

CHANGES IN ANTIOXIDANT ACTIVITY OF FERMENTED FISH SAUCE (NAM PLA-RA) FROM *OSTEOCHILUS HASSELTII* DURING FERMENTATION

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Abstract

The objective of this study was to determine the changes in antioxidative activities of fermented fish sauce (Nam Pla-ra) from *Osteochilus hasseltii* at various fermentation times. A 5 kg of fish was mixed with salt at a ratio of salt to fish of 1:4 (w/w) and packed in a plastic bag. After 1 day of fermentation, rice bran (4%) was added to the mixture and fermented under ambient conditions in a plastic bag for up to 12 months. Samples were collected at 0, 6, 9, and 12 months for antioxidant activity and chemical composition analysis. The results showed that protein content of Nam Pla-ra at 12 months of fermentation had the highest, followed by Nam Pla-ra at 9 months with protein levels of $7.82 \pm 0.72\%$ and $6.94 \pm 0.61\%$, respectively. The ABTS radical scavenging activity of Nam Pla-ra at 9- and 12-months were comparable but higher than that at 6-months. The 12-month-old Nam Pla-ra exhibited the highest ferric reducing antioxidant power (FRAP) value of 1.02 ± 0.068 mg Trolox equivalents/ml sample. The highest metal chelating activity was observed in the 9-month-old Nam Pla-ra (0.17 ± 0.014 mg EDTA equivalents/ml sample). While 6- and 9-month old Nam Pla-ra showed the highest hydroxyl radical scavenging activity. Nam Pla-ra at 9-months fermentation time contained peptides with molecular weights of around 1,537-579 and 410 Dalton. Based on these results, it is recommended that people consume Nam Pla-ra from *Osteochilus hasseltii* at a 9-month fermentation time because of its high nutrition and antioxidant properties.

Keywords: Fermented fish Product (Pla-ra), Nam Pla-ra, *Osteochilus hasseltii*, antioxidant activity, chemical composition

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Introduction

The fermented fish product, Pla-ra, is a common ingredient in Thailand, especially in the northern and northeastern parts of Thailand. Generally, Pla-ra is made from fresh water fish such as *Channa striata* (Striped snake head fish, Chon), *Trichogaster trichopterus* (Gourami, Kra-dee), *T. leeri* (Kra-dee-nang), *Cyclocheilichthys repasson* (Silver Carp, Soi), and *Puntius gonionotus* (Barb, Ta-pien) (Sangjindavong *et al.*, 2008). It is produced by fermenting fish with salt and either rice bran or roasted rice powder which is fermented in a closed container for at least six months (Phithakpol *et al.*, 1995). Pla-ra contains salt (approximately 11.5-23.9%), pH (approximately 4.33-5.64) and lactic acid bacteria (approximately $1.0^2 \times 10^7$ - 2.84×10^{10} cell/g) (Phithakpol *et al.*, 1995). In addition, Pla-ra is rich in small peptides and amino acids (Yachai *et al.*, 2008).

Reactive oxygen species (ROS) are molecules, elements, atoms or radicals composed of oxygen in their structure and are highly reactive. Overproduction of ROS can damage DNA, proteins, and lipids, leading to many serious diseases like cancer, cardiovascular disease, inflammation and Alzheimer's disease (Moskovitz *et al.*, 2002; Qian *et al.*, 2008). Free radical scavengers are preventive antioxidants. It has been reported that small peptides from fermented fishery products act as antioxidants, such as fish sauce (Harada *et al.*, 2003), fermented mussel sauce (Jung *et al.*, 2005; Rajapakse *et al.*, 2005), fermented shrimp/krill products (Faithong *et al.*, 2010), Japanese fermented chub mackerel (Kanno *et al.*, 2012) and Pla-ra from snakehead murrel (*Channa striata*, Sawatdichaikul *et al.*, 2014). Protein hydrolysis gradually occurs during fermentation by the action of both fish endogenous and bacterial proteinases to produce amino acids and small peptides (Peralta *et al.*, 2008; Sawatdichaikul *et al.*, 2014). Antioxidant activities of these peptides are based on hydrophobicity, the molecular mass of peptides, amino acid composition, sequences and fermentation conditions. In addition, peptides and amino compounds in fermented fishery products also interact with

reducing sugars to form compounds exhibiting antioxidant activity such as Maillard reaction products (MRPs) (Peralta *et al.*, 2008).

Osteochilus hasseltii (Hard-lipped Barb) is a freshwater fish in Cyprinidae family. It is found in the Mekong and Chao Phraya river basins, the Malay Peninsula, Sumatra, Java and Borneo (Kottelat, 1998). It has been traditionally used in Pla-Som and Pla-ra. Thus, the study of the antioxidant activity of fermented fish sauce (Nam Pla-ra) from *Osteochilus hasseltii* could be marketed as a health food with a high market value.

Therefore, the objective of this study was to determine the changes in antioxidative activities of fermented fish sauce (Nam Pla-ra) from *Osteochilus hasseltii* at 0, 6, 9, and 12 months of fermentation. In addition, molecular weight distribution of Nam Pla-ra showing high antioxidant activity was investigated. Finally, chemical composition of fermented fish meat (Solid Pla-ra) and Nam Pla-ra at various fermentation times was also investigated.

Materials and Methods

Materials

Osteochilus hasseltii were collected from the Huay Luang River of Nong Khai province. 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Biochemika (Buchs, Switzerland). Trinitrobenzenesulfonic acid (TNBS) and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

Preparation of Fermented Fish (Pla-ra)

The fish were descaled, headed, gutted and washed with water. Fermented fish were prepared by mixing 5 kg of *Osteochilus hasseltii* with salt at a ratio of salt to fish of 1:4 (w/w) and packed in a plastic bag. After 1 day of fermentation, rice bran was added at a ratio

of 4%. Then, fermentation was carried out under ambient conditions (27-32°C) in a plastic bag for up to 12 months. Fermenting fish started from May 28, 2015 to May 28, 2016. Nam Pla-ra (fermented fish sauce) and Solid Pla-ra (fermented fish meat) were collected at 0, 6, 9, and 12 months for chemical composition analysis. Nam Pla-ra at various time was collected for determination of α -amino acid content and antioxidant activity. Nam Pla-ra showing high antioxidant activity was selected for determination of molecular weight distribution.

Chemical Composition

Solid Pla-ra (fermented fish meat) at 0, 6, 9, and 12 months of fermentation times were cut into small pieces and used for determination of moisture, protein and ash content according to AOAC (2000). The moisture, protein and ash content of Nam Pla-ra at 0, 6, 9, and 12 months of fermentation times were also determined.

Sample Preparation for Analysis

Nam Pla-ra at 0, 6, 9, and 12 months of fermentation were centrifuged at 10,000 $\times$ g, 4°C for 20 min. Supernatants were collected to determine the α -amino acid content and antioxidant activities.

α -Amino Acid Content Assay

The α -Amino acid content of Nam Pla-ra at various fermentation times were determined based on trinitrobenzenesulfonic acid (TNBS) using L-leucine as a standard (Adler-Nissen, 1979). Absorbance was monitored at 420 nm using a spectrophotometer (SmartSpecTM Plus, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Antioxidant Activities of Nam Pla-ra

ABTS Radical Scavenging Activity Assay

ABTS radical scavenging activity assay was carried out as described by Wiriaphan *et al.* (2012). In brief, the ABTS^{•+} stock solution contained 7.4 mM ABTS solution and a 2.6 mM potassium persulfate solution in 10 mM phosphate buffer with a pH of 7.4. The mixture

was kept at room temperature in the dark for 16 h. The ABTS^{•+} working solution was prepared by diluting ABTS^{•+} stock solution with a 10 mM phosphate buffer pH 7.4 until absorbance at 734 nm reached 0.70 $\pm$ 0.02. 20 μ l of Nam Pla-ra at various fermentation times were added to a test tube, followed by adding 1,980 μ l of a fresh ABTS^{•+} working solution. After 5 min of incubation the absorbance was measured at 734 nm. Results were expressed as mg Trolox equivalents (eq.)/ml sample.

Ferric Reducing Antioxidant Power Assay (FRAP assay)

The Reducing power of samples was measured using the ferric reducing antioxidant power (FRAP) assay according to Wiriaphan *et al.* (2012). Briefly, the FRAP reagent contained 25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 2.5 ml of 20 mM Iron (III) chloride hexahydrate (FeCl₃ · 6H₂O) solution. One hundred μ l of Nam Pla-ra at various fermentation times were allowed to react with 1,000 μ l of fresh FRAP reagent and incubated at 37°C for 15 min in a water bath. Absorbance was measured at 593 nm. Results were expressed as mg Trolox eq./ml sample.

Metal Chelating Assay

Fe²⁺-chelating activity measuring the formation of a Fe²⁺-ferrozine complex followed the method reported by Decker and Welch (1990). Nam Pla-ra (100 μ l) at various fermentation times was mixed with deionized water (2.4 ml), 2 mM FeCl₂ (50 μ l) and 5 mM ferrozine (100 μ l). The mixture was then incubated at room temperature for 20 min in the dark. The colour of Fe²⁺-ferrozine complex was recorded at 562 nm. Metal chelating activity was expressed as mg EDTA eq./ml sample.

Hydroxyl Radical Scavenging Activity Assay

The hydroxyl radical scavenging activity was measured using the 2-deoxyribose oxidation method described by Sakanaka and Tachibana (2006) with some modifications.

Hydroxyl radicals were produced by a Fenton reaction. First, Nam Pla-ra (100 µl) from various fermentation times were added into a test-tube and then mixed with 0.45 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM FeSO₄-EDTA and 0.5 ml of deionized water. The reaction was started by adding 0.15 ml of 10 mM hydrogen peroxide (H₂O₂). After incubating at 37°C for 4 h in a water bath, 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% thiobarbituric acid were added. Subsequently, the test-tube was then boiled for 10 min and cooled in ice. The absorbance of the reaction mixture was recorded at 520 nm. A 100 µl of deionized water instead of the sample was used as a control. The hydroxyl radical scavenging activity was calculated using the following formula:

$$\text{Inhibition (\%)} = \left[\frac{\text{Absorbance of control}_{520} - \text{Absorbance of sample}_{520}}{\text{Absorbance of control}_{520}} \right] \times 100$$

Molecular Weight Distribution

The molecular mass distribution of peptide in Nam Pla-ra at 9 months of fermentation times was determined using a gel filtration chromatography technique. A 100 µl of diluted sample (1.6 mg leucine eq./ml) was loaded in a Superdex Peptide 10/300 GL column (10 mm×300 mm; GE Healthcare, Piscataway, NJ, USA) pre-equilibrated with 30% ACN+0.1% TFA in deionized water. Elution was carried out at a flow rate of 0.6 ml/min using 1 column volumes of 30% ACN+0.1% TFA in deionized water. Absorbance was monitored at 215 nm. Cytocrom C (12,000

Da), Aprotinin (6,512 Da), AGNQVLNLQADLPK (1,480.6 Da), MILLLFR (1,095 Da), PLL (341 Da), LL (244 Da) and tyrosine (181 Da) were used as standard. Blue dextran (2,000 kDa) was used to measure the void volume of the column.

Statistical Analyses

Statistical analyses were carried out using a PASW Statistics 18 Release 18.0.0 software. The results were analyzed using a one-way analysis of variance (ANOVA). Duncan's multiple-range test (DMRT) was used to determine significant differences at the 95% confidence level.

Results and Discussion

Chemical Composition

The moisture content of Nam Pla-ra decreased with fermentation time which correlated with an increase in protein and ash (Table 1). Nam Pla-ra at 12 months showed the highest protein and ash content which were 7.82 and 33.30%, respectively (Table 1). This could be attributed to fermentation, during which protein hydrolysis gradually occurs by the action of both fish endogenous and bacterial proteinases to produce amino acids and small peptides in Nam Pla-ra. In addition, the high content of ash in Nam Pla-ra would be due to increased solubility of salt during the prolonged fermentation time. The moisture content of Solid Pla-ra increased for up to 6 months of fermentation time and did not change with prolonged fermentation times (58.93-64.04%). This is because Solid Pla-ra

Table 1. Chemical composition of Nam Pla-ra and Solid Pla-ra at various fermentation times

Fermentation time (months)	Chemical composition (% wet basis)					
	Moisture		Protein		Ash	
	Nam Pla-ra	Solid Pla-ra	Nam Pla-ra	Solid ^{ns} Pla-ra	Nam Pla-ra	Solid Pla-ra
0	78.07±0.42 ^a	58.93±0.79 ^b	1.42±0.04 ^d	19.25±1.05	19.13±0.40 ^c	16.93±0.41 ^{ab}
6	68.79±0.46 ^b	64.04±2.55 ^a	5.82±0.64 ^c	19.17±1.63	29.71±1.60 ^b	13.52±0.50 ^c
9	63.49±6.35 ^c	63.41±2.23 ^a	6.94±0.61 ^b	17.41±2.24	28.84±0.77 ^b	15.85±0.66 ^b
12	51.49±2.16 ^d	63.48±2.07 ^a	7.82±0.72 ^a	17.40±1.84	33.30±2.37 ^a	17.51±1.67 ^a

Different letters within a column indicate with significant differences (p<0.05).

Values are expressed as means ± S.D. (n=3).

ns = non-significant

might absorb water from Nam Pla-ra resulting in no difference in moisture content after 6 months of fermentation. It has been reported that the moisture content of Pla-ra from three manufacturers of Ubon Ratchathani were 63.88 ± 0.25 - $69.90 \pm 0.19\%$ (Promchote, 2017). The protein content of Solid Pla-ra slightly decreased with increased fermentation time because of proteolysis taking place during fermentation leading to the production of small peptides and amino acids in Nam Pla-ra (Table 1). The protein content of Solid Pla-ra at 0-12 months of fermentation time were in the range of 17.40-19.25%. Rattagool and Stroem (1996) reported a protein content of 16.6% for Pla-ra. In addition, the protein content of Pla-ra for three manufacturers in Ubon Ratchathani were 19.07 ± 1.05 - $21.24 \pm 0.55\%$ on a dry basis (Promchote, 2017). Our results were similar to those reported. The ash content of Solid Pla-ra at 0-12 months of fermentation time was in the range of 13.52-17.51%. It has been reported that Pla-ra in Udon Thani and Nongkhai contained ash contents in the range of 4.10 ± 0.12 - $10.17 \pm 0.21\%$, while Pla-ra in Nakhon Phanom and Sakon Nakhon contained ash contents in the range of 10.06 ± 0.08 - $19.45 \pm 0.34\%$ (Suriyanont and Chancharoonpong, 2018). Differences in chemical composition of Pla-ra could be due to differences in fish species, size, fermentation conditions (salt, rice bran and roasted rice) and fermentation time. Our results suggested that Solid Pla-ra and Nam Pla-ra from *Osteochilus hasseltii* are a rich source of protein and minerals.

α -Amino Acid Content

The α -Amino acid content of Nam Pla-ra increased with increasing fermentation times until 6 months (Figure 1, $p < 0.05$). However, the α -amino acid content gradually increased after that ($p > 0.05$). An increase in α -amino content indicated the extent of proteolysis during fish fermentation as it reflected the formation of oligopeptides and/or amino acids (Adler-Nissen, 1979). The lack of a difference in α -Amino content of Nam Pla-ra after 6 months of fermentation could be due to the reaction of reducing sugars and amino acids and/or small peptides that led to a Maillard

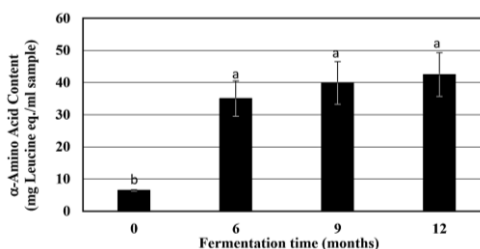


Figure 1. α -Amino acid content of Nam Pla-ra at various fermentation times. Bars with different letters indicate means with significant differences ($p < 0.05$). Values are expressed as means $\pm$ S.D. ($n = 3$)

browning reaction, which occurred to a higher degree in the prolonged fermentation process. Phisut and Jiraporn (2013) reported that an increase in Maillard browning products (MRPs) were concomitant with a decrease in free amino groups and a reduction in sugar content. In addition, free amino groups were changed to amine, volatile acids and nitrogenous compounds such as, nucleotides, ammonia, urea and TMAO (Anggo *et al.*, 2015). Promchote (2017) reported that the differences in α -Amino content of Pla-ra from three manufacturers of Ubon Ratchathani could be due to differences in fermentation processing, fish species and size of fish.

Antioxidant Activity

ABTS Radical Scavenging Activity

Figure 2 shows the changes in ABTS radical scavenging activity of Nam Pla-ra at 0, 6, 9, and 12 months of fermentation. The ABTS radical scavenging activity significantly increased for up to 9 months ($p < 0.05$) and then declined, but not significantly, at 12 months ($p > 0.05$). The results of ABTS radical scavenging activity did not correlate well with α -amino acid content (Figure 1). Therefore, the ABTS radical scavenging activity of Nam Pla-ra was most likely governed by the type and composition of the amino acids in the peptide sequences. This was in agreement with Faithong *et al.* (2010) which suggested that the antioxidant activity of fermented shrimp and krill products depended on the type and amino

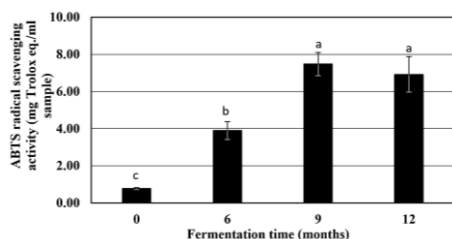


Figure 2. ABTS radical scavenging activity of Nam Pla-ra at various fermentation times. Bars with different letters indicate means with significant differences ($p < 0.05$). Values are expressed as means $\pm$ S.D. ($n = 3$)

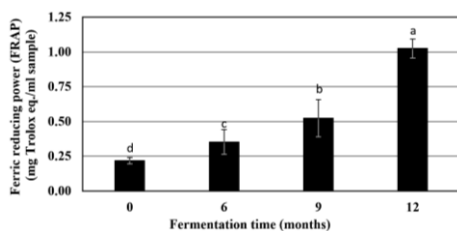


Figure 3. Ferric reducing antioxidant power (FRAP) of Nam Pla-ra sauce at various fermentation times. Bars with different letters indicate means with significant differences ($p < 0.05$). Values are expressed as means $\pm$ S.D. ($n = 3$)

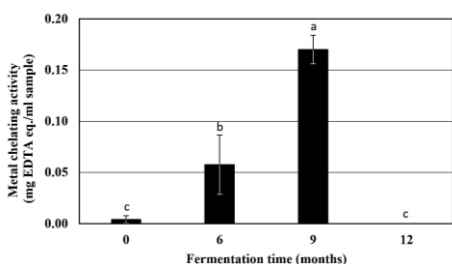


Figure 4. Metal Chelating activity of Nam Pla-ra at various fermentation times. Bars with different letters indicate means with significant differences ($p < 0.05$). Values are expressed as means $\pm$ S.D. ($n = 3$)

acid composition of the resulting peptides. In addition, Peralta *et al.* (2008) reported that the peptides or free amino acids in shrimp paste would not directly influence the observed increase in antioxidant activity but would likely participate in the production of antioxidant substances such as MRPs during a prolonged fermentation. Furthermore, the ABTS radical scavenging activity of Kapi correlated well with the increase in MRPs during fermentation (Pongsetkul *et al.*, 2017). Thus, an increase in the ABTS radical scavenging activity for Nam Pla-ra from *Osteochilus hasseltii* during fermentation might be due to both peptides and MRPs.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP value of Nam Pla-ra increased with a prolonged fermentation time (Figure 3, $p < 0.05$). Nam Pla-ra at 12-months exhibited the highest FRAP value (1.024 mg Trolox eq./ml sample). The results of the FRAP value did not correlated well with α -amino acid content (Figure 1). This result suggested that both the peptides and MRPs in Nam Pla-ra are expected to donate an electron to the Fe^{3+} -TPTZ complex.

Metal Chelating Activity

Metal chelating activity of Nam Pla-ra increased with a prolonged fermentation time up to 9 months (Figure 4, $p \leq 0.05$). However, at 12 months of fermentation time, Nam Pla-ra did not show metal chelating activity (Figure 5, $p > 0.05$). The different in iron chelating activity for water extracted from Kapi might be related to the differences in the amino acid composition of the peptides (Pongsetkul *et al.*, 2015). In addition, the iron-chelating activities of the chickpea protein hydrolysate was observed and depends on peptide sizes, amino acid compositions, and the sequences of peptides (Torres-Fuentes *et al.*, 2012). Thus, the decrease in iron-chelating activity of Nam Pla-ra at 12 months of fermentation time might be related to the differences in peptide sizes and their amino sequences.

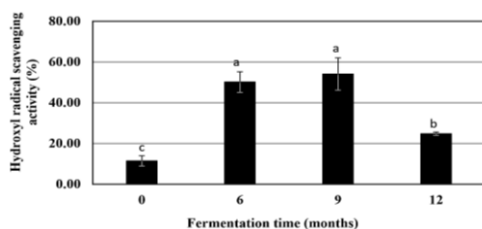


Figure 5. Hydroxyl radical scavenging activity of Nam Pla-ra at various fermentation times. Bars with different letters indicate means with significant differences ($p < 0.05$). Values are expressed as means $\pm$ S.D. ($n = 3$)

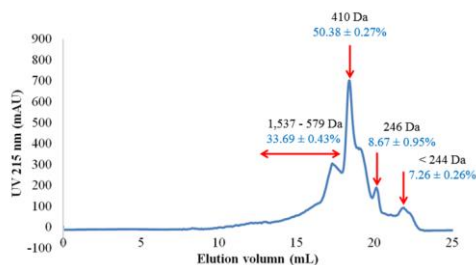


Figure 6. Molecular weight distribution of Nam Pla-ra at 9-months of fermentation

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of Nam Pla-ra increased with prolonged fermentation time up to 9 months fermentation time and declined at 12 months (Figure 5, $p \leq 0.05$). The results of hydroxyl radical scavenging activity correlated well with metal chelating activity (Figure 4). In this study, hydroxyl radicals were produced by a Fenton reaction ($\text{Fe}^{2+}/\text{H}_2\text{O}_2$ system). Therefore, decreases in the hydroxyl radical scavenging activity of Nam Pla-ra at 12 months of fermentation time might be related to a decrease in metal chelating activity. Hydroxyl radical ($\text{HO}\cdot$) is the most reactive of the oxygen radicals that can damage DNA, proteins and lipids in cell systems. Nam-Pla-ra at 9 months of fermentation time showed the strongest hydroxyl radical scavenging activity (54.02 $\pm$ 7.93%), which suggested that it could

serve as a scavenger for reducing the damage induced by hydroxyl radical in biological systems.

Molecular Weight Distribution

Based on the antioxidant activity assays, Nam Pla-ra at 9 months of fermentation exhibited a high antioxidant activity. Therefore, it was selected for the determination of molecular weight distribution. Figure 6 shows the Superdex peptide 10/300 GL chromatograms of Nam Pla-ra at 9 months old of fermentation. The major peaks 1 and 2 observed in chromatograms indicated molecular weight (MW) around 1,537-579 and 410 Da with peak area of 33.69 $\pm$ 0.43 and 50.38 $\pm$ 0.27%, respectively. In addition, MW about 246 and <244 Da was observed in Nam Pla-ra at 9 months-old. This implied that Nam Pla-ra at 9 months of fermentation contained low MW peptides, which might contribute to high antioxidant activities.

Conclusions

In conclusion, Solid Pla-ra and Nam Pla-ra from *Osteochilus hasseltii* could be an important source of protein and minerals in terms of ash content. Based on all assays, antioxidant activity of Nam Pla-ra increased with increasing of fermentation time up to 9 months and then declined, except for FRAP assay. This finding suggests that antioxidant activity of Nam Pla-ra does not necessarily increase with fermentation time. Specific amino acid composition in the peptide sequences, size of peptides and MRPs might be effect on antioxidant activity of Nam Pla-ra. Thus, it is recommended that people consume Nam Pla-ra from *Osteochilus hasseltii* at 9-months of fermentation time because of its high nutrition and antioxidant properties.

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