acids. Hydrophobic amino acids have a high affinity for lipid systems. Therefore, oil-soluble radicals such as hydrophobic peroxyl radicals generated during the oxidative reaction of unsaturated fatty acids are believed to be neutralised by hydrophobic amino acid containing antioxidant peptides. A study by Karakayaand Simsek [2] suggests that the PV of frying oils analysed decreased after 125 min of frying. The decrease can be explained by the formation of secondary oxidation products such as hydrocarbons, alcohols, ketones, and aldehydes from very unstable primary oxidation products such as hydroperoxides. Another report also suggested the PV decreases as oxidation proceeds due to the rapid decomposition of hydroperoxides [28]. Freshly refined oils regularly have a PV, lower than 1 meq/kg oil, and oil is considered to be rancid at a PV above 10 meq/kg oil [29]. Therefore, the PV of palm olein added with TFB hydrolysate and Nanox 189 does not exceed the maximum level of PV in oil and still within the acceptable range.

Total polar compound (TPC)

Formation of polar compounds, which indicates oil deterioration is heavily linked with the primary and secondary oxidation that takes place during frying. Generally, the degradation of oil during frying is accompanied by increasing the polar compounds of oil [30]. When the amounts of TPC reach 24% levels, oil is considered to be thermally degraded and should be replaced with fresh oil [11]. Polar compounds are the total of non-triglycerides of oil, including fatty acids, alkaline pollutions, sterols, tocopherols, alcohols, aldehydes, ketones, and other soluble compounds in oil that are more polar than triglycerides [31]. The amounts of total polar compounds in the oil sample after frying are shown in Figure 2c. TPC of the oil showed a slight increment with the frying cycle. In a study by Aydeniz and Yilmaz [32], the oil samples supplemented with natural antioxidant extracts had more TPC, which possibly due to the characteristics of the natural antioxidant materials itself which are polar. The smaller peptides from myofibrillar proteins of a fish protein hydrolysate, such as salmon, are expected to have proportionally more polar residues, which can affect the binding of the protein with oil [33]. Therefore, it could increase the TPC in the oil during frying. Besides, TPC content in the frying oil could be contributed from the moisture of the hydrolysate as most of the fish protein hydrolysate contained below 10% moisture content [34]. Several researchers suggested that the threshold level of TPC to discard the frying oil is 25 to 27% [35]. Therefore, the TPC for the palm olein sample in this study is still in the acceptable range, since the highest level of TPC recorded was 13%.

Viscosity

The viscosity of oil increases with frying time due to oxidation, isomerisation, and polymerisation reaction. Oxidation reaction leads to the formation of carbonyl or hydroxyl groups bonded to the carbon chain, making flux among molecules that enhance viscosity [36]. The difference between the viscosity of the palm olein sample can be seen in Figure 3. From the results obtained, the addition of TFB hydrolysate and Nanox 189 increased the viscosity of oil after 30 frying cycles with Nanox 189, showing the lowest reading. In the 5th cycle, the addition of Nanox 189 showed the lowest viscosity, with about 73.2 cP, which increased to 80.3 cP at the 30th frying cycle. This is correlated with Prasad *et al.*[37], who stated that oil

containing 200 ppm TBHQ changed the viscosity to a small extent during the frying process. The highest level of TBHQ mixed oil exhibits the lowest change in oil viscosity. This might occur due to the phenolic compounds from TBHQ, which incorporated into the fried oil during the frying process [38]. Meanwhile, the addition of TFB hydrolysate in oil showed the viscosity of 76.7 cP at the 5th cycle and 80.3 cP at the 30th cycle, which indicates the percentage of increase of 4.7%. This is much lower than the increase of the viscosity of Nanox 189 added oil, which recorded an increase of 9.6%. This could be due to the unstable degradation product from the polymerisation occurs in oil, which resulted from the polar materials from TFB hydrolysate.

CONCLUSION

TFB hydrolysate exhibited some antioxidant activities based on the DPPH radical scavenging activities and ferrous chelating observations in addition to the slight improvement of oxidative stability of frying oil in terms of the induction period, FFA, PV, and viscosity.

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