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Interrelationship between myoglobin and lipid oxidations in oxeye scad (*Selar boops*) muscle during iced storage

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ABSTRACT

The interrelationship between myoglobin oxidation, lipid oxidation and discolouration in oxeye scad fish during iced storage were investigated. The myoglobin autoxidation rate increased with increasing storage time up to 12 days ($p < 0.05$) and remained constant thereafter ($p > 0.05$). Increase in metmyoglobin correlated well with a blue shift from 410 to 408 nm for myoglobin. The soret band of myoglobin decreased with a concomitant decrease in the redness index ($p < 0.05$). During storage, the extractable haem iron decreased ($p < 0.05$), while the non-haem iron increased ($p < 0.05$). Hydrogen peroxide and ferrylmyoglobin concentrations had increased at the end of storage ($p < 0.05$). The conjugated diene (CD) and peroxide value (PV) of oxeye scad lipids tended to stabilise during the initial phase of storage, increased in the differentiation phase and had declined at the end of storage. However, thiobarbituric acid reactive substances (TBARS) increased markedly ($p < 0.05$). Overall, lipid and myoglobin oxidations in oxeye scad occurred in a concurrent manner and each process appeared to enhance the other.

1. Introduction

Myoglobin is one of the haem proteins responsible for the quality of fish muscle, particularly for colour and rancidity development. The presence of residual haem proteins in fish muscle plays an essential role in several quality parameters such as whiteness, microbial growth, shelf life and lipid oxidation (Chaijan, Benjakul, Visessanguan, & Faustman, 2005; Undeland, Kristinsson, & Hultin, 2004). Conversion of reduced myoglobin to oxidised forms yields a greyish-brown colour in fish muscle, and is associated with lipid oxidation (Undeland et al., 2004). Lipid oxidation is one of the major reactions behind a loss in quality of fish muscle during storage, with many muscle components having the potential to catalyse the reaction (Undeland et al., 2004). Myoglobin has strong lipid pro-oxidative capacities, using several mechanisms such as hydroperoxide decomposition, and the ability to act as free radicals due to a conversion to ferryl/perferryl forms (Baron & Andersen, 2002). During the oxidation of oxymyoglobin, both a superoxide anion and a hydrogen peroxide are produced, and further react with iron to produce hydroxyl radicals (Chan, Faustman, Yin, & Decker, 1997). These hydroxyl radicals have the ability to penetrate into the hydrophobic lipid region of muscle and hence facilitate lipid oxidation (Chan et al., 1997). Thus, the degree of lipid oxidation during handling, processing and subsequent storage of fish, especially small pelagic species, seems to correlate well with the haem content in the proteins presented.

Due to the shortage of lean fish, which are commonly used for surimi production, more attention has been given to dark-fleshed fish species as a raw material for surimi. Among dark-fleshed fish, oxeye scad (*Selar boops*) is one of the most abundant species commonly caught in southern Thailand, especially in the Thasala coast of Nakhon Si Thammarat (Wongwichian, Chaijan, & Klomklao, 2013). However, small pelagic fish contain large quantities of lipids and myoglobin in the muscle tissue. After capture, fish are normally kept in ice prior to unloading, and during this stage discolouration and lipid oxidation of dark-fleshed fish muscle can easily take place (Chaijan, Benjakul, Visessanguan, & Faustman, 2006; Chaijan et al., 2005). No information regarding the interrelationship between myoglobin oxidation, loss of redness and lipid oxidation of the muscle from oxeye scad caught in Thailand has been reported. Therefore, this study aimed to monitor the oxidation of myoglobin and lipid in oxeye scad muscle during extended

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iced storage for 15 days. The correlation between myoglobin oxidation and lipid oxidation in oxeye scad muscle was also determined.

2. Materials and methods

2.1. Chemicals

Bathophenanthroline disulfonic acid was purchased from Sigma–Aldrich (St. Louis., MO, USA). Trichloroacetic acid, hydrogen peroxide, iron standard solution and anhydrous sodium sulphate were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Fluka (Bunchs, Switzerland).

2.2. Fish supply and icing procedure

Fresh oxeye scad with an average weight of 110–120 g were caught from the Thasala coast along the Gulf of Thailand during March, 2014. The fish were off-loaded approximately 12 h after capture, and placed in ice, with a fish/ice ratio of 1:2 (w/w) and transported to the School of Agricultural Technology, Walailak University, Thasala, Nakhon Si Thammarat within 30 min. Whole fish (10 kg) were immediately washed with chilled water (4 °C) and kept in ice with a fish/ice ratio of 1:2 (w/v). Fish were placed and distributed uniformly between the layers of ice. The average inner temperature of the fish, measured by Testo 104 thermometer, was 4.14 ± 0.26 °C. The polystyrene box containing fish and ice was kept at 4 °C for 15 days. To maintain the ice content, melted ice was removed and replaced with an equal amount of ice every 2 days. During storage, 10 fish were randomly taken as a composite sample, at 0, 3, 6, 9 and 15 days, for analyses. The fish samples were manually filleted and whole muscle was collected. The muscles were minced to uniformity using a mincer (4-mm hole diameter; Panasonic MK-G20MR, Japan) and used for analyses. The muscles were kept on ice during preparation and analysis.

2.3. Extraction of myoglobin, determination of the proportions of the three myoglobin forms, determination of myoglobin autoxidation and absorption spectra of myoglobin

The myoglobin from whole muscle of oxeye scad was extracted by the method of Benjakul and Bauer (2001). A minced sample (2 g) was weighed into a 50 ml polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer, pH 6.8 were added. The mixture was homogenised at 13,500 rpm for 10 s (using an IKA® homogeniser, Model T25 digital ULTRA-TURRAX®, Germany), followed by centrifuging at 3000*g* for 30 min at 4 °C (using a Sorvall Centrifuge, Model Heraeus Biofuge Stratos, Germany). The supernatant was filtered with Whatman No. 1 filter paper and subjected to spectrophotometric measurement at 503, 525, 582, 557 nm using a UV visible spectrophotometer (Shimadzu, Model UV-16001, Tokyo, Japan). The proportions of the three myoglobin forms (deoxymyoglobin, oxymyoglobin and metmyoglobin) were calculated using a modified Krzywicki's equation (Tang, Faustman, & Hoagland, 2004) as follows:

$$[\text{Deoxymyoglobin}] = 0.543R_1 + 1.594R_2 + 0.552R_3 - 1.329$$

$$[\text{Oxymyoglobin}] = 0.722R_1 + 1.432R_2 + 1.659R_3 - 2.599$$

$$[\text{Metmyoglobin}] = -0.159R_1 + 0.085R_2 + 1.262R_3 - 0.520$$

where R_1 = A582/A525, R_2 = A557/A525 and R_3 = A503/A525.

To follow the myoglobin autoxidation rate over time, the loss of ferrous myoglobin (deoxymyoglobin plus oxymyoglobin) was calculated using the formula from Tajima and Shikama (1987) with a slight modification, as follows:

$$\text{Autoxidation rate} = \ln([\text{ferrous myoglobin}]_t/[\text{ferrous myoglobin}]_0)$$

where $[\text{ferrous myoglobin}]_t$ is the fraction of ferrous myoglobin at time *t*, and $[\text{ferrous myoglobin}]_0$ is the fraction of ferrous myoglobin at the start of the experiment (day 0).

The absorption spectra of extracted myoglobin was measured using a V-530 UV/vis double beam spectrophotometer (Jasco, Tokyo, Japan). The spectra were recorded from 250 to 750 nm, at a scanning rate of 1000 nm/min using 40 mM phosphate buffer, pH 6.8 as blank (Chaijan et al., 2005). Changes in the soret peak (380–450 nm) were monitored.

2.4. Colour measurement

The colour of oxeye scad mince formed within a small petri dish (5-cm dia, 1-cm depth) was determined by measuring the L^*, a^* and b^* values using a Minolta Chromameter (Model CR-400, Osaka, Japan), calibrated with a standard white plate. The illuminant used was *C (D65) and the standard observer angle was 2°. The measuring area was 8 mm. The redness index (a^*/b^*) of the mince was determined as described by Chen, Chiu, and Huang (1997).

2.5. Determination of haem iron content

The haem iron content was determined as described by Benjakul and Bauer (2001). Sample solutions were prepared by the method previously described for myoglobin extraction. The total haem pigment content was determined by direct spectrophotometric measurement at 525 nm. Haem iron content was calculated based on myoglobin, which contains 0.35% iron. The haem iron content is expressed as mg/100 g sample.

2.6. Determination of non-haem iron content

Non-haem iron content was determined as described by Schricker, Miller, and Stouffer (1982), with a slight modification. The fish mince samples (1.0 g) were weighed into a screw cap test tube and 50 μl of 0.39% (w/v) sodium nitrite were added. A mixture (4 ml) of 40% TCA and 6 MN HCl (ratio of 1:1 [v/v], prepared freshly) was then added. The tightly capped tubes were placed in an incubator shaker at 65 °C for 22 h and then cooled down to room temperature (28–30 °C) for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-haem iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenanthroline (0.162 g, dissolved in 100 ml of double-deionised water with 2 ml thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution. The non-haem iron content was calculated from an iron standard curve. The iron standard solution, ranging from 0 to 2 ppm (400 μl), was mixed with 2 ml of the non-haem iron colour reagent. The concentration of non-haem iron is expressed as mg/100 g sample.

2.7. Determination of hydrogen peroxide content by the ferrous ion oxidation-xylenol orange (FOX) assay

The concentration of hydrogen peroxide was measured according to the method of Long, Evans, and Halliwell (1999). FOX reagent was prepared by adding nine volumes of reagent 1 to one volume of reagent 2, where reagent 1 is 4.4 mM 2,6-di-t-butyl-4-methylphenol (BHT) in methanol, and reagent 2 is 1 mM xylenol orange plus 2.56 mM ammonium ferrous sulphate in 250 mM H_2SO_4. 100 μl of

sample solution, prepared by the method previously mentioned for myoglobin extraction, were added to the FOX reagent (3 ml) and vortexed for 5 s. After incubation for 30 min at room temperature and centrifugation at 5000*g* for 30 min at 25 °C, the absorbance at 560 nm was measured using a spectrophotometer. The FOX assay was calibrated using a standard hydrogen peroxide solution whose concentration was determined by using a molar extinction coefficient of 43 $M^{-1}cm^{-1}$ at 240 nm. The concentration of hydrogen peroxide is expressed as mmol/kg sample.

2.8. Determination of ferrylmyoglobin content

A sample solution prepared using the method previously described for myoglobin extraction was subjected to spectrophotometric measurement at 550 and 630 nm using a UV visible spectrophotometer (Shimadzu, Model UV-16001, Tokyo, Japan). Ferryl myoglobin concentration in the sample solution was calculated according to the following formula (Giulivi, Romero, & Cadenas, 1992):

$$[\text{Ferrylmyoglobin}](\mu M) = (249 \times A550) - (367 \times A630)$$

The concentration of ferrylmyoglobin is expressed as μmol/kg sample.

2.9. Lipid extraction

Lipid was extracted by the Bligh and Dyer method (1959). The sample (25 g) was homogenised in 200 ml of a chloroform: methanol: distilled water mixture (50:100:50) at a speed of 9500 rpm for 2 min at 4 °C (using an IKA® homogeniser, Model T25 digital ULTRA-TURRAX®, Germany). The homogenate was treated with 50 ml of chloroform and homogenised at 9500 rpm for 1 min. Then, 25 ml of distilled water were added and the mixture homogenised again for 30 s. The homogenate was centrifuged at 3000*g* at 4 °C for 15 min (using a Sorvall centrifuge, Model RC 55 Plus, USA) and transferred into a separating flask. The chloroform phase was drained off into a 125 ml Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulphate, shaken well, and decanted into a round-bottom flask through Whatman No. 4 filter paper. The solvent was evaporated at 25 °C using a rotary evaporator (Eyela, Model N-100, Tokyo, Japan), and the residual solvent was removed by flushing with nitrogen. The extracted lipid was subjected to analysis of CD and PV.

2.10. Determination of CD

CD was measured according to the method of Frankel, Huang, Prior, and Aeschbach (1996). Oxeye scad oil (0.1 g) was dissolved in 5 ml of isooctane and the absorbance was measured at 234 nm. CD is expressed as mmol/kg sample.

2.11. Determination of PV

PV was determined according to the method of Low and Ng (1978). The lipid sample (1.0 g) was treated with 25 ml of organic solvent mixture (chloroform:acetic acid mixture, 2:3). The mixture was shaken vigorously, followed by the addition of 1 ml of saturated potassium iodide solution. The mixture was kept in the dark for 5 min, and then 75 ml of distilled water were added and the mixture was shaken. To the mixture, 0.5 ml of starch solution (1%, w/v) was added as an indicator. The PV was determined by titrating the iodine liberated from potassium iodide with standardised 0.01 N sodium thiosulphate solution. The PV is expressed as milliequivalents of free iodine per kg of lipid.

2.12. Determination of TBARS

TBARS assay was performed as described by Buege and Aust (1978). A ground sample (0.5 g) was homogenised with 2.5 ml of a solution containing 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min to develop a pink colour, then cooled with running tap water and centrifuged at 3600*g* at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 10 ppm. TBARS was calculated and expressed as equivalents of mg malonaldehyde/kg sample.

2.13. Statistical analysis

For each experiment, fish minces from 10 randomly selected fish were used as the composite sample. The composite sample was divided into 3 replications, each of which were considered experimental units ($n = 3$). Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test ($p < 0.05$; Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Autoxidation of myoglobin, metmyoglobin formation, changes in redness index and changes in absorption spectra in the soret region

The formation of metmyoglobin in oxeye scad muscle during iced storage is shown in Fig. 1. The metmyoglobin content of oxeye scad muscle increased with increasing storage time up to day 12 ($p < 0.05$) and remained constant until the end of the storage period ($p > 0.05$). Normally, the increase in metmyoglobin formation can be observed during iced storage in many dark-fleshed fish species (Chaijan et al., 2005; Sohn et al., 2005). An increased metmyoglobin proportion in fish samples indicated that ferrous myoglobin species (oxymyoglobin plus deoxymyoglobin) underwent oxidation to form ferric metmyoglobin during extended storage (Thiansilakul, Benjakul, & Richards, 2010). From the autoxidation rate determination (Fig. 1), fast and slow rates of myoglobin autoxidation could be identified. During the first 12 days of storage the autoxidation of myoglobin occurred at a fast rate, as indicated by a distinctly sharp curve. A slow rate of

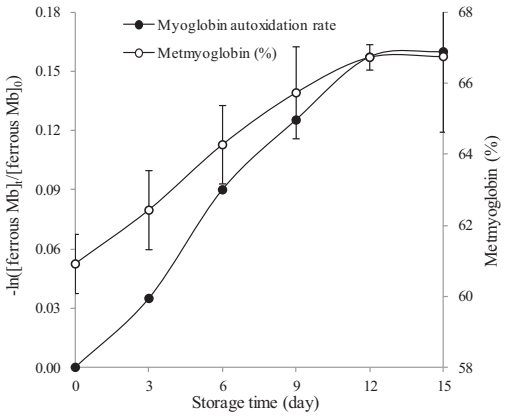

Fig. 1. Autoxidation rate of ferrous myoglobin and metmyoglobin formation (%) of oxeye scad muscle during iced storage. The bars indicate standard deviation from triplicate determinations.

autoxidation was observed at the end of storage period (day 15) as visualised by a plateau in the curve.

The constant metmyoglobin proportion at the final stage of storage was probably due to the action of an endogenous oxidising agent, such as hydrogen peroxide, which can oxidise metmyoglobin to other forms such as ferrylmyoglobin species. At day 15, the rate of metmyoglobin formation should balance with the rate of metmyoglobin oxidation. Thus, no change in metmyoglobin content was observed. Additionally, radicals generated by lipid oxidation can promote the accumulation of metmyoglobin (Faustman, Sun, Mancini, & Suman, 2010), which can then be reduced back to the oxygen binding form, while metmyoglobin reducing activity (MRA) is still active. However, in the degradative, oxidative environment of muscle with free radicals formed by lipid oxidation, the NADH depletes faster, reducing MRA activity and leading to metmyoglobin accumulation (Ladeira et al., 2014).

The absorption spectra in the soret region of myoglobin from oxeye scad muscle are depicted in Fig. 2. It has been reported that the soret peaks of oxymyoglobin and metmyoglobin purified from tuna (*Thunnus albacares*) were noticeable at 414 and 406 nm (Smulevich et al., 2007). From these results, the intense peak in the soret band was found at 410 nm, and decreased with increasing storage time. A soret band results mainly from the interaction of the haem moiety with apomyoglobin. Hence it can be used to monitor the unfolding of haem proteins (Benjakul & Bauer, 2001). The disappearance of the soret absorption peak indicated the destruction of the haem protein, or the detachment of the porphyrin moiety from the globin (Baron & Andersen, 2002). This result was in agreement with a decreased haem iron content and increased non-haem iron content in the muscle (Fig. 4). From these results, non-haem iron was mostly liberated from the haem proteins, particulary myoglobin during storage. In addition, a blue shift from 410 to 408 nm at day 12 coincided with a noticeable increase in metmyoglobin content (Fig. 1). From day 12 to day 15, no change in soret absorption peak was found, and this was in agreement with the constant metmyoglobin content at that period. Antonini and Brunori (1971) reported that a blue shift from 418 to 409 nm was observed when sperm whale oxymyoglobin was changed to metmyoglobin. From the results, it was presumed that, during iced storage, the degradation and oxidation of haem proteins in oxeye scad muscle occurred simultaneously.

The relative (percent) change in redness index (a^*/b^* ratio) of oxeye scad muscle during iced storage are shown in Fig. 3. The absolute redness index data of oxeye scad muscles is also depicted in the inset of Fig. 3. From the results, the relative redness index decreased when the storage time increased ($p < 0.05$). The redness index can be used to monitor the apparent changes in redness (Chen et al., 1997) and to evaluate the discolouration in tuna meat during storage (Lee, Joo, Alderton, Hill, & Faustman, 2003). The decrease in the redness index was associated with the darkening

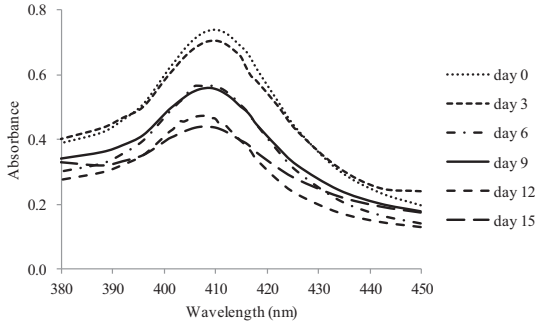

Fig. 2. Changes in the absorption spectra in the soret region (380–450 nm) of myoglobin from oxeye scad muscle during iced storage.

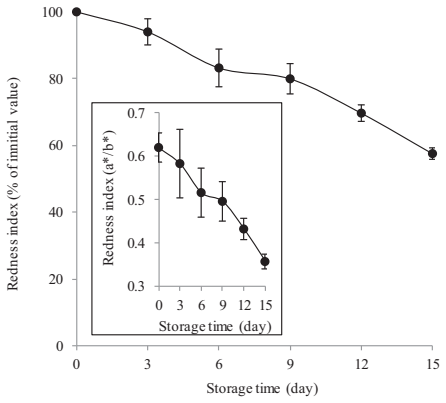

Fig. 3. Relative (percent) changes in redness index (a^*/b^*) of oxeye scad muscle during iced storage. (Inset) Absolute redness index (a^*/b^*) data. The bars indicate standard deviation from triplicate determinations.

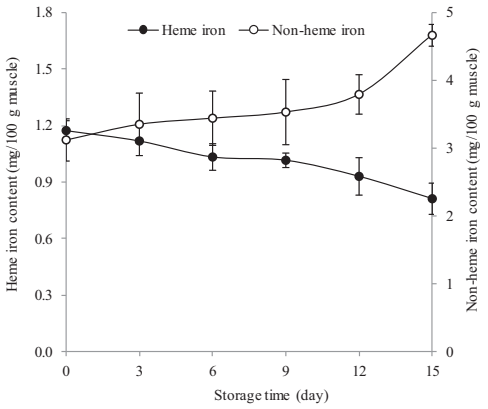

Fig. 4. Changes in haem iron and non-haem iron contents of oxeye scad muscle during iced storage. The bars indicate standard deviation from triplicate determinations.

of the meats, resulting from the formation of metmyoglobin (Fig. 1) and was also coincidental with the disappearance of the soret absorption band, as well as the shift of soret peak (Fig. 2). At day 15, the redness index of oxeye scad muscle decreased by 43%, compared to that obtained in fresh muscle. The decline of the meat colour indices during extended storage in ice can be attributed to the oxidation of ferrous-myoglobin (deoxymyoglobin and/or oxymyoglobin) to ferric-metmyoglobin and the loss of metmyoglobin reducing activity (Faustman et al., 2010). In addition, the loss of myoglobin into the melted ice can be taking place, resulting in the discolouration of oxeye scad muscle. Thus, the loss of redness index can be used as a tool to follow the discolouration of oxeye scad muscle during iced storage. Wetterskog and Undeland (2004) hypothesised that the observed loss of redness occurring simultaneously as trout haemoglobin-catalysed lipid oxidation was due to the oxidation of the haem iron from the ferrous to the ferric state under production of a brownish grey colour.

3.2. Changes in extractable haem iron and non-haem iron contents

Changes of extractable haem iron and non-haem iron contents in oxeye scad muscle during iced storage are shown in Fig. 4. Fresh oxeye scad muscle contained 1.17 mg haem iron per 100 g of muscle. Generally, haem iron content gradually decreased as the storage time increased ($p < 0.05$). The marked decrease in haem iron content during storage was coincidental with an increase in

non-haem iron content (Fig. 4). Benjakul and Bauer (2001) reported that the decrease in haem iron content in the muscle was inversely related to non-haem iron content. Higher content of soluble haem pigment could be found in fresh meat, and this might contribute to the greater extractability of haem pigments (Chaijan et al., 2005). The decrease in haem iron content observed with extended storage time, was presumably due to the release of free iron from haem. As a result, less haem iron was retained. Additionally, the lowered haem pigment extractability with increasing storage time, was also caused by the interaction between haem pigments and muscle components e.g., myofibrillar proteins (Chaijan, Benjakul, Visessanguan, Lee, & Faustman, 2007) and/or cellular membranes (Shviro, Zilber, & Shaklai, 1982). At day 15, haem iron content of oxeye scad muscle decreased by 31%, compared with fresh muscle samples. Thiansilakul et al. (2010) reported that the higher rate of decrease in haem iron content observed in seabass muscle with extended storage time was related to the higher disruption of haem proteins and higher release of iron from haem when compared with red tilapia muscle. Haemoglobin, myoglobin, as well as mitochondrial iron-containing enzymes, are the sources of iron in fish muscle (Decker & Hultin, 1990). Benjakul and Bauer (2001) reported that haem iron content in fresh catfish (*Silurus glanis* Linne) fillet was 0.72 mg/100 g and decreased to 0.5–0.6 mg/100 g after 3 days of chilled storage. Generally, haem iron content was most likely correlated with total pigment content in fish muscle (Chaijan et al., 2005).

Non-haem iron content in oxeye scad muscle tended to increase during iced storage ($p < 0.05$). At day 15, non-haem iron content of oxeye scad muscle increased by 33%, compared to that obtained in fresh muscle. The increase in non-haem iron content was in accordance with the decrease in haem iron content (Fig. 4.). The results suggested that the haem pigment or other iron-containing proteins, are possibly denatured/degraded with increased storage time, resulting in the release of iron Decker & Hultin, 1990. Benjakul and Bauer (2001) and Chaijan et al. (2005) reported an increased non-haem iron in catfish, sardine and mackerel muscles with extended iced storage times. Wongwichian et al. (2013) reported that the autolytic degradation of muscle proteins from oxeye scad, as measured by TCA-soluble peptide, increased with increasing iced storage time. Thus, the degradation of muscle proteins with the disruption of the porphyrin ring can take place during extended storage, leading to the release of free iron. This free iron can act as a pro-oxidant in fish muscle, and was associated with the enhanced lipid oxidation (Thiansilakul et al., 2010).

3.3. Changes in hydrogen peroxide and ferrylmyoglobin contents

Hydrogen peroxide is a non-radical oxidising species that can be formed through the oxidative processes. It can be further reacted with a transition metal ion or iron-containing haem proteins (e.g., myoglobin and haemoglobin) to produce hydroxyl radicals, a non-selective oxidant, by the Fenton reaction (Burns et al., 2012). Halliwell and Gutteridge (1990) suggested that low concentrations of hydrogen peroxide can be found in aerobic cells as a metabolite under physiological condition. From the results, hydrogen peroxide content generally showed an increasing trend for oxeye scad muscle during iced storage ($p < 0.05$) (Fig. 5). Hydrogen peroxide content increased to a maximum around day 9 and then dropped by day 12 ($p < 0.05$). However, after day 12, an increase in hydrogen peroxide was noted ($p < 0.05$). Generally, hydrogen peroxide can be generated during the oxidation of oxymyoglobin (Chan et al., 1997). From the results, ferrous myoglobin oxidised spontaneously to from metmyoglobin (Fig. 1) and hydrogen peroxide could be found simultaneously as a potential by-product. Alternatively, a superoxide anion radical generating system would be expected to yield hydrogen peroxide by non-enzymatic or superoxide dismutase-catalysed dismutations. The latter can react with the metmyoglobin concurrently generated in this oxidation sequence to form an activated metmyoglobin complex capable of enhancing lipid oxidation attributed to ferrylmyoglobin (Tajima & Shikama, 1987). Metmyoglobin-hydrogen peroxide complex and ferrylmyoglobin have been documented as potentially powerful contributors to lipid oxidation in meat (Baron & Andersen, 2002; Faustman et al., 2010). Hydrogen peroxide can perform most of its damaging effects by the generation of more reactive species, such as hydroxyl radicals by the catalysis of ferrous ions (Chan et al., 1997). In addition, hydrogen peroxide can denature haem proteins to release free iron and a haem group, or convert haem proteins into ferryl or perferryl radicals, depending on its concentration (Baron & Andersen, 2002). From the results, the increase in hydrogen peroxide content corresponded well with the increase in non-haem iron content (Fig. 4) and ferrylmyoglobin content (Fig. 5). Furthermore, in the presence of hydrogen peroxide, metmyoglobin can form cross-links with myosin (Hanan & Shaklai, 1995).

Regarding the ferrylmyoglobin content (Fig. 5), during the first 3 days of storage, the ferrylmyoglobin content increased markedly ($p < 0.05$). Thereafter, no changes were observed up to 9 days of storage ($p > 0.05$) and subsequently increased ($p < 0.05$) until the end of storage time ($p < 0.05$). During iced storage, the ferrylmyoglobin content of oxeye scad muscle tended to increase as the storage time increased ($p < 0.05$). This was coincidental with the increase in metmyoglobin (Fig. 1) and hydrogen peroxide contents during storage (Fig. 4). Kanner and Harel (1985) suggested that ferrylmyoglobin can be generated by the interactions between hydrogen peroxide and metmyoglobin. This ferryl species can initiate lipid peroxidation in muscle tissue. It has been suggested that hydrogen peroxide alone or metmyoglobin alone could not stimulate the oxidation reaction in a sarcosomal fraction of turkey dark muscle, whereas membrane lipid peroxidation was readily promoted in the presence of hydrogen peroxide and metmyoglobin (Kanner & Harel, 1985). The ratio of hydrogen peroxide to metmyoglobin seemed to be important for the generation of the ferryl species (Min & Ahn, 2005). Rhee, Ziprin, and Ordonez (1987) demonstrated that the catalytic activity of metmyoglobin-hydrogen peroxide was highest at the molar ratio of ~1:0.25 in raw microsomal systems of beef, and at the molar ratio of 1:1.5 or 1:2 in cooked systems, although metmyoglobin alone had little catalytic activity. Moreover, the non-haem iron concentration in that system was shown to increase as the concentration of hydrogen peroxide increased. In addition to the pro-oxidative contribution of the myoglobin oxidation process and generation of ferrylmyoglobin, the dissociation of both haem from myoglobin, and iron from haem

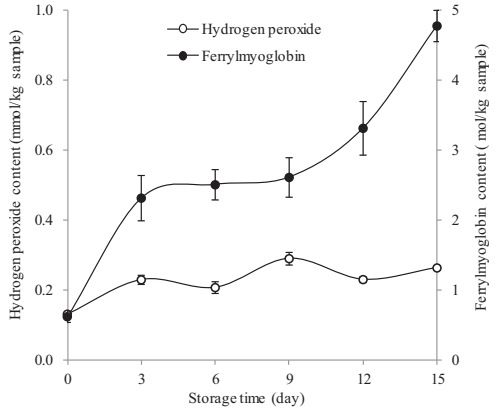

Fig. 5. Changes in hydrogen peroxide and ferrylmyoglobin contents of oxeye scad muscle during iced storage. The bars indicate standard deviation from triplicate determinations.

may also contribute to the mechanism by which myoglobin enhances lipid oxidation (Faustman et al., 2010).

3.4. Changes in CD, PV and TBARS

The conjugated compounds reflect the degree of primary products formed during lipid oxidation (Guillén & Ruiz, 2004). Initiation reactions take place either by the abstraction of hydrogen from an allylic methylene group of an unsaturated fatty acid or by the addition of a radical to a double bond. The rearrangement of the double bonds results in the formation of conjugated double bonds (Frankel et al., 1996). From Fig. 6, the CD of lipids extracted from oxeye scad muscle remained constant up to day 9 ($p > 0.05$) and sharply increased to a maximum value on day 12 ($p < 0.05$). Thereafter, the CD decreased until the end of the storage period (day 15) ($p < 0.05$). The same trend has been reported in Atlantic pomfret muscle, where no change in CD was found within the first 9 days, followed by a gradual increase up to 19 days of chilled storage (Pérez-Alonso, Arias, & Aubourg, 2003).

During the first 9 days, the rate of CD formation could be equal with the decomposition rate, leading to the constant CD accumulated in the lipid fraction (Chaijan et al., 2006). At day 12, the formation rate would be significantly higher than the decomposition rate and therefore an obviously increased CD was noted. At the end of storage, the CD was excessively decomposed to secondary oxidation products, resulting in the decreased CD. It has been proposed that the decrease in CD can be explained by the instability of these primary compounds, and then their degradation could promote the formation of secondary products (Chaijan et al., 2006).

Fig. 6 also shows the changes in PV of oxeye scad muscle during iced storage. The PV of oxeye scad remained constant during the first 6 days ($p > 0.05$) and then a marked increase in PV was found at day 9 ($p < 0.05$). After this period, the PV of oxeye scad was constant up to day 12 ($p > 0.05$) and then decreased considerably up to day 15 ($p < 0.05$). Thiansilakul et al. (2010) reported that an increase in PV was noticed in red tilapia within the first 3 days of iced storage, and gradually decreased thereafter. Hydroperoxide is the primary product of lipid oxidation, and the determination of the peroxide value can be used as an oxidative index for the early stage of lipid oxidation (Ramadan & Mörsel, 2004). The decreased PV observed with extended storage time was presumed to be due to the decomposition of hydroperoxides to form low molecular weight compounds e.g., aldehydes and ketones.

TBARS generally showed an increasing trend for oxeye scad muscle over the period studied ($p < 0.05$) (Fig. 6). In general, the increase in TBARS indicated the formation of secondary lipid oxidation products. The marked raise in TBARS, especially at the end of storage, was coincidental with the decreases in CD and PV (Fig. 6). This was probably due to the destruction of conjugated compounds and hydroperoxides into secondary oxidation products, especially aldehydes in the final stage of lipid oxidation. Aubourg, Lehmann, and Gallardo (2002) reported that the gradual increase in TBARS was found during the frozen storage of horse mackerel (*Trachurus trachurus*). Generally, it was noted that a higher rate of lipid oxidation of fish muscle usually took place at the end of the storage period (Chaijan et al., 2006). This was probably due to the greater release of free iron and other prooxidants from the muscle, which was excessively degraded when storage time increased. Additionally, the loss of natural antioxidants during extended storage might contribute to the increased lipid oxidation (Chaijan et al., 2006). Generally, increased lipid oxidation has been linked to the changes in myoglobin and other haem compounds. Apart from myoglobin, haemoglobin in fish muscle was an effective catalyst of lipid oxidation (Undeland et al., 2004; Wetterskog & Undeland, 2004).

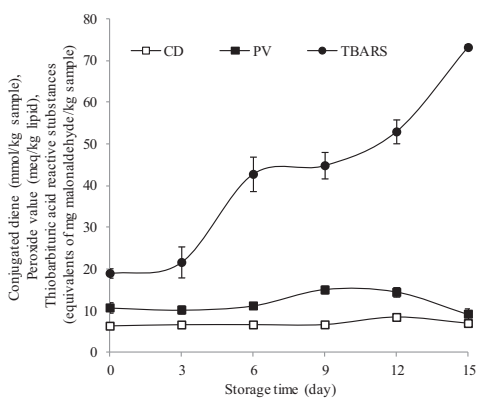

Fig. 6. Changes in CD, PV and TBARS of oxeye scad muscle during iced storage. The bars indicate standard deviation from triplicate determinations.

3.5. Correlations between myoglobin and lipid oxidations

Lipid oxidation in fish muscle could be governed by the contents and forms of myoglobin as well as non-haem iron (Grunwald & Richards, 2006; Lee et al., 2003; Sohn et al., 2005). The release of non-haem iron from fish muscle during extended iced storage can enhance the oxidation process in muscle (Chaijan et al., 2005). Haem dissociation, haem destruction and iron release also play a crucial role in the acceleration of lipid oxidation (Grunwald & Richards, 2006). The accumulation of non-haem iron had a good correlation with the TBARS development in oxeye scad muscle during iced storage ($r^2 = 0.8604$). In addition, an increase in ferrous myoglobin autoxidation, as well as an increase in metmyoglobin content (Fig. 1) during iced storage was coincidental with an increase in lipid oxidation (Fig. 6) and a decrease in redness index (Fig. 3). Autoxidation of ferrous myoglobin was overall highly correlated with TBARS development ($r^2 = 0.8675$) and a decrease in redness index ($r^2 = 0.8970$). Autoxidation of oxymyoglobin results in the formation of metmyoglobin and superoxide, which rapidly dismutates to hydrogen peroxide and oxygen. The interaction of hydrogen peroxide with metmyoglobin led very rapidly to the generation of an active ferryl radical, which can initiate lipid oxidation (Baron & Andersen, 2002). A strong correlation between TBARS formation and the decrease in redness index was also noticeable ($r^2 = 0.9781$). This phenomenon was in accordance with Wetterskog and Undeland (2004) who suggested that the loss of redness was directly correlated with the lipid oxidation in washed cod mince fortified with haemoglobin, and the loss of redness can be use as a tool to follow haem protein-mediated lipid oxidation. Therefore, the autoxidation of myoglobin and the release of non-haem iron were associated with enhanced lipid oxidation and discolouration of oxeye scad muscle during iced storage.

4. Conclusion

Myoglobin and lipid oxidations concurrently occurred in oxeye scad muscle during iced storage. Progressive formation of metmyoglobin and the release of non-haem iron were observed, along with an increased TBARS and a decrease in redness index. Thus, myoglobin and lipid oxidations were coupled and each process appeared to enhance each other.

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